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SELECTION AND IDENTIFICATION OF BACTERIA FROM TONGKAT LANGIT BANANA (*Musa troglodytarum* L.) TO CONTROL THE BLOOD DISEASE BACTERIA

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ABSTRACT

'Tongkat Langit' Banana (*Musa troglodytarum* L.) is a type of banana plant which is only found in the Moluccas and Papua Indonesia. Beneficial bacteria, such as endophytic or rhizosphere bacteria isolated from plants, can be utilized to promote plant health and to control plant pathogens. The research was conducted from November 2012 to October 2013 in the Laboratory of Plant Bacteriology, Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University, to select the candidates of biocontrol bacteria from *M. troglodytarum* to overcome blood disease bacteria (BDB) in banana. There were 252 bacterial isolates consisting of 102 isolates of endophytic bacteria and 150 isolates of rhizosphere bacteria that were successfully isolated. These were screened against BDB *in vitro* and evaluated for their effects on plant growth. The banana plants infested with the bacteria, after inoculation with BDB, were observed for the disease incidence. Four isolates of rhizosphere bacteria were able to stimulate the growth of the banana plants. These isolates were RTI 2, RTI 3, and RTI 4 with index suppression up to 80 %. The most prospective isolates are EAI 26 (root endophytic bacteria) and RTI 4 (rhizophere bacteria). Thoee isolates were identified as *Bacillus* sp. and *Bacillus subtilis*, respectively. Both of the isolates will be tested further as biocontrol agents to suppress BDB in the field.

Key words: *Bacillus subtilis,* biological control, endophytic bacteria, Plant Growth-Promoting Rhizobacteria

INTRODUCTION

'Tongkat Langit' or 'Fe'i' banana (*Musa troglodytarum* L.) is a banana species in Indonesia that only be found in eastern Indonesia, such as Maluku and Papua (Ploetz et al. 2007). This plant has fruit bunches that grew straight upwards, no banana flowering spike, with pseudostem height of more than 82 cm with 3 cm in diameter, pigmentation for stem and pseudostem is green and the number of saplings are more than six (Sutanto and Edison 2005). As a local and endemic plant, this banana is a germplasm source, thus its potential needs to be developed. One of advantage potential is the beneficial bacteria that is associated with the banana plant. Endophytic and rhizosphere bacteria can be used as biological agents to enhance the growth and resistance of plant against diseases.

Endophytic bacteria grow in the intercellular space of plants without giving negative effect to their hosts (Hallman et al. 1997), while rhizospheric bacteria live in the rhizosphere and suppress soilborne pathogen and stimulate plant growth (Rengel and Marschner 2005; Lemessa and Zeller 2007). Nawangsih (2007) reported that the endophytic bacterial isolate was able to suppress the blood disease pathogen *in vitro*.

Blood disease caused by blood disease bacteria (BDB) is one of the most dangerous diseases that spread widely across the tropical and subtropical region. The symptoms started with color changing of young leaves from green to yellow or brown and finally withered. A specific symptom is the presence of bacterial slime which is smelly, white greyish to brown reddish in color that comes out from fruit, hump, fruit stalk, rod cluster and stem. The loss caused by this disease can be up to 10-42% even up to 93.1% during severe attack (Aeny et al. 2007). According Suastika (2010), this disease suppresses banana production in Bali for more than 50% in two years starting from year 1997 to 1999. Thus, efforts to suppress pathogenic bacteria development as well as to increase banana production are needed. BDB is a pathogen with complex variation and so it is important to integrate control stategies and one of these is using biological agent (Supriadi 2011). This study was conducted to select and identify the endophytic and rhizosphere bacteria from *Musa troglodytarum* L. which have the potential to control blood diseases bacteria (BDB) in banana.

MATERIALS AND METHODS

Isolation of the endophytic bacteria

The roots and rhizospheric soils of banana were taken from Ambon Island, Subdistrict Nusaniwe (Village Seilale, Siwang, and Tuni) and Subdistrict West Leihitu (Village Alang). All of the areas have the higher population of "Tongkat Langit" banana relatively compared with another area. Soil samples were taken from the rhizosphere of healthy plant roots at the reproductive phase. The BDB was belonged to the collection of Professor Supriadi, The Spices and Aromatics Plants Research Center (BALITTRO), Bogor, Indonesia. Experiments were conducted from November 2012 to October 2013, in the Laboratory of Plant Bacteriology, Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University.

The endophytic bacteria was isolated from roots of healthy banana plant at the generative phase. Endophytic bacteria were isolated from surface sterilized root following procedure described by Hallmann et al. (1997). The banana roots were washed under flowing water to remove adhered soil particles. A portion of the roots was cut with a knife into approximately 1 cm then 5 gram were weighed. The pieces were surface sterilized with potassium hypochlorite 5% and Tween 20, 0.25% for 5 minutes and then washed four times with sterilized distilled water. Samples were macerated using a sterile mortar and pestle until it was subtle, then placed inside an Erlenmeyer flask that contained 45 ml sterilized distilled water. After a serial dilution from 10^{-1}-10^{-3}, 100 µl of each suspension was plated on 50% of triptic soy agar (TSA) and the plate was incubated at room temperature for 24-48 hours (Marwan *et al* 2011).

Isolation of the rhizosphere bacteria

About 200 grams of soil sample were taken from the rhizosphere of banana at 20-30 cm depth. From each sample, 10 gram of soil was suspended in Erlenmeyer flask containing 50 ml sterilized distilled water and shaken at 100 rpm for five minutes. Ten milliliters of soil suspensions were serially diluted up to 10^{-10}. One-hundred microliters of each diluted suspension was inoculated on Petri dishes containing King's B medium and chitin medium. The rest of soil suspension was

heated at 80°C for 10 minutes, and after a serial dilution, 100 µl of each diluted suspension was plated on TSA medium. All plates were incubated at room temperature for 24-48 hours.

Pathogenicity Test (Hypersensitive Reaction Test)

The endophytic and rhizosphere bacteria were cultured at 5 ml of Luria Berthani Broth (LBB) and shaked at 100 rpm for 48 hours. Two milliliters of bacterial suspension was injected to the lower side of tobacco leaves using sterile syringe. One leave was injected on separate location with six isolates of bacteria. Hypersensitive Reaction (HR) was stated as positive if the injected area shown necrotic or yellowing symptom after 24-48 h. Isolates with HR positive were not tested further.

Antibiosis test *in vitro*

The antibiosis mechanism of endophytic and rhizosphere bacteria toward BDB was tested using agar diffusion method reported by Marwan et al (2011). One hundred microliters of BDB suspension containing 10^8-10^9 cfu/mL (OD600 = 0.16) was spread on sucrose pepton agar (SPA) medium and after the agar sirface was air dried, two wells were made on the plates using cork borer (0.5 cm in diameter). Twenty milliliters suspension of the endophytic and rhizosphere bacteria in tryptone sucrose broth (TSB) with population of 10^8-10^9 cfu/mL (OD600 = 0.16) were inserted into one well. The other one well in the same plates was inserted with sterilized distilled water. All the plates were incubated at room temperature. Diameter of the inhibition zone (mm) was measured every day up to 4 days after inoculation.

Effects of the bacteria on the growth of banana plant

Bacterial isolates with inhibition zone and some selected bacteria without inhibition zone but growing faster were tested for their ability to enhance the growing of banana plants in the green house. One month plant of 'Kepok' banana produced by tissue culture and already acclimatization were used as tested plant. Each of the bacterial isolates was cultured in TSB medium at room temperature for 24-48 h, and the population was adjusted at 10^8-10^9 cfu/ml.

The application of rhizophere bacteria was conducted by pouring 10 ml of bacterial suspension around the roots of banana plant at the time of transplanting. The application of root endophytic bacteria on banana seedling was done by soaking the root of each seedling in 60 ml suspension of each bacterial isolates for 4 hours. The control plant was soaked in sterilized distilled water. Seedling with inoculated root endophytic and rhizophere bacteria were planted in polybag with diameter 30 cm filled with sterilized soil and husk charcoal (2:1) (Marwan *et al*. 2011).

The effect of root endophytic and rhizosphere bacteria treatment to the growth of the plants was observed based on the height of the plants and the diameter of stem every week up to four weeks after treatment (WAT). Data were plotted into plant growth curve with time (WAT) as x-axis and height and diameter of stem as y-axis.

Total *Area Under Plant Height Growth Curve* (AUPHGC) and *Area Under Stem Diameter Growth Curve* (AUSDGC) were counted using the following:

$$\text{AUPHGC} = \sum_{i=1}^{n} \left[\frac{x_{i+1}+x_i}{2}\right] \mathbf{x} \left[t_{i+1} + t_i\right] \quad \text{and} \quad \text{AUSDGC} = \sum_{i=1}^{n} \left[\frac{y_{i+1}+y_i}{2}\right] \mathbf{x} \left[t_{i+1} + t_i\right]$$

where: xi : weekly growth of height plant
yi : weekly growth of stem diameter
ti : time of observation (days)

Effect of the bacteria on blood disease incidence

Inoculation of Blood Disease Bacteria was conducted on 4 weeks after treatment of endophytic and rhizospheric bacteria. BDB was cultured in TSB medium at room temperature for 24-48 h and the population was adjusted at 10^8-10^9 cfu ml^{-1}. Two milliliters of BDB suspension was taken and injected into banana corm which already inoculated with root endophytic bacteria (Kasutjianingati 2004). Aplication of BDB on the plants inoculated with rhizosphere bacteria was conducted by cutting some of the roots and pour 10 ml of bacterial suspension to the roots after transplanting.

Disease incidence was calculated every week and disease suppression (%) and was counted using the formula:

$$\text{Disease Suppression} = \frac{Yc - Yt}{Yc} \times 100\%$$

Yt : disease incidence at the plants treated with endophytic or rhizosphere bacteria
Yc : disease incidence at control plants

Data Analysis

Data of blood disease bacterium suppression, AUPHGC, and AUSDGC were statistically analyzed by ANOVA as completely randomized design. Treatment means were compared using Dunnet test (comparison of each treatment with control) at 5% level of significance. All statistical analyses were done using SAS Program version 9.1.3.

Molecular identification of selected bacteria

Molecular identification of the selected isolates, one of endophytic and one of rhizosphere bacteria based on the highest index suppression, were conducted based on sequence of 16S-rRNA. Isolation and purification of DNA was done using Genomic DNA Mini Kit (Geneaid). Isolated DNA was amplified using primer 63f (5' -CAG GCC TAA CAC ATG CAA GTC-3') and 1387r (5'-GGG CGG CGT GTA CAA GGC-3') (Marchesi *et al*. 1998). PCR amplification was done in 30 cycles of predenaturation at 94^{o}C for two minutes, denaturation at 94^{o}C for 30 seconds, annealing at 55 ^{o}C for 30 seconds, elongation at 72^{o}C for one minute and post PCR at 72^{o}C for seven minutes. Amplification results were visualized using electrophoresis in agarose gel 1% for 45 minutes at 70 volt/cm with TAE buffer one at a time. Sequence data of 16S-rRNA genes were analized using BlastN program to see the similarity with other bacterial genes at *Genbank*.

RESULTS AND DISCUSSION

Abundance and pathogenic character of endophytic and rhizosphere bacteria

A total of 102 isolates of endophytic and 150 isolates of rhizosphere bacteria were successfully isolated from the rootlets and rhizosphere soil of 'tongkat langit' banana. Those bacterial isolates were classified based on the differentiation of colony morphology such as color, shape and the edge of colony. The endophytic bacterial isolates were isolated from samples collected at Siwang Village (27 isolates), Tuni Village (22 isolates), Seilale Village (21 isolates), Alang 2 Village (20 isolates), and Alang 1 Village (12 isolates). The rhizosphere bacterial isolates were classified into 3 groups, i.e. heat tolerant bacteria, fluorescence bacteria, and chitinolytic bacteria, which contained of 74, 54, and 22 isolates, respectively. The abundance and number of isolates of the bacteria at each sampling area were presented in Table 1.

The abundance of endophytic bacteria in the roots of 'tongkat langit' banana was in the range of 10^4-10^5 cfu g^{-1} roots, while the rhizosphere bacterial range was 10^3-10^5 cfu g^{-1} soil (Table 1). The range of abundance of endophytic bacteria was narrower compared with rhizosphere bacteria. Bacterial abundance at rhizosphere area were very diverse and different among regions because rhizosphere area contains a lot of organic materials and root exudates which also affect rhizosphere microbial growth including bacteria (Rangel and Marschner 2005, Lynch 1983). Each source of sampling location has different vegetation and altitude. Siwang and Tuni Village were located at higher area, while Seilale Village was on the lower land (coastal area). Samples from Alang 1 (in Alang Village) were taken on area close to the river, while samples from Alang 2 were collected from an area which is far from the river.

Table 1. Abundance and number of bacterial isolates successfully isolated from Fei banana.

Bacterial groups	Origin of Sample	Bacterial abundance (cfu g^{-1} sample)	Number of isolates
Endophytic bacteria	Siwang	1.18×10^5	27
	Tuni	9.45×10^4	22
	Alang 2	1.88×10^5	20
	Seilale	5.85×10^4	21
	Alang 1	4.05×10^4	12
Total endophytic bacterial isolates			102
Rhizosphere bacteria			
Heat tolerant bacteria	Siwang	1.03×10^5	12
	Tuni	6.25×10^4	19
	Alang 2	4.85×10^4	15
	Seilale	4.65×10^4	11
	Alang 1	2.70×10^4	17
Fluorescence bacteria	Siwang	1.35×10^4	8
	Tuni	7.00×10^3	11
	Alang 2	1.40×10^4	12
	Seilale	1.95×10^4	13
	Alang 1	1.30×10^4	10
Chitinolytic bacteria	Siwang	1.05×10^4	7
	Tuni	6.50×10^3	2
	Alang 2	1.70×10^4	4
	Seilale	1.50×10^4	7
	Alang 1	1.20×10^4	2
Total rhizosphere bacterial isolates			150

The abundance of endophytic bacteria in one place was affected by some factors such as host plant species, age of plant, type of plant tissue, time of sampling, temperature of host growth, host genetic and environment condition (Bacon and Hinton 2007, Hallman 2001). Based on the pathogenicity test (Hypersensitive Reaction test), 25 isolates of endophytic and rhizosphere bacteria

were successfully selected with negative results. These isolates were used as the candidate biocontrol agents for further experiments.

Effects of the bacteria on the growth of banana plants and on the suppression of blood disease bacterium

The effects of the bacteria on the suppression of colony growth of blood disease bacterium are presented in Table 2, while effects on the height of banana plants are presented in Table 3. There were 16 isolates which produced inhibition zone with diameter ranging from 2.25 to 25.00 mm. The widest inhibition zone diameter was produced by the rhizosphere isolate, heat tolerant bacteria, RTT 1 with inhibition zone diameter up to 25.00 mm. Among the endophytic bacteria, the widest inhibition zone was 15.75 mm, which was produced by isolate EAT 13.

Based on the data of disease incidence and index suppression in Table 3, the isolates RTI 1, RTI.2, and RTI caused the highest index suppression at the end of experiment, at 28 DAI, with index suppression of 80%. These bacteria also produced inhibition zone *in vitro* but the diameters of inhibition zone were relatively low, i.e. 6.75 mm, 3.00 mm, and 20.00 mm, respectively. On the other hand, isolates which produced the widest inhibition zone caused relatively lower suppression to the blood disease, such as isolate RTT 1. This result showed that suppression of biocontrol agents *in vitro* was not always positively correlated with suppression *in planta*.

Table 2. Diameter of inhibition zone (mm) produced by the endophytic and rhizosphere bacteria from 'tongkat langit' banana against blood disease bacterium *in vitro*

Bacterial Groups	Code of Isolate bacteria	Diameter of inhibition zones (mm)[a)]
Endopytic bacteria	EAI 23	2.25 d
	EAI 26	2.25 d
	EAI 27	3.25 d
	EAS 1	13.75 bc
	EAT 13	15.75 b
	EAA 2.13	14.25 bc
Rhizosphere bacteria		
Heat tolerant bacteria	RTI 2	6.75 cd
	RTI 3	3.00 d
	RTI 4	20.00 a
	RTT 1	25.00 a
	RTT 2	3.50 cd
	RTT 3	15.50 b
	RTA 1.9	8.75 bcd
	RTA 2.2	13.75 bc
	RTA 2.13	9.00 bcd
Fluorescence bacteria	RFA 2.1	11.75 bc

[a] Means followed by the same letter were not significantly different at $\alpha = 0.05\%$.

Endophytic and rhizosphere bacteria ae able to suppress pathogen directly through antibiosis mechanism and space competition, and indirectly through plant resistance induction mechanism (Haggag 2010). Antibiotic compounds produced by endophytic bacteria are suspected to be more of elicitors which induce plant resistance compared to acting directly as bactericide (Marwan et al. 2011).

Table 3. Disease incidence and index suppression of blood disease on 'kepok' banana affected by application of endophytic or rhizosphere bacteria isolated from 'tongkat langit' banana

Bacterial groups	Code of isolate	Disease Incidence (%)		Index Suppression (%)	
		14 DAI[a)]	28 DAI	14 DAI	28 DAI
Endophytic bacteria	EAI 1	80	80	20	20
	EAI 23	80	100	20	0
	EAI 26	40	40	60	60
	EAI 27	90	90	10	10
	EAS 1	70	70	30	30
	EAS 4	80	80	20	20
	EAS 7	70	70	30	30
	EAT 13	70	80	30	20
	EAA 2.13	80	100	20	0
	EAA 2.23	70	100	30	0
Rhizosphere bacteria					
Heat tolerant bacteria	RTI 2	20	20	80	80
	RTI 3	20	20	80	80
	RTI 4	20	20	80	80
	RTT 1	40	40	60	60
	RTT 2	40	40	60	60
	RTT 3	40	40	60	60
	RTA 1.9	40	60	60	40
	RTA 2.2	50	60	50	40
	RTA 2.13	40	40	60	60
Chitinolytic bacteria	RKT 1	40	40	60	60
Fluorescence bacteria	RFA 2.1	40	40	60	60
	Control	100	100	0	0

[a)] DAI = days after inoculation of blood disease bacterium

The effects of application of root endophytic and rhizosphere bacteria in the green house to the height and diameter of banana plants are presented in Table 4. Some isolates significantly increased the growth of plant height, but for the diameter of stem, there were no significant difference between the plants treated with endophytic and rhizosphere bacteria. Isolates of the bacteria which significantly increased the AUPHGC value were RTI 3, RTI 4, RTT 3, and RTA 2.2 with AUPHGC values were 109.59, 124.95, 102.90, and 102.64, respectively. The AUPHGC value of the control was 43.58.

These isolates of rhizosphere bacteria have high ability to stimulate the growth of plant height that could be described as plant growth-promoting rhizobacteria (PGPR). Rhizosphere bacteria can be classified as plant growth promoting rhizobacteria (PGPR) because they can stimulate plant growth and produce phytohormones (Kumar et al. 2012, Hallman 2001). Almaghrabi et al. (2013), reported that the application of some PGPR isolates on tomato plant can stimulate weight of dry bud and plant height.

Table 4. Means of Area Under Plant Height Growth Curve (AUPHGC) and Area Under Stem Diameter Growth Curve (AUSDGC) values of banana plants affected by the application of endophytic and rhizosphere bacteria from 'tongkat langit' banana

Bacterial groups	Code of isolate	AUPHGC values	AUSDGC values
Endophytic bacteria	EAI 1	32.38	6.51
	EAI 23	32.01	2.25
	EAI 26	40.42	4.10
	EAI 27	20.90	1.83
	EAS 1	38.33	5.21
	EAS 4	65.17	2.56
	EAS 7	74.31	4.13
	EAT 13	74.10	4.66
	EAA 2.13	62.27	5.36
	EAA 2.23	45.54	3.50
Rhizosphere bacteria			
Heat tolerant bacteria	RTI 2	88.62	4.80
	RTI 3	109.59*	6.13
	RTI 4	124.95*	6.83
	RTT 1	69.58	6.02
	RTT 2	93.03	5.64
	RTT 3	102.90*	5.25
	RTA 1.9	53.42	5.25
	RTA 2.2	102.62*	4.80
	RTA 2.13	84.56	7.09
Chitinolytic bacteria	RKT 1	59.47	5.67
Fluorescence bacteria	RFA 2.1	72.14	5.29
	Control	43.58	5.18

*Significantly different compared with control by Dunnet test at $\alpha = 0.05$

Molecular identification of selected bacteria

Based on the index suppression for endophytic bacteria and index suppression and AUPHGC value, two isolates were molecularly identified. Both of the isolates were EAI 26 (endophytic bacteria) and RTI 4 (rhizosphere bacteria, heat tolerant bacteria). Identification was conducted based on the sequences of 16S rRNA. Results of RNA amplification were visualized using electrophoresis agarose gel 1% resulting in DNA band sized ~1400 basepair (Fig. 1).

The percentage of homology of 16S rRNA gene sequence of both isolates are presented in Table 5. Isolate EAI 26 has 98% similarity with *Bacillus* sp EPI-RI and RTI 4 has 95% similarity with *Bacillus subtilis* site 7S. *B. subtilis* and other *Bacillus* spp have long known as biocontrol agents that can control plant diseases. These bacteria commonly found as endophytic bacteria or PGPR that play a role in stimulating plant growth and control plant diseases (Piggot and Hilbert 2004; Guo et al. 2004). Lemessa and Zeller (2007) reported that rhizosphere bacteria *Bacillus subtillis* strain B2G can supress withering disease in tomato with 63% inhibition percentage.

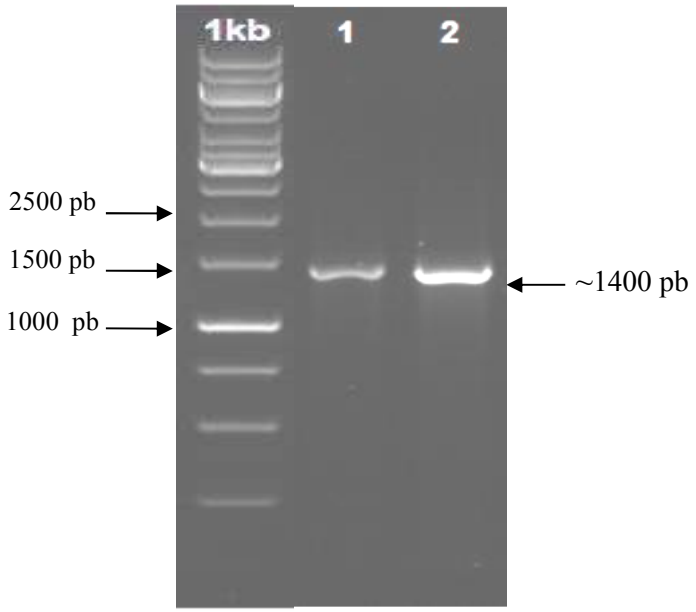

Fig. 1. Bands of 16S rRNA amplified from DNA of isolates EAI 26 (1) and RTI 4 (2)

Table 5. Homology and percentage of identical 16S rRNA sequence of EAI 26 and RTI 4 isolates with BlastN program

Homology and percentage of identical sequence (%)			
Isolate EAI 26		**Isolate RTI 4**	
Bacillus sp EPI-RI	98	*Bacillus subtillis* site 7S	95
Bacterium JP25	98	*Geobacillus stearothermophilus* strainB107	95
Bacillus cereus strain BM1	97	*Bacillus subtilis* strain DNEB42	95
Bacillus thuringensis	97	*Bacillus subtilis* strain KPC	94
Bacillus sp RSP-VIW 54	97	*Bacillus subtilis* strain BN1	94
Bacillus anthracis strain M51	96	*Bacillus subtilis* strain IARI-C8-53	95
Bacillus cereus strain FB	96	*Bacillus* sp M64 (2010) strain M64	95
Bacillus thuringensis strain G6	96	*Bacillus subtilis* strain BE-91	95
Bacillus thuringensis strain E1-12	97	*Bacillus subtilis* strain E9-1	95
Bacillus sp GPTSA100-6	96	*Bacillus subtilis* 1769	95

CONCLUSION

There were 252 isolates of bacteria isolated from 'tongkat langit' banana plant which contained 102 isolates of root endophytic bacteria and 150 isolates of rhizosphere bacteria. Four isolates, which belonged to rhizosphere bacteria, gAve significant index suppression toward blood disease under green house conditions compared to control. These isolates are RTI 3, RTI 4, RTT 3, and RTA 2.2 which also stimulated the height of banana plants, and the highest was given by isolate RTI4. Among the endophytic bacteria, isolate EAI 26 gave the highest index suppression and was identified as *Bacillus* sp while isolate RTI 4 was identified as *Bacillus subtilis*. Both of the bacteria will be tested further for their potential as biocontrol agents of blood disease on banana and the other banana diseases. .

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