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The Use of Genetic Variability Analysis of *Fusarium oxysporum* f. sp. *cubense* for Breeding Resistance of Banana against Fusarium Wilting Disease

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Abstract

Fusarium wilting on banana crop caused by *Fusarium oxysporum* f. sp. *cubense* is one of the important disease in banana plant in Indonesia. This disease can cause plant to wilt and die, therefore bringing loss to the banana farmer and entrepreneur.

F. oxysporum f. sp. *cubense* genetic variability analysis techniques can be done by in vitro or in vivo. One of *F. oxysporum* f. sp. *cubense* genetic variability analysis techniques by in vitro is RAPD-PCR. In this research, analysis is continued with pathogen test. Genetic variability analysis by in vivo is needed to determine the level of pathogen and the race.

The result of genetic variability techniques by RAPD-PCR done by this writer indicates that there is a big relation/ link difference between isolats from different island. Isolat from Mojokerto (East Java) is 100% genetically different compared to the one from West Sumatera. Later, result of pathogen test shows that Pisang Ambon Kuning is the most resilient compared to Pisang Raja and William Cavendish. Based on the level of pathogen, there are two race grouping, which are race 1 that attacks Pisang Ambon Kuning and race 4 that attacks Pisang Raja and William Cavendish. Scott-Knott analysis on 26 isolats results in no real difference between isolats tested.

Keywords : Genetic variability, banana, Fusarium wilting disease

Introduction

Banana is one of the important fruit commodity with high economic values. More than 70% of the provinces in Indonesia yield banana. Big plant areas can be found in South Sulawesi, North Sumatera, Riau, and Balinamun.

Fusarium wilting, which also called Panama Disease, is one of important diseases in banana farming in Indonesia. The disease is caused by *Fusarium oxysporum* Schlect f. sp. *cubense* (E. F. Smith) Snyd & Hans. Until now many reasearches are intended to find the most effective way to handle the disease. One way to handle it is by planting the resistant variety. Problems with this kind of handling are : 1) the number of Fusarium resistant banana variety is very limited, 2) producing Fusarium resistant banana variety is very difficult because this fungi is known to have several physiology races, 3) *Fusarium Oxysporum* can infect more than one plant. According to Kim and friends (1992) up to now there are more than 90 forms of *Fusarium Oxysporum* which almost all cause wilting on host plant. Therefore, it is important to conduct study on *F. oxysporum* f. sp. *cubense* genetic variability as the foundation for genetic improvement of banana plant against fusarium wilting disease.

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Materials and Method

Materials that are needed in *in vitro* and *in vivo* genetic variability analysis test are 26 *Fusarium oxysporum* f. sp. *Cubense* fungi isolat, 3 banana varieties resulted from cul-

ture multiplying of non-root tissue, size ± 5 cm, and not yet aclimatisized, which are Ambon Kuning, Raja dan William Cavendish, aquabides, PDA media and V8, *juice-broth*, buffer solution consists of buffer lisis, buffer TBE, buffer loading, buffer TE, buffer PCR (Table 1). Other materials needed are chemicalis including chloroform, phenol, NaOAc, isopropanol, EtOH, enzym DNA Taq polymerase, oligonukleotida primer (Table 1), deoksiribonukleotida trifosfat, liquid N2, l kb DNA marker and mineral oil, Murashige- Skoog media, and additional materials for pathogen test which are NAA, BA, adenin sulfat, sukrosa and vitamin C.

Table 1. Buffer, Media and Primer that for the research

No.	Buffer, Media and Primer	Composition
1.	Bufer Lisis (Innis, dkk., 1990)	50 mM Tris-HCl pH 7.2 50 mM EDTA 3% SDS 1% 2-Mercaptoetanol
2.	Bufer TE (Innis, dkk., 1990)	10 mM Tris-HCl 0,1 mM EDTA
3.	Bufer TBE 5x (Bassam, dkk., 1995)	0,45 M Trisma 0,45 Borac Acid 20 ml 0,5 M EDTA pH 8,1
4.	Solid Media (PDA)	15 g Jelly 200 g Potato 20 g Dextrose 1 l Aquades
5.	Liquid Media (V8 juice-broth)	50 ml V8 juice 2 g CaCO3 1 l Aquades pH 6
6.	Primer	1. M7802 : ACG ATC GTC A 2. M7810 : CAT GTC CGA G 3. M8132 : TCT GGT GAG G 4. M8134 : AGG GGT CTT G 5. M3936 : GAC GTC CCG T

The tools needed are LFC (Laminar Flow Cabinet), erlenmeyer, petridis, pinset, jam bottle, bunsen, scalpel, micropipet, gloves, analytic scale, thermometer, fridge, submarine electrophoresys, eppendorf tube, *vortex* (Model VM 2000), *centrifuge* (Biofug 13), *waterbath*, PCR machine (*MiniCycler*--PTC I50), UV Transilluminator and polaroid DS 34 camera.

Genetic Variability Analysis with RAPD-PCR technique

Genetic Variability Analysis with RAPD-PCR technique is done through five series of steps as follow :

a.Preparation of Miselium *Fusarium oxysporum* f. sp. *cubense*

F. oxysporum f. sp. *cubense* fungi is inoculated at PDA media on petri dish. After 5 days of incubation at room temperature, fungi that grows is taken to multiply its misellium at V8 *juice-broth* media in erlenmeyer 1000 ml. Misellium that grows on the surface of the media is harvested, washed with sterile aquades 4 times.

b.Isolation of Total DNA

Isolation of total DNA was done by Innis method (1990). Harvested fungi which has been harvested and washed, then taken about 20-40 mg, added with *liquid* N2 then grinded in mortar using *pestle*. The result is put into eppendorf tube 1,5 ml, added with buffer lisis (400 µl) then vortexed for 10 seconds, and incubated in *waterbath* 55°C for an hour. Into this solution then is added klorofonm solution : fenol : iso amil alchohol with 24:25:1 volume ratio (400 µl) and vortexed for 10 seconds, and sentrifused at 10.000 rpm, at 20°C temperature for 20 minutes. Obtained supernatan is then moved to new eppendorf tube and added with 10 µl NaOAc and 0,54 volume isopropanol. Tube is shuffled gently about 2-3 times so that it shows white DNA precipitation, then sentrifuged at 10.000 rpm at 20°C temperature for 2 minutes. Obtained pellet was washed with 1 ml cold ethanol dingin (4°C) 70% then sentrifused (10.000 rpm) at 20°C temperature for 5 minutes. Supernatan was thrown away and eppendorf tube with pellet is turned upside down with tissue base. Pellet is then added with 100 µl Tris EDTA.

c.Electrophoresis for Total DNA

1.6 gr of agarose is dissolved in 200 ml buffer electrophoresis (TBE), then heated until boiled. Solution is let to cool down (50°C), then poured into electrophoresis plate. Brush is put on a straight posision. Gel is let to be solified then taken again with brush.. 5 µl DNA sample which has been mixed with 3 µl loading buffer is put into electrophoresis hole. Electrode is connected to the power supply of 56V for 1.5 hours from negative to positive. When it's done, gel is taken and put into 0,5 ppm ethidium bromide solution for 15 minutes. Gel is then washed with running water for 10 minutes. The existence of DNA in the gel is watched on UV Transilluminator, and photographed for documentation using DS-34 polaroid camera with Fuji FP-3000B black and white film.

d.RAPD-PCR

Metode RAPD-PCR method is done in 50 µl total volume in sterilized eppendorf tube size 0,5 ml. The steps of RAPD-PCR are as follow:

1.Subsequently put 35 µl H20 steril, 5µl buffer PCR 10x, 2 µl $MgCl_2$ 50 mM, 1 µl DNA template, 4 µl dNTP-*mix* 2 mM, 1µl DNA *Taq* polymerase 5U, and 2 µl primer into 0,5 µl sterilized eppendorf using micropipet.

2.To reduce evaporation during the reaction add 75 µl of mineral oil on the mix. Eppendorf tube is then closed and put in the PCR machine (*Minicycler*-PTC 150) which has been programmed as follow :

Phase	Temperature	Time
1	94 °C	3 minutes
2	35 °C	1 minute
3	75 °C	3 minutes
4	94 °C	1 minute
5	35 °C	1 minute
6	75 °C	3 minutes
7	Back to phase 4 for 44 times	
8	94 °C	1 minute
9	35 °C	1 minute
10	75 °C	10 minutes
11	4 °C	30 minutes
12	End	

e.Electrophoresys for Amplified DNA

Amplified DNA is analysed with electrophoresys in the same process as above. The difference is in the concentration of used agarose which is 1,5%, and the voltage used 40v for 4.5 hours. dan voltage yang digunakan sebesar 40V selama 4,5 jam. Simultaneously, 1 kb marker DNA is also electrophoresized as measurement.

Pathogen Test on Banana

Pathogen test is done throught factorial experiment, using Complete Random Design with 2 repeats and consisting of 2 factors : Factor I is banana variety, consist of V 1 (Ambon Kuning), V2 (Raja), and V3 (William Cavendish).Factor II is the origin of *F. oxysporum* f. sp. *cubense* isolat, consist of 26 isolat as follow

Table 2. List of origin of *F. oxysporum* f. sp. *cubense* isolates

No.	Origin of Isolat	No.	Origin of Isolat
1.	Desa Kumango 2, Tanah Datar, Sumbar	14.	Desa Koto Tinggi 2, Bukit Tinggi Sumbar
2.	Desa Kumango 2, Tanah Datar, Sumbar	15.	Desa Koto Tinggi 3, Bukit Tinggi Sumbar
3.	Desa Dakan Sinayan, Bukit Tinggi, Sumbar	16.	Desa Koto Tinggi 4, Bukit Tinggi Sumbar
4.	Desa Lima 2, Tanah Datar, Sumbar	17.	Kp. Aripan, Balihorti, Solok 1
5.	Desa Lima 1, Tanah Datar, Sumbar	18.	Kp. Aripan, Balihorti, Solok 1
6.	Desa Angkat Cadung 1, Bukit Tinggi, Sumbar	19.	Kp. Aripan, Balihorti, Solok 1
7.	Desa Angkat Cadung 1, Bukit Tinggi, Sumbar	20.	Kp. Aripan, Balihorti, Solok 1
8.	Desa Baso 1, Bukit Tinggi, Sumbar	21.	Kp. Aripan, Balihorti, Solok 1
9.	Desa Kt. Tinggi, Baso 1, Bukit Tinggi, Sumbar	22.	Mojokerto, Jatim
10.	Desa Pintu Koto, Baso 1, Bukit Tinggi, Sumbar	23.	Mojokerto, Jatim
11.	Desa Kt. Tinggi,Baso 1, Bukit Tinggi, Sumbar	24.	Lumajang, Jatim
12.	Desa Kacang, Solok	25.	Banyuwangi, Jatim
13	Desa Banu Hampu, Bukit Tinggi Sumbar	26.	Probolinggo, Jatim

Pathogen test on banana is done by inoculating *F. oxysporum* f. sp. *cubense* on media MS + BA 5 ppm + NAA 2 ppm in jam bottle. Meanwhile banana plantlet is prepared. Five days after incubation, plantlet is planted on media MS which is inoculated with fungi.

Observation is done on the tenth day after planting with observation parameters the percentage of colour change in xilem (%).

Experiment Result Analysis

Genetic similarity coefficient between two isolat at *similarity matrix* is calculated using Dice coefficient (Sokal dan Sneath, 1963). Relative connection between isolat is analyzed using *Numerical Tazonomy System* program (NTSYS-pc) at *Phenogram* branch diagram type (Rohlf, 1992). Grouping to form the Phenogram branch diagram is calculated using UPGMA method (*unweighted pair group method with arithmatic mean*).

Data from *F. oxysporum* f. sp. *cubense* pathogen test on Ambon Kuning, Raja and William Cavendish banana is first transformed using angle transformation (arcsin "y) (Steel dan Torie, 1991), then analysed using Variety Analysis. Later all *F. oxysporum* f. sp. *cubense* isolat is grouped using Scott-Knott analysis, while banana variety is differentiated based on BNT test (Gaspersz, 1991).

Result

RAPD-PCR analysis result based on the existence of amplified *F. oxysporum* f. sp. *cubense* DNA fragment shows good result which are isolat number 1, 2, 6, 7, 10, 16, 17, and 23. Results of DNA amplification with 5 different primer are shown on Figure 1-7.t

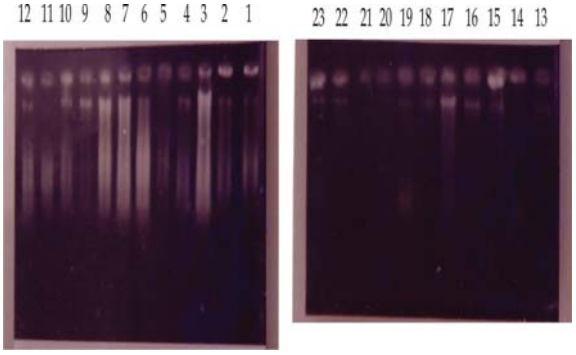

Figure 1. Results Total DNA Isolation

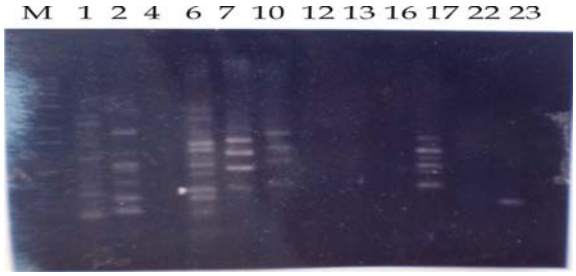

Figure 2. The total DNA amplification isolat no. 1, 2, 4, 6, 7, 10, 12, 13, 16, 17, 22, 23 using primary M 7802

Figure 3. The total DNA amplification isolat no. 1, 2, 4, 6, 7, 10, 12, 13, 16, 17, 22, 23 using primary M 7810

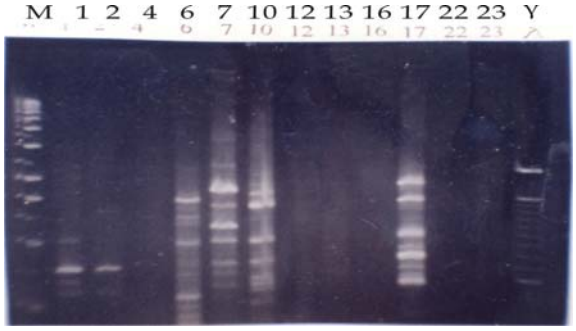

Figure 4. The total DNA amplification isolat no. 1, 2, 4, 6, 7, 10, 12, 13, 16, 17, 22, 23 using primary M 8132

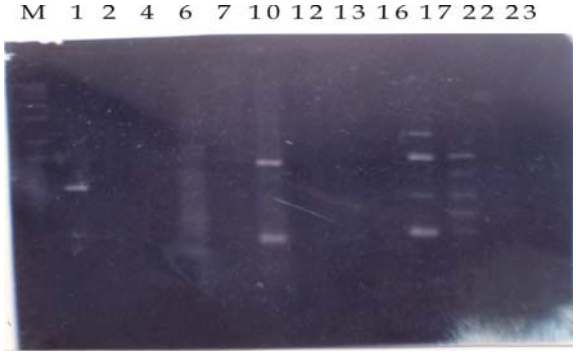

Figure 5. The total DNA amplification isolat no. 1, 2, 4, 6, 7, 10, 12, 13, 16, 17, 22, 23 using primary M 8134

Figure 6. The total DNA amplification isolat no. 1, 2, 4, 6, 7, 10, 12, 13, 16, 17, 22, 23 using primary M 3936

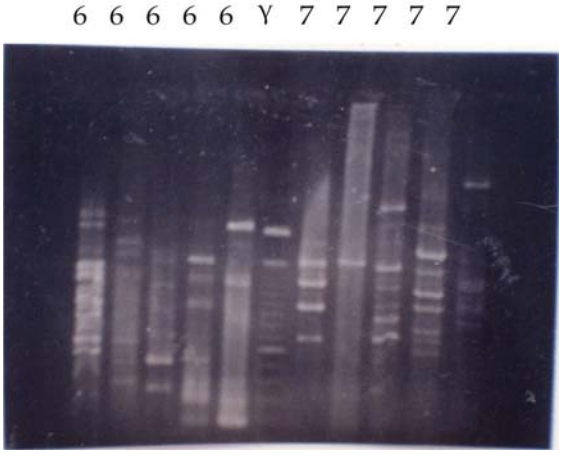

Figure 7. The total DNA amplification isolat no. 6, 7 using primary M 3936

Fusarium oxysporum f. sp. *cubense* grouping using NTSYS-pc program at *phenogram* branch diagram type shows that isolat number 1, 6, 7, and 17 which are amplified using five primer (M7802, M7810, M8132, M8134 dan M3936) consist of two groups. Group I consists of isolat number 6 and 7, while group II are isolat no. 1 and 17. Isolat number l, 6, 7, 10, and 17 which are amplified with four primer (M7802, M7810, M8134 dan M3936) consist of two groups, Group I consists of isolat number 1, 6, and 17, while Group II isolat number 7 and 10. Isolat number 1, 2, 6, 7, 10, and 17 which are amplified with two primer (M7802 dan M3936) consist of two groups, Group I are isolat no. 1, 2, 6, and 17, and Group II isolat no. 7 and 10. Isolat number 1, 2, 6, 7, 10, 17, and 23 which are amplified with M7802 primer consist of three groups, Group I are isolat no. l, 6, and 10, Group II isolat no. 2, 7, and 17, and Group III isolat no. 23. Isolat number 1, 6, 7, 10, 16, and 17 which are amplified with M7810 primer consist of two groups, Group I are isolat no. 6, 10, 16, and 17, whhile Group II isolat no. 1 dan 7.

Genetic variability analysis result using RAPD-PCR technique shows that there is big difference in relative connection between isolat from different islands. Isolat no. 23 from Mojokerto 100% genetically different compared to isolat 1, 2, 6, 7, 10 and 17 that come from West Sumatera. Based on pathogen test, it is known that Ambon Kuning banana is the most resistant compared to Raja and William Cavendish banana. Race grouping based on pathogen level has two races : race 1 that attacks Ambon Kuning banana and race 4 that attacks Raja and William Cavendish banana. Scott-Knott analysis result on 26 isolat shows no real difference in pathogen level among tested isolat.

Discussion

In improving crop's resistance against pathogen fungi, there are two critical things, variation in pathogen's capability to induce disease to host and resistance difference of crop species against pathogen infection (Allard, 1989). Besides that, condition of environment also has effect to the crop and disease causing organism (Crowder, 1990). In this experiment, the three factors are studied throught genetic variability analysis with 26 isolat *F. oxysporum* f. sp. *cubense* pathogen test against Ambon Kuning, Raja and William Cavendish banana variety and RAPD-PCR study on *F. oxysporum* f. sp. *cubense*.

RAPD-PCR analysis results ini 8 ampli-

fied isolat. It is suspected due to low quality of isolated DNA because of high contamination or less suitable primer used. Bassam *et al*. (1990) said that high intensity of DNA template is a must because PCR can amplify only small portion of DNA fragment. Primer for PCR analysis must be complementary with DNA template that contains G+C until 40 to 50%. Besides that, good preparation is important including sterilization of tools and the media to be used and careful execution to avoid contamination. If possible, PCR reaction is conducted inside Laminar Flow Cabinet equipped with ultra violet light.

Genetic variability can be confirmed further if there are more amplified DNA fragments on each isolat. The assumption is that the more DNA fragment known, the more sure about trait of each isolat. The chance becomes larger as more primers anneal at total DNA. Test result shows that isolat that can be amplified with primer 4 and 5 generate fragments in high quantity and almost the same, which are 94 and 95. In race characterization test by Grajal-Martin *et al*. (1995) using 14 primer types, each of 10 alkali gets 52 bands and this result can already be used to conclude that there is a high variability among tested isolat.

From semblance matrix, we know that the biggest genetic semblance coefficient is 0,741. The number derives from isolat no. 7 and 10 amplified using two dua primer : M7808 and M3936 primer. The number shows closest relative relation between eight amplified isolat. Genetic semblance coefficient between the two isolat is still the biggest if total DNA of both are amplified with four primer : M7802, M7810, M8134 and M3936. Meanwhile, isolat that genetically has no relation at all is isolat no. 23. Genetic semblance coefficient of isolat no. 23 compared to other isolat (l, 2, 6, 7, 10, 16, 17) is 0,00. This result shows that there is a high variability of *F. oxysporum* f. sp. *cubense* that comes from different areas but still inside one province, West Sumatera.

Grouping of *F. oxysporum* f. sp. *cubense* isolat is known from relative connection phenogram among isolat. The phenogram shows that grouping based on the type of primer that can amplify is different. Genetic variability among all isolat is high, where most have semblance coefficent under 0,50. The closest relative connection is between isolat no. 7 and 10 with genetic semblance coefficient of 0,741.

F. oxysporum f. sp. *cubense* pathogen test on Ambon Kuning, Raja and William Cavendish banana shows that fungi isolat that has been kept for a long time can still infect the three banana varieties with different attack levels (Figure 8).

Figure 8. Condition banana plants infected by the *F. oxysporum* with different levels a) control plants (healthy), b) the infected plant is less than 20% (resistant), c) plants infected with the 50% (slightly resistant), d) dead plants (sensitive).

This confirmed by Sastrahidayat (1983) that miselium in xilem tube generates 3 kinds of toxin : *fusaric acid, dehidrofusaric acid* dan *lycomarasmin*. These toxins change the permeability of plant cell's plasma membran to cause dehydration. F. *oxyaporum* also emits polyphenol compound which then oxydized by polyphenolocsidase enzyme into quinon and polymerization which results in brownish melanin. This result in change of plant colour into brown.

Fungi comes in through the wounded plant base as a result of the separation of

each plantlet. Miselium then breaks through intraseluler tissues into the upper region of banana crop (Figure 9). Miselium in xilem tube produces toxin and polyphenol which cause the tissue to die and become brownish.

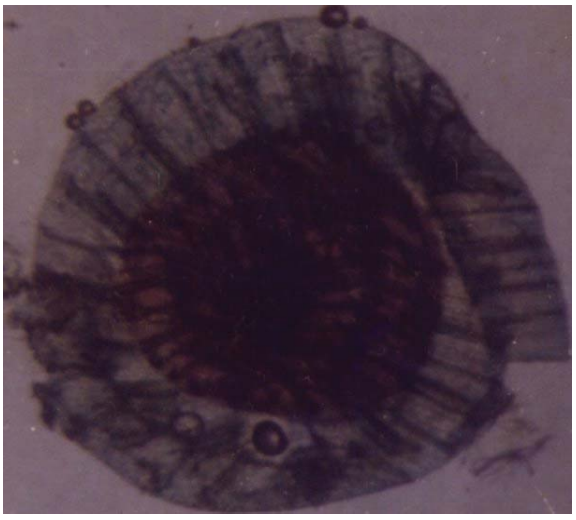

Figure 9. Transverse incision in the xylem tissue of infected banana plants

Miselium in xilem tube produces microkonidia and macrokonidia, as well as in floem tube, so that the attack escalates (Figure 10).

At the next attact, miselium breaks through intraseluler tissue (Figure 11) and grows in floem tube, causing the plant to die slowly.

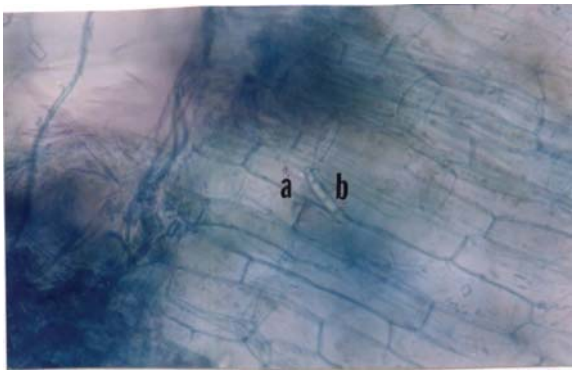

Figure 10.Microkonidia and macrokonidia, in floem tube

Figure 11. Miselium penetrate intraseluler tissue toward floem

Variety analysis result shows that there is no interaction between the origin of isolat with variety, but there is a real difference in each treatment. There is no real difference in Scott-K nott analysis result on 26 isolat. While BNT analysis on banana variety shows difference in groups : Group I is Ambon Kuning banana is more resistant than Raja and William Cavendish banana, Group II is Raja and William Cavendish banana. Pisang Ambon is known to be susceptible to *F. oxysporum* f. sp. *cubense* race l (Stover, 1993), while William Cavendish is prone to race 4 (Stover, 1993). Which race is Pisang Raja susceptible to is not yet known but based on pathogen test, it is suspected that it is susceptible to race 4 because its pathogen level based group is the same as William Cavendish. Allard (1989) said that physiology race can be differentiated based on its pathogen level against different host varieties of the same species.

As the conclusion of this research, genetic variability analysis with RAPD-PCR technique continued with pathogen test is necessary as the foundation of genetic improvement of banana crop. Result of genetic variability analysis with RAPD-PCR technique shows that there is a big difference in relative connection between isolat from different islands. Then from pathogen test result, we know that Pisang Ambon Kuning is the most resistant compared to Pisang

Raja and William Cavendish. Race grouping based on pathogen level consists of two races : race 1 that attacks Pisang Ambon Kuning and race 4 that attacks Pisang Raja and William Cavendish. Based on Scott-Knott analysis result, there is no real difference in pathogen level between tested isolat.

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