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ISOLATION OF FLUORESCENT PSEUDOMONADS, HEAT TOLERANT AND CHITINOLYTIC BACTERIA IN BANANA RHIZOSPHERE WITH ANTAGONISTIC ACTIVITIES AGAINST *Fusarium oxysporum* f. sp. *cubense* *IN VITRO* AND MOLECULAR IDENTIFICATION OF SELECTED ISOLATES

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ABSTRACT

Fusarium wilt disease caused by *Fusarium oxysporum* f. sp. *cubense* (Foc) race 4 is the most destructive disease of banana in Indonesia. Ecological studies on the disease in relation to the bacterial population in the rhizosphere, is still limited. The rhizobacteria could be exploited as the biocontrol agents to suppress the growth of the pathogen. This experiment was conducted to observe the abundance of fluorescent Pseudomonads, heat tolerant, and chitinolytic bacteria in the banana rhizosphere and assess their antagonistic activities against *Foc in vitro*. The bacterial population was calculated by serial dilution and plate-count method. The antagonistic test was observed using dual culture method. The average populations of non-fluorescent, heat tolerant, and chitinolytic bacteria in healthy banana rhizosphere were significantly higher compared with those in the diseased plants, while fluorescent Pseudomonads population did not show significant difference from control (the diseased plants). Among the fluorescent Pseudomonads, isolate KB2 produced the widest inhibition zone against *F. oxysporum* f.sp. *cubense*, while among the isolates of chitinolytic and heat tolerant bacteria, isolate CH4 and TSA3 caused the highest inhibition of mycelial growth and the least mycelial growth of *F. oxysporum* f.sp. *cubense* at 3 days after inoculation (DAI), respectively. Those three isolates have the potential as biocontrol agents against fusarium wilt disease of banana and should be tested for effectiveness *in planta* and in the field. Based on the partial sequence of 16S rDNA, TSA3 and CH4 were identified as *Bacillus subtilis*, and *B. pumilus*, respectively.

Key words: *Bacillus subtilis*, *B. pumilus*, PGPR, Fusarium wilt disease, biological control

INTRODUCTION

Fusarium wilt disease caused by *Fusarium oxysporum* f.sp. *cubense* (Foc) is a major disease, one of the important diseases of banana in Indonesia. The pathogen attacked almost at all banana area. Nasir and Jumjunidang (2002) reported that at least 5,200 ha of Cavendish banana growing areas commercial plantations in Indonesia were destroyed by this disease. In West Sumatera Province diseased area covered about 140 ha, North Sumatera Province was up to 40 ha, Jambi Province 150 ha, Riau Province about 300 ha, Lampung Province 1,700 ha, and Halmahera Province was up to 3,000 ha. Wibowo et al. (2011) reported that race 4 strains of *F. oxysporum* f.sp. *cubense* are presented in Indonesia, attacking local banana, while Riska et al. (2012) reported that 47 isolates of *Foc* collected from West Sumatra were belonged to five groups of Vegetative Compatibility Groups (VCG), i.e. VCG 01213/16, VCG 0121, VCG 01219, VCG 01218 and VCG 0120 and seven isolates are unknown VCGs. In India, based on isozyme banding patterns of Foc, showed 46 scoreable markers and cluster analysis with UPGMA using genetic distance showed that the isolates

belonged to three main groups. Group 1 contained isolates 1, 2, 4, 5, 7 and isolate 3 and 6 were placed in group 2 and 3 (Kumar et al. 2010).

Stover (1990) reported that *F. oxysporum* f.sp. *cubense* is a soil borne fungi, as root inhabitant and they have different physiological race. The disease caused by the fungi was monocyclic. The fungi produce chlamidospores which are resistant in the soil for about 30 years without host. According to Agrios (2005) the pathogen spreads fast in soil with light texture or sandy soil, acid soil and soil with bad drainage.

Disease control efforts have been used by farmers, i.e. fungicide application, plant rotation, organic material amendment, growing resistant varieties and biological control (Pegg et al. 1996; Djatnika and Wakiah 1995). Nasir et al. (2003) reported that invasion of banana pseudostems in the sawdust and solarisation treatments was not significantly different from invasion in the respective control treatment. Fusarium wilt disease development on the other plants was correlated with soil physics, soil chemistry and soil microbiology. The importance of soil biodiversity in control soil borne disease was reported by Lehman-Danzinger (2003). Soil biodiversity was affected by the aerial biodiversity (plant rotation, growing more than one variety), soil organic material, soil pH, nutrition balance, humidity, and soil component material. Soil unbalance caused plants more attractive for pathogens or pests, while soil rich with organic material will be able to suppress the pathogen. Based on some statements in Roper and Ophel-Keller (1997), population size and microbe composition have been used for observation on the changes of soil microbe as response of soil tillage and become the indicator status of soil health. Population of cultivable bacteria has been used in some study to see those changes.

The information on correlation between populations of culturable rhizobacteria in banana rhizosphere with fusarium wilt disease is still limited. This experiment was conducted to observe the abundance of fluorescent Pseudomonads, heat tolerant, and chitinolytic bacteria in the banana rhizosphere and their antagonistic activities against Foc in order to get one or more biocontrol agent(s) to support the sustainability of banana production.

MATERIAL AND METHODS

Isolation of *F. oxysporum* f.sp. *cubense* race 4

The isolate of *F. oxysporum* f.sp. *cubense* race 4, used in this experiment belongs to the collection of the Laboratory of Plant Mycology, Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University (IPB), Indonesia, which was isolated from heavily infected banana fields in Bogor, West Java. The culture was preserved on Potato Dextrose Agar (PDA) slant covered by sterilized mineral oil. Before treatment, the isolate was re-cultured by inoculating the mycelia on the PDA plate.

Isolation and quantification of fluorescent Pseudomonads, heat tolerant, and chitinolytic bacteria

Estimation of the bacterial abundance was conducted by counting the growing colony of each group of the bacteria using plate-count technique. Samples of rhizosphere soil were collected from the diseased and healthy banana in some banana areas belonged to the farmers in Pasir Eurih Village, Tenjolaya Village, Loa Village, Ciapus Village, and the experimental field in Pasir Kuda Village which is belonged to The Research Center of Tropical Fruits, Bogor Agricultural University, Bogor, West Java Province. From each location or area, there were assorted two banana plants which close each other (3-5 m), one plant with wilt symptom and the other without wilt symptom. Soil

samples were taken from 5-25 cm depth, 0.5-1.0 kg per each rhizosphere at 4 points (east, west, north, and south of plant). Samples were mixed before isolation of bacteria.

Isolation of the rhizosphere bacteria was conducted by suspended 10 gram of soil sample into 100 ml of sterilized distilled water in Erlenmeyer flask and shaking them using shaker on 300 rpm for 5 minutes. After a serial dilution, 0.1 ml (100 µl) of each suspension was spread out using glass beads with diameter 0.25 cm on the King's B Agar (KB) plates and Chitin Agar. To observe the population of heat tolerant bacteria, the rest of the suspensions in Erlenmeyer flask were then heated until the temperature of the suspension was up to 80^oC for 10 minutes. After a serial dilution, 0.1 ml (100 µl) of each suspension was spread out using glass beads with diameter 0.25 cm on the Tryptic Soy Agar (TSA) plates. Each dilution was plating twice (duplo), and the plates were incubated in room temperature for 24-48 hours. Colonies of the bacteria were counted and some different colonies were isolated on new agar plates to get pure culture. The pure isolates were maintained in glycerol 20% and keep in temperature -20^oC or -17^oC.

Antagonistic test of the rhizosphere bacteria: fluorescent Pseudomonads, heat tolerant, and chitinolytic bacteria

One of the selection methods to get the candidates of biocontrol agents is by the detection of antagonistic activity. Isolate of *F. oxysporum* f.sp. *cubense* was inoculated on PDA plates and after incubation for seven days, the mycelia and the agar were cut into pieces using cork borer with diameter 5 mm. Each piece of mycelia was placed on PDA plate, 3 cm from the edge. On the opposite of the fungi, about 3 cm from the fungi, a loop full of one isolate of rhizobacteria was streaked to make a line about 2 cm long (Fig. 1).

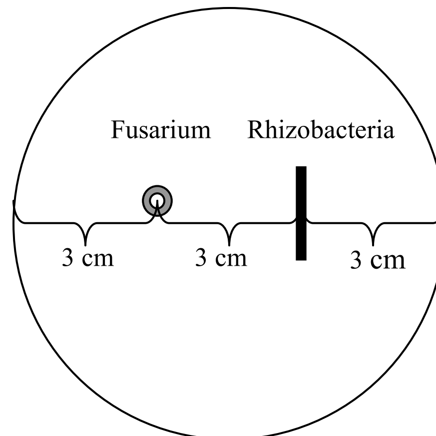

Fig. 1. Composition of pathogen and rhizobacteria on PDA plate in antibiosis activity test

The isolate of rhizobacteria was replaced with sterilized distilled water as control treatment. Inoculated plates were incubated in room temperature for 5-7 days. The diameter colony (DC) of the fungi was measured every day from 3 days after inoculation. The diameter of inhibition zones were calculated by measuring the clear zone between the bacterial colony and the mycelia of fungi when the growth of mycelia toward the edge of petri dish were maximum (3 cm). All treatments were replicated three times. The percentage suppression was calculated using the formula as follows:

$$\text{Percentage Suppression (\%)} = \frac{\text{DC control} - \text{DC treatment}}{\text{DC control}} \times 100\%$$

Characterization of the rhizosphere bacteria

Three isolates of the rhizosphere bacteria, one isolate from fluorescent Pseudomonads, one isolate from heat tolerant bacteria, and one from chitinolytic bacteria, were characterized based on colony morphology and Gram Reaction (Rudolph et al. 1990; Hayward 1990; Schaad et al. 2001). Identification was conducted by sequencing of 16S rDNA. DNA was extracted from log phase culture using phenol-chloroform extraction procedure (Sambrook and Russel 2001). Sequencing of 16S rDNA was conducted using the primers 16 SF: CAGGCCTAACACATGCAAGTC and 1387 R: GGGCGGWGTGTACAAGGC. Total volume reaction was 20 µl which contained 91.5% (v/v) Ex-Taq buffer, 8% (v/v) pNTP or 10-20 ng DNA, and 0.5% (v/v) Ex-Taq. Start condition PCR was denaturizing at 94 ^{o}C for 4 minutes, annealing at 64 ^{o}C for 30 seconds, and extention at 72 ^{o}C for2 minutes followed by 35 cycles of denaturizing at 94 ^{o}C for 30 seconds, annealing at 64 ^{o}C for 30 seconds, and extention at 72 ^{o}C for 30 seconds and the last extention was at 72 ^{o}C for 7 minutes. The PCR products were sent to MACROGEN Inc., Geumchun-gu, Seoul, Korea. Sequencing data were tested for similarity to the data in the Gene Bank using BLAST Program.

Data analysis

All data were statistically analyzed by analysis of variance with DMRT test using Statistical Analysis System (SAS) Program.

RESULTS AND DISCUSSION

Abundance of fluorescent Pseudomonads, heat tolerant, and chitinolytic bacteria in banana rhizosphere

Logarithmic values of the bacterial colony population were presented in Table 1. The table showed that there were differences on the abundance of rizospheric bacteria from banana with wilt symptom (diseased plant) and from those without symptom (healthy plant). The average of total colony forming unit per gram soil of non-fluorescent Pseudomonads, heat tolerant, and chitinolytic bacteria on healthy plants was not significantly different compared with those on diseased plants, but the result was different for the fluorescent Pseudomonads. Logarithmic value of the average population of fluorescent Pseudomonads on diseased plants was higher compared with those on healthy plants, which were up to 6.54 and 6.28 respectively. Those logarithmic values were similar with antilog of total population 2.82 x 10^{6} and 1.91 x 10^{6} cfu g^{-1} soils.

Antilog value of the average of total colonies of non-fluorescent Pseudomonads on diseased plants was up to 1.05 x 10^{7} cfu g^{-1} soils, but it was not significantly different compared with those on healthy plants, which was up to 1.82 x 10^{7} cfu g^{-1} soils. The average of total colonies of heat tolerant bacteria on diseased plants was up to 3.47 x 10^{7} cfu g^{-1} soils, while on healthy plants was up to 3.63 x 10^{7} cfu g^{-1} soils. Average of total colonies of chitinolytic bacteria on diseased plants was lower compared with those on healthy plants, which were 6.30 x 10^{6} cfu g^{-1} soils and 9.10 x 10^{6} cfu g^{-1} soils, respectively.

The result was different with those reported by Devi and Chhetry (2012) that the antagonistic fungal population was higher in rhizosphere of pigeonpea than non-rhizosphere irrespective of healthy and diseased plant due to availability of nutrients released by the root exudates around the vicinity of root zone of pigeon pea. The fungi known for antagonistic activity such as *T. viride, A.niger, G. virens* was higher in the rhizosphere of healthy plants as compared to diseased and non- rhizosphere

Table 1. Logarithmic value of the population of fluorescent Pseudomonads, heat tolerant, and chitinolytic bacteria in diseased and healthy banana rhizosphere

Sample Code*	Population of bacteria (log cfu gram soil^{-1})							
	Fluorescent		Non-Fluorescent		Heat tolerant		Chitinolytic	
	Diseased plant	Healthy plant	Diseased plant	Healthy plant	Diseased plant	Healthy plant	Diseased plant	Healthy plant
C1	6.00	6.00	7.00	7.27	7.55	7.07	7.00	6.95
C2	6.30	6.30	6.00	6.69	6.84	7.46	6.47	7.47
C3	6.47	6.00	7.32	7.74	7.85	8.17	6.77	6.84
C4	6.47	6.30	7.23	7.14	8.17	7.86	6.84	7.00
C5	6.30	6.00	6.30	7.46	7.32	6.69	7.04	6.69
C6	6.77	6.60	7.36	7.63	7.36	7.00	6.47	7.07
C7	6.47	6.30	7.20	7.04	7.14	7.78	6.60	7.04
T1	6.84	6.60	7.51	7.57	6.77	7.38	6.69	6.95
T2	6.47	6.00	6.69	7.32	7.84	7.72	6.69	6.47
T3	6.47	6.00	6.00	6.60	7.56	6.47	6.00	7.07
T4	6.69	6.47	6.95	7.36	8.17	8.17	6.69	6.84
L1	6.77	6.77	7.72	8.22	8.17	8.21	6.69	6.90
L2	6.47	6.30	7.14	7.14	7.86	7.77	7.14	7.44
L3	6.00	6.30	7.32	7.23	7.43	8.11	6.69	7.00
Pk1	6.47	6.00	7.32	7.25	7.84	8.19	7.00	6.90
Pk2	6.84	6.60	7.04	7.14	6.77	6.30	7.32	7.47
R1	6.30	6.30	7.36	7.65	7.23	8.02	7.36	6.60
R2	6.30	6.30	7.04	6.47	7.86	7.99	7.00	6.47
Pe	6.30	6.30	6.90	7.17	7.57	7.44	6.90	7.23
Means**)	6.46 **b**	6.29 **a**	7.02 **A**	7.27 **A**	7.54 ***a***	7.56 ***a***	6.80 ***A***	6.96 ***A***

* C = Ciapus; T = Tenjolaya; L = Loa; Pk = Pasir Kuda; R = Jalan Raya Ciapus; Pe = Pasir Eurih

** Means followed by the same letter on the same row are not significantly different by Duncan test at α = 0.05

Antagonistic activity of the fluorescence Pseudomonads, heat tolerant, and chitinolytic bacteria against *F. oxysporum* f.sp. *cubense*

Based on some characters of the rhizosphere bacteria, there were 12 isolate selected to be tested for their antibiosis activity to *F. oxysporum* f.sp. *cubense*. The characters to be compared were: colony morphology, fast growing, easy to re-culture, produced fluorescent pigment or chitinolytic zone strongly for fluorescence Pseudomonads and chitinolytic bacteria. From each group of bacteria there were four assorted isolates. Some effect of the isolates on the diameter colonies of *F. oxysporum* f.sp. *cubense* and percentage suppression are presented in Table 2 and Table 3.

The colony diameter of *F. oxysporum* f.sp. *cubense* on the agar plates inoculated with the bacteria at 5, 6, and 7 days after treatment were significantly lower compared with those in control. The effect was inconsistent at 3 and 4 days after treatment. The suppression was consistently showed by the isolate CH4 from 3 to 7 days after treatment. The lowest diameter colony (Table 2), which reflected the highest suppression (Table 3), was demonstrated by isolate CH4. Suppression was up to 42.47% at 6 days after treatment. At 3 and 4 days after treatment, some of the bacteria, i.e. TSA1, TSA4, KB1, KB4, CH1, and CH3, showed the growth-promoting activities to the colony of *F. oxysporum* f.sp. *cubense* but at 5, 6, and 7 days, their effect changed to suppression of the growth of *F. oxysporum* f.sp. *cubense*.

The rhizobacteria not only suppressed the mycelia growth of the pathogen, but these also caused some changes in the morphological characteristics of the colony of *F. oxysporum* f.sp. *cubense*. One of the heat tolerant bacteria, i.e. isolate TSA2, showed the smallest diameter growth at

7 DAI, however, it has no significant difference from that of TSA3. The fluorescence Pseudomonads isolate KB3 caused a change in the colony color of *F. oxysporum* f.sp. *cubense* that became plain white, while controls were pink in color in the center of the colony.

Table 2. Some effect of fluorescence Pseudomonads, heat tolerant, and chitinolytic bacteria on the diameter colony of *F. oxysporum* f.sp. *cubense* in antibiosis test *in vitro*

Isolate Code[1]	**Diameter colony of fungi (cm)**				
	3 DAI[2]	**4 DAI**	**5 DAI**	**6 DAI**	**7 DAI**
Control	1.90 ab[3)]	2.16 ab	3.36 a	3.76 a	4.00 a
TSA1	2.10 ab	2.50 a	2.93 b	3.13 b	3.33 b
TSA2	1.80 ab	2.13 ab	2.40 d	2.56 cde	2.86 bc
TSA3	1.76 ab	2.10 ab	2.46 cd	3.00 bc	3.16 bc
TSA4	2.06 ab	2.50 a	2.86 bc	3.10 b	3.23 b
KB1	2.23 a	2.46 a	2.66 bcd	2.86 bcd	3.00 bc
KB2	1.66 ab	2.06 ab	2.33 de	2.43 de	2.70 bc
KB3	1.73 ab	2.10 ab	2.43 cd	2.60 cde	2.86 bc
KB4	1.66 ab	2.30 ab	2.63 bcd	2.76 bcd	2.93 bc
CH1	1.90 ab	2.30 ab	2.50 cd	2.73 bcd	2.96 bc
CH2	1.73 ab	2.03 ab	2.23 de	2.53 cde	2.80 bc
CH3	1.93 ab	2.30 ab	2.50 cd	2.66 bcd	2.86 bc
CH4	1.53 b	1.76 b	1.96 e	2.16 e	2.53 c

[1] TSA = heat tolerant bacteria, KB = fluorescent Pseudomonads, CH = chitinolytic bacteria
[2] DAI = Days after Inoculation
[3)] Means followed by the same letter on the same column are not significantly different by Duncan test at α = 0.05

Table 3. Percentage suppression caused by fluorescence Pseudomonads, heat tolerant, and chitinolytic bacteria on the diameter colony of *F. oxysporum* f.sp. *cubense* relatively compared with control .

Isolate Code[1]	**Percentage of Suppression (%)**				
	3 DAI[2)]	**4 DAI**	**5 DAI**	**6 DAI**	**7 DAI**
TSA1	0 abc	0 b	12.9 e	16.8 e	16.7 b
TSA2	5.3 abc	1.5 ab	28.7 bc	31.9 abc	28.3 ab
TSA3	7.0 abc	3.1 ab	26.7 bcd	20.4 cde	20.8 ab
TSA4	0 abc	0 b	14.9 de	17.7 de	19.2 b
KB1	0 abc	0 b	20.8 cde	23.9 bcde	25.0 ab
KB2	12.3 ab	4.6 ab	30.7 abc	35.4 ab	32.5 ab
KB3	8.8 abc	3.1 ab	27.7 bc	31.0 abc	28.3 ab
KB4	12.3 ab	0 b	21.8 bcde	26.5 bcde	26.7 ab
CH1	0 abc	0 b	25.7 bcd	27.4 bcde	25.8 ab
CH2	8.8 abc	6.2 ab	33.7 ab	32.7 ab	30.0 ab
CH3	0 abc	0 b	25.7 bcd	29.2 bcd	28.3 ab
CH4	19.3 a	18.5 a	41.6 a	42.5 a	36.7 a

[1] TSA = heat tolerant bacteria, KB = fluorescence Pseudomonads, CH = chitinolytic bacteria
[2] DAI = Days after Inoculation
[3] Means followed by the same letter on the same column not significantly different by Duncan test at α = 0.05

Four isolates of the bacteria, all are fluorescence Pseudomonads, produced inhibition zone between colony of the bacteria and colony of the fungi. The width of inhibition zone produced by each isolate of bacteria against *F. oxysporum* f.sp. *cubense* were presented in Table 4. The widest inhibition zone was produced by the isolate KB2. Production of the inhibition zone is the indicator of antibiosis mechanism by the rhizosphere bacteria against the fungi. Mohammed et al. (2011) reported that *Pseudomonas fluorescence* showing high antifungal activity against FOC in vitro (Dual Culture).

In this experiment, the percentage suppression caused by the bacteria to the growth of the fungus colony was relatively high that was up to 42.47% caused by the chitinolityc bacteria isolate CH4. This isolate has potential as biocontrol agent of *F. oxysporum* f.sp. *cubense* and the activities could be improved by another effort, such as making formulation of this bacteria by amended with some nutrition to encourage their activities and their survival during colonization of the banana rhizosphere. Research to observe the ability of the bacteria to suppress the pathogen in planta or in the green house should be conducted to get the best candidate as biocontrol agent.

The chitinolytic bacteria isolate CH4 caused the highest suppression to the mycelial growth of *F. oxysporum* f.sp. *cubense* even thought they did not produced inhibition zone. The possible mechanisms of suppression against the fungus are production of volatile compound or the production of chitinolytic enzymes. Both of the possibilities should be proofed, such as by analyzing of the existence of volatile compound. This isolate positively produced chitinolytic enzyme that dissolved the cell wall of fungi. This bacteria was isolated from the banana rhizosphere using a medium containing colloidal chitin; chitinolytic activity was shown by production of clear zone around the colony of this bacteria, thus, it was grouped into chitinolytic bacteria. It was shown by the limited growth of the fungus on the agar plate inoculated with this isolate compared with the growth of the fungus in control treatment which demonstrated thick mycelial growth.

Table 4. Width of the inhibition zone produced by fluorescence Pseudomonads against *F. oxysporum* f.sp. *cubense in vitro*

Isolate code	Width of inhibition zone (mm)	
	6 DAI[1]	7 DAI
KB1	3.6 c[2)]	4.0 c
KB2	8.3 a	10.0 a
KB3	4.6 cd	5.3 cd
KB4	8.0 ab	9.0 ab

1) DAI = Days after Inoculation

2) Means followed by the same letter on the same column not significantly different by Duncan test at $\alpha = 0.05$

Identification and characterization of the rhizosphere bacteria

Three isolates of the rhizobacteria with different criteria were selected for identification based on the 16S rDNA sequence. The chitinolytic bacteria isolate CH4 was selected based on its highest inhibition activity to the diameter colony of the pathogen, the fluorescence Pseudomonads isolate KB2 was selected because it produced the widest inhibition zone against the pathogen, and the heat tolerant bacteria isolate TSA3 was selected in correlation with their effect on the tiny mycelial growth on the colony of pathogen beside suppression on the diameter colony.

Based on the alignment of the 16S rDNA sequences to the Gene Bank, the chitinolytic bacteria isolate CH4 has 99% similarity with *Bacillus pumilus*, and the heat tolerant bacteria isolate TSA3 has 99% similarity with *Bacillus subtilis*.

The fluorescent Pseudomonad isolate KB2 was not yet molecularly identified. Based on the Gram test, the fluorescent Pseudomonad isolate KB2 was Gram negative while the chitinolytic bacteria isolate CH4 and the heat tolerant bacteria isolate TSA3 were Gram positive. The maximum scores and E values of the sequences producing alignment using BLAST Program is presented in Table 5, and the position of both isolates in phyllogenetic tree is presented in Figure 2.

Table 5. Maximum scores, E values, and percentage of similarities of chitinolytic and heat tolerant bacteria

Isolate	Species homolog	Identity	Max Score	Query Cover	E value	Accession
CH4	*Bacillus pumilus* strain MB7 NIOT 16S ribosomal RNA gene, partial sequence	95%	1188	99%	0.0	HQ858063.1
TSA3	*B. subtilis* strain EGY-SCS3 16S ribosomal RNA gene, partial sequence	94%	1186	99%	0.0	KC573510.1

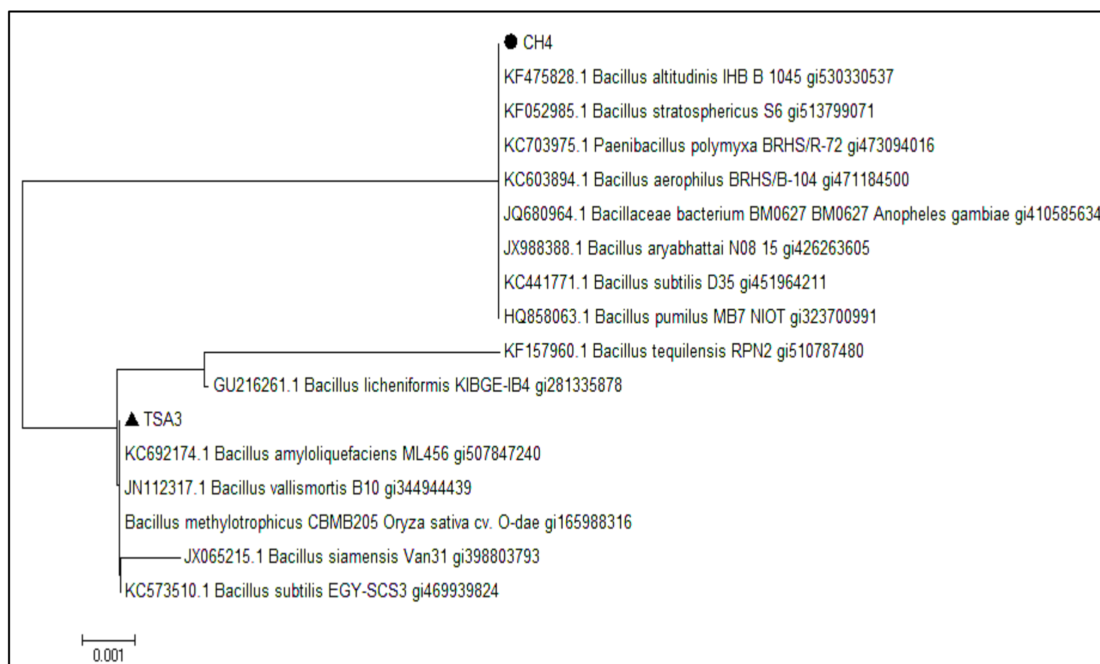

Fig. 2. The phyllogenetic tree based on the sequence of 16S rDNA of the isolates CH4 (*Bacillus pumilus* strain MB7 NIOT gi323700991) and TSA3 (*B. subtilis* strain EGY-SCS3 gi469939824).

Bacillus spp. is a group of bacteria that are commonly found in the plant rhizosphere and many members of this group have already been used as biocontrol agents against plant pathogens. Besides being a hydrolytic enzyme, *B. subtilis* also produced siderophore which is named as bacillobactin (Crosa and Walsh 2002). Kremer (2006) based on previous publications reported that there were some rhizosphere bacteria successfully isolated, i.e. *Pseudomonas*, *Burkholderia*,

Aeromonas, *Agrobacterium*, *Alcaligenes*, *Acenitobacter*, *Azospirilum*, *Bacillus*, *Chryseomonas*, *Klebsiella*, *Enterobacter*, *Flavobacterium*, *Stenitrophomonas*, *Xanthomonas*, *Lysobacter*, *Pantoea*, and *Serratia*. Podile and Kishore (2006) stated one of the important communities of microbe live in rhizosphere which give beneficial effects to the plants. These were first defined by Joseph Kloepper and Milton Schroth in 1978 and named as "Plant Growth-Promoting Rhizobacteria (PGPR).

Suppressive soil is well known to control soil-borne diseases. The soil suppressive to fusarium wilt is the best known. The suppressive soil limited the incidence or severity of wilt in banana, carnation, cucumber, cotton, flax, muskmelon and tomato (Alabouvette 1990). One of the components which have a role in suppressive soil are microorganisms that live in the rhizosphere and in the soil or microflora. In this experiment it was shown that in the healthy plants, the total population of some bacteria was higher than those in the diseased plants. The rhizosphere bacteria might play some role in the suppression of fusarium wilt. It was proven by the suppression activity of some isolates to the *F. oxysporum* f.sp. *cubense* in vitro. Saravanan et al. (2004) reported that all strains of *Pseudomonas fluorescens* isolated from banana rhizosphere had significant inhibitory action on the growth of *F. oxysporum* f.sp. *cubense*. Among the strains, Pfm of *P. fluorescens* had higher inhibitory action compared with other strains.

In diseased plants, the lower population of some saprophytic bacteria might cause lower competitiveness against the pathogen. One of the successful factors of biological control agents in disease control is the ability to occupy the root surface or the competitiveness in nutrition uptake. Higher population of the saprophytic bacteria gives competitive advantage against *F. oxysporum* f.sp. *cubense*. Some of the selected bacteria produced an antifungal compound that was shown by the production of inhibition zone. Munimbazi and Bullerman (1998) reported *Bacillus pumilus* produced extracellular antifungal metabolites which inhibited mycelial growth of many species of *Aspergillus*, *Penicillium* and *Fusarium*. The effect of antifungal metabolites produced by bacilli on growth of *Aspergillus*, *Penicillium* and *Fusarium* species has not been investigated extensively. The compound(s) are heat-stable and active over a wide range of pH values. Chitarra et al. (2003) reported the antifungal metabolite produced by *B. subtilis* YM 10-20 was highly similar to iturine A. A higher population of this group of bacteria might produce a higher concentration of the antifungal compound. The populations' ratio of saprophytic bacteria compared with inocula of pathogen, *F. oxysporum* f.sp. *cubense*, in the rhizosphere of suppressive soil is an interesting topic to be observed, as well as the endophytic bacteria. Cao et al. (2005) reported that 18.3% of 131 isolates of endophytic actinomycetes from banana roots inhibited the growth of pathogenic *F. oxysporum* f.sp. *cubense*.

CONCLUSIONS

The population of heat tolerant and chitinolytic bacteria in the healthy banana rhizosphere were significantly higher compared with those in diseased plants while the population of fluorescent Pseudomonads was not significantly different. Among 12 isolates selected for the antibiosis test, four isolates, all belonged to the fluorescent Pseudomonads, produced inhibition zone. The highest inhibition of mycelial growth of *F. oxysporum* f.sp. *cubense* race 4 was demonstrated by the chitinolytic bacteria isolate CH4. Based on the sequence of 16S rDNA, isolate CH4 was identified as *Bacillus pumilus* (Accession Number HQ858063.1), while isolate TSA3 was identified as *B. subtilis* (Accession Number KC573510.1). Both of the isolates could be tested for their effectiveness to control the fusarium wilt disease of banana in planta and in the field as biocontrol agents.

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