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Isolation and screening of Antagonistic Bacteria to *Colletotrichum musae*

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Abstract: *The present experiment was conducted at the microbiology unit of post-harvest laboratory, department of horticulture, PSTU, during January to December, 2017 to identify the Antagonistic bacteria against Colletotrichum musae. To identify Antagonistic Bacteria different local verities viz ''Kathali", "Anaji", "Sobri", "Bitchi" was used. Total fifty five (55) bacteria were isolated from banana fruits in the Applied Microbiology unit of Postharvest Laboratory, Department of Horticulture, PSTU. Of the 55 bacterial isolates, 18 were inhibitory towards C. musae on PDA. Out of these, three isolates namely KA-5, KA-6 and SBA-15 had significantly higher inhibitory effect towards C. musae than the others and strongly inhibited the mycelial growth on PDA medium. These three bacterial species were then molecularly identified as Bacillus cereus strain PSTU HORT-10, Stenotrophomonas maltophilia and Bacillus cereus strain PSTU HORT-11. The resulting phylogenetic tree indicated that KA-5 and Bacillus cereus strain PPB6 shared the same cluster. So, based on the 16S rDNA gene sequence and phylogenetic tree KA-5 strain could be identified as Bacillus cereus strain PSTU HORT-10. As having highest PIRG (%) in dual culture test, Bacterium species Bacillus cereus strain PPB6 was selected for postharvest application.*

Keywords: *Colletotrichum musae, Antagonistic bacteria, Isolation, Screening*

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I. Introduction

Colletotrichum musae, plant pathogen, affecting banana Fruits; infections by this pathogen causes severe post-harvest losses (Abd-Elsalam *et al*., 2010). This pathogen was control by diffenent chemical treatment. Presently, Fungicidal treatment is considered to be the most effective method, but it often gives rise to resistant pathogens (Reimann and Deising, 2000) and also associated with environmental health risks (Janisiewicz and Korsten, 2002; Conway et al., 2004). Besides, non-chemically treated bananas are more appealing to the consumers. Therefore, all of these issues impose a necessity to develop new sustainable alternatives to synthetic fungicides for eco-friendly management of postharvest diseases (Droby et al. 2009).

During the last few decades, there have been increased efforts to develop a number of alternatives to chemical fungicides (Smilanick et al. 1999; Droby et al. 2009). Meanwhile, biological control using microbial antagonistic has emerged as a desirable and rapidly developing alternatives due to increasing global chemo-phobia and environmental social awareness (Emmert and Handelsman, 1999). Among the bio-control agents, antagonistic bacteria are attracting research focus, with their wide antimicrobial spectrum, good antagonistic effects, genetic stability, low nutrition requirements, and high security (Zhou et al., 2007).Currently, several promising biological control approaches that include antagonistic microorganisms, compounds of natural origin and induced resistance have been proposed as potential alternatives to synthetic fungicides for postharvest disease control, among the proposed alternatives, development of antagonistic microorganisms has been the most studied one. The substantial progress has been made in this area (EI-Ghaouth *et al.* (2002). Spotts and Sanderson (1994) mentioned that biological control is a possible alternative to fungicides in a postharvest environment, where temperature and relative humidity are controlled. Unfortunately, there was little information concerning bio-control of postharvest diseases of fruit in Bangladesh. As far as knowledge goes, controlling of anthracnose using biocontrol agent is nearly absent in our country. Therefore, this research has been taken to isolation and selection of bacteria antagonistic to ***Colletotrichum musae*** and dentification of promising antagonistic bacteria through molecular method.

II. Materials And Methods

The present experiment was conducted at the microbiology unit of post-harvest laboratory, department of horticulture, PSTU, during January to December, 2017 to study the antagonistic activity.

Methods

The following methods were used for the present investigation, each for specific purposes. Those were:

1) **Isolation of pathogen:** Agar plate method in Potato Dextrose Agar (PDA).
2) **Isolation of bacteria:** Grow in Nutrient Medium (NA).
3) **Selection of best antagonistic bacteria against pathogen:** Dual culture method in according to Percent Inhibition Radial Growth (PIGR) determination.

Experimental materials

The materials used for the experiment were the freshly harvested banana fruits of cv. Amrito sagor. The fruits were purchased from local grower of Jailsha, Dumki, Patuakhali. The fruits at the mature green stage with uniform size, and free of any visible defects, disease symptoms, and insect infestations were harvested and transported to the postharvest processing and analysis unit of Postharvest laboratory of the Department of Horticulture, PSTU, with careful handling to avoid damage and injury.

Source of bacteria

Patuakhali is rich in biodiversity of banana.To collect the antagonistic bacteria, organic plants (local varieties of banana plants) were selected in different place of dumki, patuakhali. Among the local varieties kathali, Anaji, Sobri, and bitchi were choose as source of antagonistic bacteria. The fruits of these varieties were collected from Germplasm center(GPC), Department of horticulture (PSTU), Dhumki, Jalisha and patabunia. Fruits are immediately sealed by sterilized polythene bag and transfer in microbiology unit post-harvest laboratory to prevent contamination during transportation.

Isolation of bacterial epiphytes

Bacterial epiphytes were isolated from the surface of green banana fruit in the post-harvest laboratory. Surface tissues of fruit skins were taken randomly using a sterile scalpel. The tissues pieces were washed in running tape water for 5 minutes and blotted dry on sterilized filter paper. The tissues of fruit surfaces were cut (5 mm^2 size) from the healthy area. 15 grams of sliced tissues were soaked in 100 ml of sterilized water and rotary shake at 100 rpm for 24 hour at room temperature. Fold serial dilutions (10^{-6}) suspensions were made in test tubes. Then 100 microliter suspensions from each tube were spread on nutrient agar (NA). Differentsize and shapes of bacterial colonies were formed and then transfer into Petri dishescontaining fresh NA. Bacteria were purified on NA plates by making streaks in four right angle directions, flaming the loop after each directional streak. After 24h incubation at 25ºC, a single colony well separated from other colonies was re-streaked on a new NA plates to be sure the bacterium was pure. A single colony was then isolated on a NA plate and stored in a low temperature freezer.

Isolation of Colletotrichum musae

Infected banana fruits showing symptoms of anthracnose disease were collected from Dumki sadar and brought in microbiology unit of post-harvest laboratory, PSTU. Fruits were kept in sterilized polythene bag for 25 hours to maintaining high humidity and observed the anthracnose disease symptoms clearly. Infected tissues were taken using cork borer from middle and end portions of fruits. Infected tissues were sterilized with 0.1% ethanol solution for 30 seconds and plated on center of PDA (Potato Dextrose Agar). The plates were then incubated at room temperature in order to encourage fungal growth.

Sub-culture of C. musae

Mycelia disk (5mm^2) were taken by using cork-borer from the margin of growing portions of *C. musae*. The inoculated plates were then incubated at 26±2ºC for 7 days. The 7 days old plates were stored at 4ºC for further use.

Identification of C. musae

C. musae, the causal organism of anthracnose disease of banana was identified on the basis of morphological characteristics such as the shape, color, and size of conidia produce on PDA plates.

In vitro screening of antagonistic bacteria

Bacteria were obtained by the dilution method from fruit surfaces of banana. These isolates were evaluated for in vitro antagonism toward *C. musae* by dual incubation method (Fokkema, 1978). Isolates with inhibitory characteristics against the test fungus were selected for further screening by means of dual and concomitant test.

Dual culture activity in vitro

Mycelial plugs, 6 mm in diameter, were taken from the edge of 7-day-old fungal colonies maintained on PDA plates and placed on the center of a 9 cm diameter petri dish containing PDA. A loop full bacterial isolates from 24 hours culture was then streaked on PDA 1.5 cm from the edge of plate. Plates inoculated only with the pure pathogens served as control. Plates were then incubated at room temperature for seven days. The experiment was repeated twice with three replications of each treatment. After the incubation period, percent inhibition of radial growth (PIRG) was recorded based on following formula (Sariah, 1994):

$$PIGR(\%) = \frac{\text{R1}-\text{R2}}{R1} \times 100$$

(R_1 = Radial growth of *C. musae* in control plate, R_2 = Radial growth of *C. musae* interacting with antagonistic bacteria).

The microbial isolates with the highest percentage inhibition were selected as candidates for the in vivo study.

Preparation of aqueous antagonistic suspension

Isolates of bacteria were selected based on antagonistic activity. In preparing aqueous antagonistic suspension, isolates were grown on NA at 28±2ºCfor 24h. A loop of each culture was then transferred to a 250 ml conical flask containing 50 ml of nutrient broth (NB, Difco, MI) and incubated on a rotary shaker at 150 rpm for 72h at 28±2ºC. The isolates were re-cultured in fresh nutrient broth for another 72h before used. The bacterial suspension was applied on the banana fruits to observed Physio-chemical character, shelf life and post-harvest diseases.

Identification of antagonistic bacteria

Conventional method

The antagonistic bacteria was conventionally identified with the help of members of plant pathology department, PSTU considering morphological characteristics viz. size, form, margin, elevation, texture, opacity, color, gram staining, and motility.

Molecular method

a) Genomic DNA extraction

1 ml aliquots of bacterial cells from well-grown overnight cultures in LB medium (e.g. OD = 1.2 - 1.4) at a salt concentration of 0.5 M NaCI and pH 8 were transferred into 1.5 ml Eppendorf tubes and then harvested by centrifugation in a bench top centrifuge at 8,000 rpm for 4 minutes at room temperature.

The supernatant was poured off immediately and then each pellet was suspended (washed) two times in 1 ml sterile distilled water (this step was carried out to remove all residual salt as it may affect the quality of DNA). Chloroform isoamyl alcohol was added to fill up the tube. It was capped tightly and mixed by shaking for 2-3 minutes. It was centrifuged at a maximum speed of 8000 rpm for 4 minutes. A new 1.5ml tube containing 600 µl of isopropanol. The aqueous portion (upper portion) was transferred to the tube containing using a micropipette. The tube was then gently inverted for few times. It was then again centrifuged and the solution was decanted, and it was hanged on a test tube rack to keep the tube upside down. An aqueous drop was wiped out. 800 µl of 75% ethanol with 10 MM ammonium acetate was added. The tube was then gently inverted for several times, the bottom of the tube was tapped with a finger to detach DNA pellet from the tube surface into solution. The tube was let for 30 minutes to sit. It was again centrifuged briefly, and the solution was decanted (care was taken not to throw away DNA pellet), and tube was hanged on a test tube rack to keep the tube upside down. Then, an aqueous drop was wiped out, and 800 µl of 75% ethanol was added. It was then gently inverted. The tube was again centrifuged for 4 minutes. The solution was decanted and hung on a test tube rack until DNA is dried. 100 µl of TE buffer and 2 µl of RNase was added to resuspend DNA pellet and to digest RNA. The tube was left until DNA was completely dissolved (tapping the tube after a few hours is helpful). The DNA samples was stored at 4ºC.

b) Polymerase chain reaction (PCR)

Amplification of 16S rRNA gene following extraction of genomic DNA, polymerase chain reaction (PCR) was carried out in order to amplify the 165 rRNA gene. The reaction was performed in a final volume of 50 µl using universal primers for Bacteria.

Primer information

Sequencing primer Name Primer Sequences

785F 5' (GGA TTA GAT ACC CTG GTA) 3'

907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'

PCR primer name Primer sequences

27F 5' (AGA GTT TGA TCM TGG CTC AG) 3

1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

The amplification (PCR) mixture contained the following reagents in a 0.2 ml thin walled PCR tube: 39 µl of sterile double distilled (deionized) water, 5 µl 10X PCR Buffer, 2.5 µl 50 mM MgCI2, 0.5 µl Forward Primer, 0.5 µl Reserve Primer, 1 µl (25 mM dNfPs), 1 µl genomic DNA, 0.5 µl Taq DNA polymerase.

Amplifications were carried out in an automated thermo cycler device using the following parameters: 30 cycles of denaturation at 94ºC for 30s, extension at 58^0C for 1 minute and 72ºCfor 1 minute and one cycle of final extension at 72^0C for 7 minutes with annealing temperatures 60^0C for 16S rDNA. The PCR amplified products were then sent to the Macrogen Company, Seoul, South Korea for molecular identification.

III. Results And Discussions

Isolation of bacteria

Total fifty five (55) bacteria were isolated from banana fruits in the Applied Microbiology unit of Postharvest Laboratory, Department of Horticulture, PSTU. Among those, 27 were creamy white and shiny, 10 dry and creamy white, and 17 were white. Pure culture of each isolate was maintained for screening test against *C. musae*.

Screening of antagonistic bacteria

The isolates with inhibitory characteristics were selected and screened by means of dual culture tests. In dual culture test of 55 bacterial isolates, 18 were inhibitory towards *C.musae* on PDA. Out of these, three isolates namely KA-5, KA-6 and SBA-15 had significantly higher inhibitory effect towards *C. musae*than the others and strongly inhibited the mycelial growth on PDA medium. The PIRG of isolates KA-5, KA-6 and SBA-15 were 73.3%, 70.01% and 69.25% (Table 1), respectively with respect to the control after 7 days of incubation. Moreover, no mycelial growth was observed after 4 days of incubation, when PDA plugs from the interaction zone were re-cultured on fresh PDA plates.

Table 1: Screening the antagonistic bacteria against ***Colletotrichum musae*** in Banana

Isolate No.	Antagonism (PIRG %)
KA-5	73.30 a
KA-6	70.01 b
SBA-15	69.25 c
SBA-17	54.67 f
KA-1	53.56 g
SA-42	48.37 i
SBA-7	45.50 l
SBA-11	43.07 m
KC-44	56.09 d
SBB-43	49.89 h
SA-40	34.47 n
SA-26	47.43 j
Anaji-45	45.87 k
SBA-2	55.47 e

Co-efficient of variation = 0.156, Critical difference (0.05) = 0.178

* Means in a column followed by different letter (s) are significantly different by Duncan Multiple Range Test (DMRT)

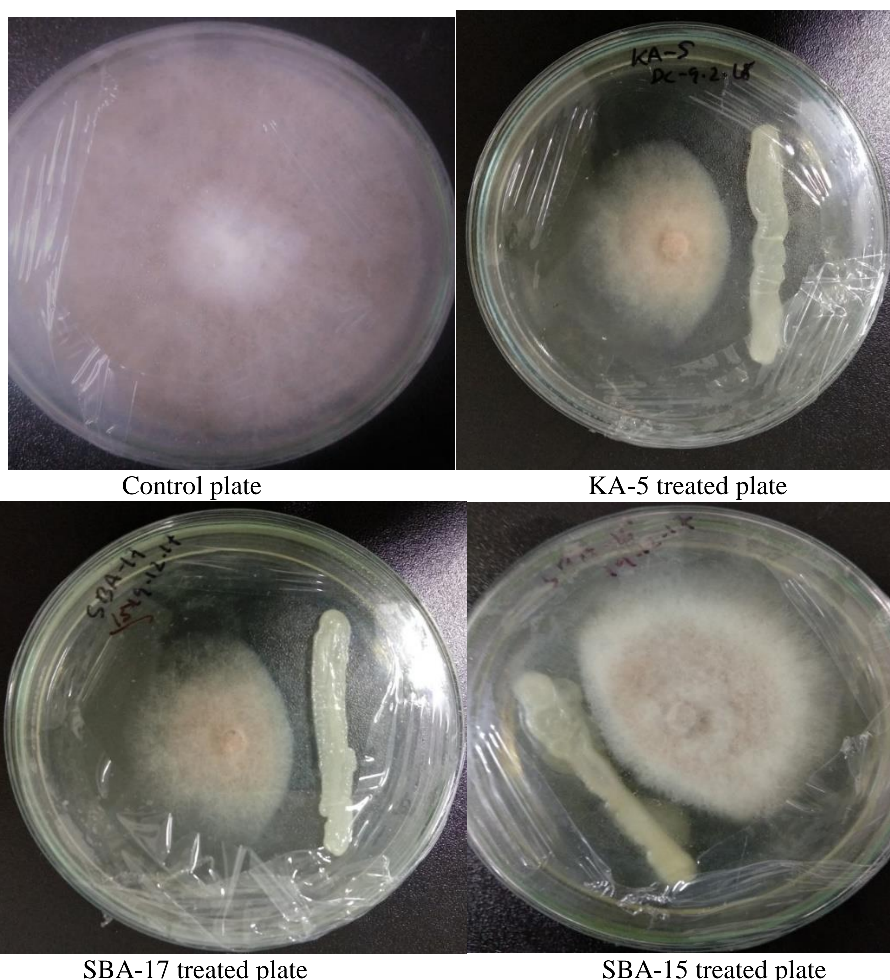

Plate1: Dual culture assay on the inhibition of mycelial growth of C. musae by three isolates (KA-5, SBA-17 and SBA-15) of bacteria after 7 days incubation at 26±2ºC

Identification of antagonistic Bacteria

The identification of antagonistic bacterium was the most important step for the application as bio-control agent. The antagonistic bacterium (KA-5) was initially identified considering morphological characteristics that it might be Bacillus genus.

The results obtained from sequence (Plate 2) comparison with the National Centre for Biotechnology Information (NCBI) data base showed the strain sequence similarity 98% to the published *Bacillus cereus* strain PSTU HORT-10. The resulting phylogenetic tree (Plate.3) indicated that KA-5 and Bacillus cereus strain PPB6 shared the same cluster. So, based on the 16S rDNA gene sequence and phylogenetic tree KA-5 strain could be identified as Bacillus cereus strain PSTU HORT-10.

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1      TTTGATCCTG CTCAGGATGA ACGCTGGCGG CGTGCCTAAT ACATGCAAGT CGAGCGAATG
61     GATTAAGAGC TTGCTCTTAT GAAGTTAGCG GCGGACGGGT GAGTAACACG TGGGTAACCT
121    GCCCATAAGA CTGGGATAAC TCCGGGAAAC CGGGGCTAAT ACCGGATAAC ATTTTGAACC
181    GCATGGTTCG AAATTGAAAG GCGGCTTCGG CTGTCACTTA TGGATGGACC CGCGTCGCAT
241    TAGCTAGTTG GTGAGGTAAC GGCTCACCAA GGCAACGATG CGTAGCCGAC CTGAGAGGGT
301    GATCGGCCAC ACTGGGACTG AGACACGGCC CAGACTCCTA CGGGAGGCAG CAGTAGGGAA
361    TCTTCCGCAA TGGACGAAAG TCTGACGGAG CAACGCCGCG TGAGTGATGA AGGCTTTCGG
421    GTCGTAAAAC TCTGTTGTTA GGGAAGAACA AGTGCTAGTT GAATAAGCTG GCACCTTGAC
481    GGTACCTAAC CAGAAAGCCA CGGCTAACTA CGTGCCAGCA GCCGCGGTAA TACGTAGGTG
541    GCAAGCGTTA TCCGGAATTA TTGGGCGTAA AGCGCGCGCA GGTGGTTTCT TAAGTCTGAT
601    GTGAAAGCCC ACGGCTCAAC CGTGGAGGGT CATTGGAAAC TGGGAGACTT GAGTGCAGAA
661    GAGGAAAGTG GAATTCCATG TGTAGCGGTG AAATGCGTAG AGATATGGAG GAACACCAGT
721    GGCGAAGGCG ACTTTCTGGT CTGTAACTGA CACTGAGGCG CGAAAGCGTG GGGAGCAAAC
781    AGGATTAGAT ACCCTGGTAG TCCACGCCGT AAACGATGAG TGCTAAGTGT TAGAGGGTTT
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841     CCGCCCTTTA GTGCTGAAGT TAACGCATTA AGCACTCCGC CTGGGGAGTA CGGCCGCAAG
901     GCTGAAACTC AAAGGAATTG ACGGGGGCCC GCACAAGCGG TGGAGCATGT GGTTTAATTC
961     GAAGCAACGC GAAGAACCTT ACCAGGTCTT GACATCCTCT GAAAACCCTA GAGATAGGGC
1021    TTCTCCTTCG GGAGCAGAGT GACAGGTGGT GCATGGTTGT CGTCAGCTCG TGTCGTGAGA
1081    TGTTGGGTTA AGTCCCGCAA CGAGCGCAAC CCTTGATCTT AGTTGCCATC ATTAAGTTGG
1141    GCACTCTAAG GTGACTGCCG GTGACAAACC GGAGGAAGGT GGGGATGACG TCAAATCATC
1201    ATGCCCCTTA TGACCTGGGC TACACACGTG CTACAATGGA CGGTACAAAG AGCTGCAAGA
1261    CCGCGAGGTG GAGCTAATCT CATAAAACCG TTCTCAGTTC GGATTGTAGG CTGCAACTCG
1321    CCTACATGAA GCTGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG AATACGTTCC
1381    CGGGCCTTGT ACACACCGCC CGTCACACCA CGAGAGTTTG TAACACCCGA AGTCGGTGGG
1441    GTAACCTTTT TGGAGCCAGC CGCCTAAGGT GGGACAGATG ATTGGGGTGA AGTCGTAACA
1501    AGGTAGCCGT ATCGGAAGGT GCGGCTGGAT CACCTCCTTT T
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Plate 2: Partial sequence of 16S ribosomal RNA gene of strain KA-5

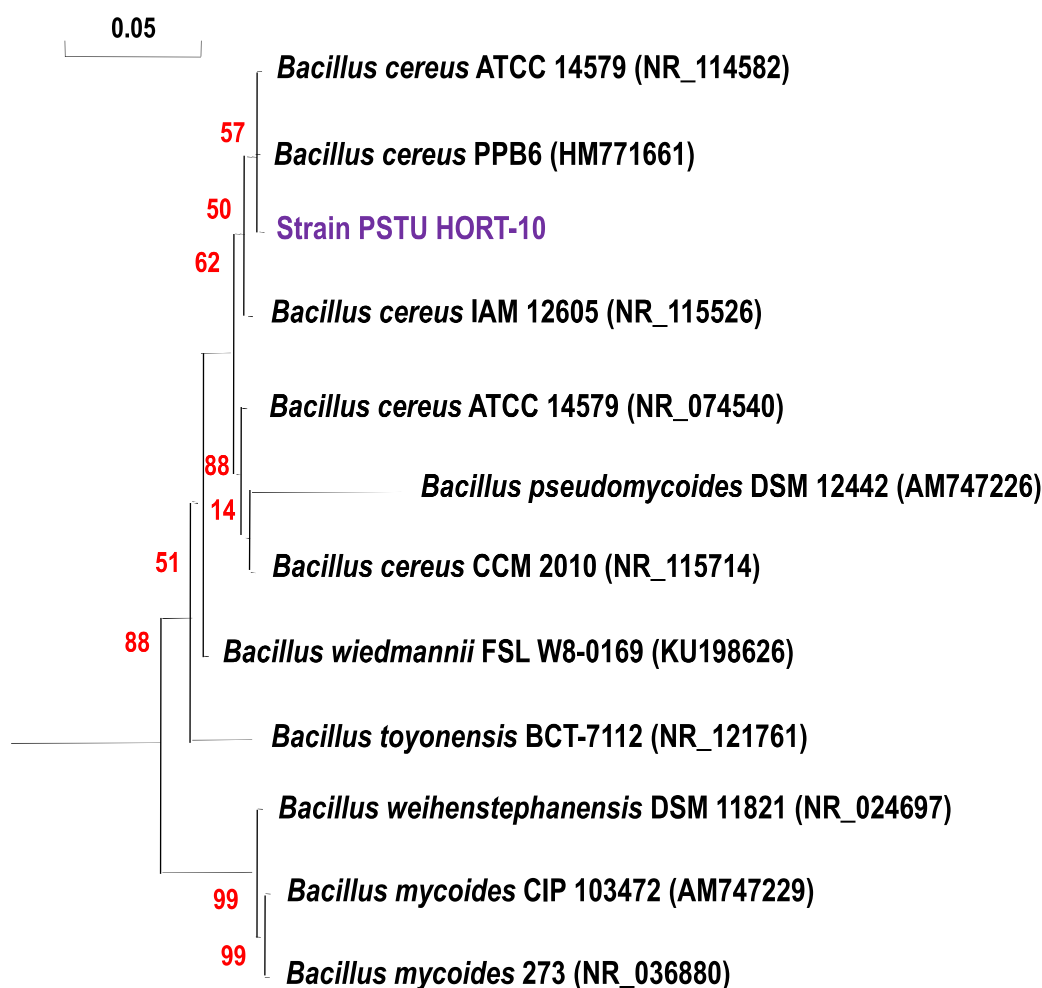

Plate 3: Phylogenetic tree based on of 16S rDNA sequences of Strain KA-5 and reference sequences from the "National Center for Biotechnology Institute" within the bracket. Numbers above each node indicate percentage of confidence levels generated from 1,000 bootstrap trees. The scale bar is in fixed nucleotide substitutions per séquence position.

IV. Conclusions

Bacteria were isolated from local banana fruit skin. These isolated bacteria were tested as bio-control agent against C. musae.Total fifty five (55) bacteria were isolated from banana fruits in the Applied Microbiology unit of Postharvest Laboratory, Department of Horticulture, PSTU. Of the 55 bacterial isolates, 18 were inhibitory towards *C. musae* on PDA. Out of these, three isolates namely KA-5, KA-6 and SBA-15 had significantly higher inhibitory effect towards *C. musae* than the others and strongly inhibited the mycelial

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