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Virulence and genetic diversity of *Fusarium oxysporum* f.sp. *cepae* as the cause of root rot in garlic

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Abstract. *Choiruddin MR, Fatawi ZD, Hadiwiyono. 2019. Virulence and genetic diversity of* Fusarium oxysporum *f. sp.* cepae *as the cause of root rot in garlic. Bioteknologi 16: 31-36.* Garlic (*Allium sativum* L.) is a well-known name in the community. The development of garlic in an area intensively and continuously has a positive impact on increasing farmers' income but also has a negative impact with a significant increase in garlic disease attacks. Root rot caused by *Fusarium oxysporum f.* sp *cepae* is one of the factors causing garlic yield loss, both in the field and during storage. Recently, this disease has also become endemic in the garlic production center in Tawangmangu, Indonesia. This study aims to obtain information about the virulence and genetic diversity of *Fusarium oxysporum* f. sp. *cepae* from endemic and non-endemic areas of garlic based on RAPD analysis. This research was conducted from August to December 2009 in Indonesia. Based on the virulence test, there is no significant difference in the damage caused by various isolates between endemic and non-endemic areas. There is no difference in the pattern of DNA fragments in endemic and non-endemic areas, but 3 groups of DNA strains can be obtained from these results. Still, the grouping is unrelated to the virulence and origin of endemic or non-endemic isolates. The grouping between lines does not include differences between endemic and non-endemic areas. In line 1, all isolates are isolates from endemic areas; in line 2, there are isolates from endemic areas (FCp2 isolates) and non-endemic (FCp7 isolates); while strain 3 is also a mixture of isolates from endemic areas (FCp3 isolates) and non-endemic areas. (FCp5, FCp6, and FCp8) isolates. It proves that there is genetic diversity in *F. oxysporum* f. sp. *cepae* isolated from Tawangmangu. However, the genetic diversity of these pathogens is not related to the disease status of the area of origin of the isolates.

Keywords: *Fusarium oxysporum* f.sp. *cepae,* garlic, genetic, root rot, virulence

INTRODUCTION

Garlic (*Allium sativum* L.) is a well-known name in the community. This tuber vegetable is one of the main kitchen spices. Garlic with the surname *Allium* is a descendant of the wild onion *Allium longicurpis* Regel, which grows in Central Asia with a subtropical climate (Wibowo 2003). High public demand for garlic has caused many farmers to plant this vegetable. However, domestic garlic production has not been able to cover this demand, so garlic imports are still an option. According to *Dinas Pertanian Daerah Istimewa Yogyakarta* (2008), Indonesian garlic imports amounted to 295 thousand tons with a value of not less than US$ 103 million. Increasing domestic awareness to reduce dependence on imported garlic has encouraged the development of garlic in Indonesia. This increase in production can also support the price of garlic in the market.

In general, garlic is only suitable for cultivation in the highlands, although some lowland tolerant varieties are now found. Tawangmangu, Indonesia, is one of the central areas for garlic production. The development of garlic in an area intensively and continuously has a positive impact on increasing farmers' income but also has a negative impact with a significant increase in garlic disease attacks. In garlic cultivation, the disease becomes an important obstacle. Root rot caused by *Fusarium oxysporum f.* sp *cepae* is one of the factors causing garlic yield loss since 1973, both in the field and during storage (Widodo et al. 2008).

Recently, this disease has also become endemic in the garlic production center in Tawangmangu. More than 92% of the area's garlic planting area has been infected with *F. oxysporum* sp. f. *cepae* (Hadiwiyono et al. 2009). Based only on experience, the current farming practice can lead to changes in the genetic character of pathogens that can trigger an explosion of pathogenic attacks. Therefore, there is a need for research on various aspects of the ecology and epidemiology of the disease, including its genetic character and its relationship to the pathogen's virulence.

The phenotype testing and observation showed that the explosion of *F. oxysporum* sp. f. *cepae* in some areas is caused by changes in the genetic virulence character of the pathogen. For example, the continuous cultivation of garlic mixed with shallots and garlic and the intensive use of agrochemicals are thought to be the cause of the explosion of base rot disease in Tawangmangu (Fatawi et al. 2003). In addition, continuous garlic cultivation can also cause changes in the phenotype of *F. oxysporum* sp. f. *cepae*. However, phenotype characterization has a weakness; namely, the phenotype is strongly influenced by the environment, so the characters that appear are often pseudonymous. Therefore, it causes the information obtained to be often biased. Therefore, to know more about changes in phenotype due to changes in genotype, it is necessary to carry out the genetic characterization of the Fusarium pathogen, for example, by DNA fingerprinting.

The molecular characterization of pathogens through DNA fingerprinting is more reliable because it is directly based on the source of genetic character information, namely DNA. One of the DNA fingerprinting methods that have been proven to be highly discriminatory and high reproducibility but inexpensive is random amplified polymorphism DNA (RAPD) analysis (Ruiz et al. 2000). This PCR-RAPD method was used for genetic characterization of *F. oxysporum* f. sp. *cepae* to detect genetic changes.

This study aims to obtain information about the virulence and genetic diversity of *F. oxysporum* f. sp. *cepae* from endemic and non-endemic areas of garlic based on RAPD analysis.

MATERIALS AND METHODS

Research time and place

This research was conducted from August to December 2009 in Indonesia at the Laboratory of Plant Pests and Diseases, Universitas Sebelas Maret, Surakarta, and the Laboratory of Agricultural Biotechnology, Universitas Gadjah Mada, Yogyakarta.

Materials and Tools

The materials used in this study were plants infected with the fungus *Fusarium oxysporum f.* sp. *cepae* garlic, distilled water, lactic acid 25%, furelox 5%, sublimate 0.1%, alcohol 90%, CTAB 2% Tris-HCl 50 mM, Chloroform Isoamyl Alcohol (CIAA), EDTA 100 mM pH 8, NaCl 1.4 M, Mercapto Ethanol 1%, quartz sand, TBE (Tris-Boric-EDTA) 0.5X, Ethidium bromide 0.1%, Mega Mix Royal (MMR), Miliq water, Potato Dextrose Broth (PDB) medium and Potato Dextrose Agar (PDA) medium.

The tools used in this study were a digital camera, cooling flask, microscope, refrigerator, Laminar Air Flow (LAF), autoclave, inoculation needle, ose needle, 250 mL, and 100 mL Erlenmeyer tubes, sterile petridish, denatured alcohol lamp, razor blade, tweezers, beaker, label paper, filter paper, cotton, 1.5 mL Eppendorf tube, electrophoresis bath, oven, PCR tube, and ThermoCycler PCR machine (BioRad).

Surveys and collections of *F. oxysporum* f. sp. *cepae*

The survey was conducted to obtain endemic and non-endemic garlic plantations for root rot disease. The criteria for endemic areas were crops that were attacked by pathogens with more than 30%, while non-endemic areas have a disease intensity of less than 1%. Each of these areas was isolated from *F. oxysporum* f. sp. *cepae*. The survey was conducted by looking for plants that were attacked or had symptoms of root rot attack with the characteristics of the plants drying out, and there was white and rotten mycelium when removed from the tubers. Plants that experience attack symptoms were brought for isolation and collection of pathogens.

The *F. oxysporum* f. sp. *cepae* isolated from plants showing symptoms of infection by cutting necrotic tissue. The tissue pieces were placed on acidified PDA medium by adding 2 mL of 25% lactic acid L-1 and incubated for 7 days (Kim et al. 2001). A total of 32 isolates were collected for identification to obtain isolates with special characteristics. A total of 32 isolates were grouped based on the origin, and isolates were selected for use in the virulence test.

Identification of colonies of *F. oxysporum* f. sp. *cepae* of each isolate was carried out under a microscope and identified as *F. oxysporum* f. sp. *cepae* transferred to the PDA. Pure isolate from *F. oxysporum* f. sp. *cepae* stored in sterile liquid paraffin solution and stored at room temperature as a collection and can be used for virulence testing and genetic diversity analysis. The isolates obtained were then grouped based on the similarity of colony structure, color, and colony growth.

Virulence test

This virulence test used garlic seeds. The test was carried out by 2 methods as follows:

(i) Inoculation of garlic seeds on colonies of *F. oxysporum* f. sp. *cepae* on PDA medium. The garlic seeds to be inoculated were washed with 70% alcohol and rinsed with sterile distilled water, and then the seeds were dried and wiped with a tissue. Garlic seeds were cut on one side with a sterile ose needle. Afterward, the seeds were placed on the colonies of *F. oxysporum* f. sp. *cepae* with the injured side in direct contact with the pathogenic colony. The test was arranged according to a Completely Randomized Design (CRD) with 4 replications. Observations were made 2 weeks after inoculation by observing the intensity of root rot disease in garlic bulbs by measuring the percentage of tuber tissue with rot symptoms and colonized by pathogens. (ii) Inoculation of garlic seeds on soil infested with *F. oxysporum* f.sp. *cepae*. Garlic seeds were planted on soil infested with *F. oxysporum* f. sp. *cepae*. There are 2 varieties of seeds, namely the Chinese garlic variety and the Tawangmangu local variety. The soil was placed in a 10 x 20 cm tray with a soil depth of 5 cm, then watered until the soil was wet. The seeds were cleaned from the dry outer layer of the tubers, washed with 70% alcohol, rinsed with sterile distilled water, then planted in the soil in trays. Half of the tuber was immersed in the soil infested with the pathogen, especially the base of the tuber, to accelerate growth. Treatment was done by watering every morning to maintain soil moisture. The test was arranged in a completely randomized design with 3 replications. Observations were made 2 weeks after inoculation. Observations were made by observing the intensity of disease in the seeds planted by dismantling the planted tubers and then measuring the percentage of tuber tissue showing signs of rot. The percentage of damage was calculated by looking directly at the amount of damage that occurred in the seeds, and the results were converted into a percentage to make it easier to see the virulence. The amount of damage was calculated from the number of parts of garlic rotted or colonized by *F. oxysporum* f. sp. *cepae* and expressed in percent between 0-100%.

Analysis of the results of the virulence test observations using analysis of variance based on the F test at levels of 5% and 1%, if there is a significant difference, then proceed with the DMRT test.

Genetic diversity test of *F. oxysporum* f. sp. *cepae*

Breeding of F. oxysporum *f. sp.* cepae *for DNA extraction*

Selected pathogen isolates tested for virulence were cultured in a PDB medium. DNA extraction was initiated by preparing the culture of *F. oxysporum* f. sp. *cepae* from the existing PDA medium. Preparation for making a PDB medium is to sterilize 50 mL of PDB medium in a 100 mL Erlenmeyer tube for 15 minutes at 121°C. Next, the available cultures of *F. oxysporum* f. sp. *cepae* were put into the PDB medium, which had been cold and incubated in a rotary shaker for 5 days. The fungus that had grown was separated from the PDB medium by centrifugation and then washed with sterile distilled water until no medium was carried away. Next, the fungus suspension that had been cleaned was filtered with filter paper to separate the fungus and liquid. Finally, the fungus was ready for the DNA extraction process with CTAB.

The next process was the extraction of DNA from *F. oxysporum* f. sp. *cepae* with CTAB, starting with weighing as much as 0.5 grams of fungus mycelium. Next, the mycelium fungus was ground in a mortar with the addition of 2% CTAB 300 L and a little quartz sand to make it smooth quickly. Next, the finely ground mycelium was transferred to an Eppendorf tube to be heated in a water bath of 65°C for 30 minutes and then shaken for 10 minutes. Next, the Eppendorf tube was centrifuged for 5 minutes at a speed of 5000 rotations, the supernatant (top clear liquid) was transferred to another Eppendorf tube, and the pellet was left in the tube.

The precipitate or pellet in the Eppendorf tube was added with CIAA until the tube was full and shaken until homogeneous, then centrifuged at 12000 rotations for 10 minutes, and the supernatant contained in the tube was transferred to another Eppendorf tube while the pellet was left in an Eppendorf tube. The pellets were then added with absolute ethanol/alcohol until the tube was full and stored in a refrigerator at -20°C for 1-3 hours.

The alcohol (supernatant) was discarded, and the pellet was diluted again with 70% alcohol until the tube was full. The Eppendorf tube was centrifuged at 12000 rotations for 10 minutes. The alcohol (supernatant) was discarded, and the DNA pellet was dried in LAF for ± 2 hours. The DNA pellet was dissolved in a suspension of Miliq water and stored in a refrigerator at -20°C. Extraction results were seen by electrophoresis on 0.8% gel with ethidium bromide dye to ensure the presence of DNA in the pellet.

PCR-RAPD

The DNA amplification fingerprinting (DAF) system was carried out according to Bentley and Bassam (1996) with minor modifications, including amplification reactions by PCR, thermal cycle conditions, and electrophoresis to be carried out. The primary sequence used in the RAPD analysis was 5''-GATGAGCC-3'' (Bentley et al. 1998).

The PCR kit used was Mega Mix Royal (MMR). In one PCR process, 5 µL of MMR and other mixtures, namely 5 µL Primer, 2 µL template, and 8 µL Miliq water were needed. PCR-RAPD was performed using a Thermal cycler (BioRad) machine. The extracted DNA would be directly carried out by the PCR process in a PCR machine with an initiation program of denaturation at a temperature of 94°C for 5 minutes for 1 time, denaturation at a temperature of 94°C for 30 seconds for 30 times, annealing (separation) at a temperature of 35°C for 30 seconds for 30 times, extensions at 72°C for 30 seconds for 30 times, final extensions at 72°C for 5 minutes for 30 times.

The PCR program process ended with equilibration at a temperature of 20°C until the process ended on its own. After all processes end, the DNA from the PCR-RAPD would be directly electrophoresed or stored at 4°C until electrophoresis was carried out.

Electrophoresis for visualizing PCR-RAPD results

Electrophoresis began with the manufacture of an electrophoretic gel. Preparation began with weighing 0.8% agarose as much as 0.325 g (for 40 mL TBE gel molds with 17 gel wells) and heated in the oven for a maximum of 3 minutes. After it was not too hot, 40 µL of 0.1% ethidium bromide was added, and the agarose was shaken until homogeneous and then poured into a mold fitted with a perforation comb. After more than 20 minutes or hardened, the comb was removed. Next, the gel with the gel mold base was transferred to an electrophoresis bath to be used in the electrophoresis process.

The tub was filled with 0.5X TBE until the gel was immersed in the solution. Then, each gel well was filled with 5-20 µL of PCR result DNA, and the final well was filled with 10 µL of marker (DNA marker). Finally, the electrophoresis machine was closed, and the power supply was turned on at 100 volts for 35 minutes. After the electrophoresis process was complete, the DNA fragment pattern was seen, and the characteristics of the DNA fragment similarity pattern were tested using the NTYSIS program.

Analysis of PCR-RAPD results

The similarity analysis of DNA fragment patterns was analyzed with the NTYSIS software. In addition, analysis of gel electrophoresis data was carried out using FreeTree software (Hampl et al. 2001) with the Unweighted Pair Group Method with Arithmetic mean (UPGMA) method to determine the similarity of patterns between isolates and grouping and relationships between isolates in the form of a dendrogram. The similarity pattern was between 0.0-1 (0-100%) based on the numbers that appear in the NTYSIS program and the UPGMA method.

RESULTS AND DISCUSSION

Fusarium survey results and collection

The survey results showed that endemic garlic plantations were from the Gondosuli area, while non-endemic garlic plantations were from the Blumbang area. The results of the isolation of *F. oxysporum* f. sp. *cepae* from the two areas produced 32 isolates. A number of these isolates were grouped into 8 groups based on growth structure and colony color with extracellular. Each group was represented by 1 isolate to test for virulence and genetic diversity. Those 8 isolates consisted of 4 from endemic areas and 4 from non-endemic areas (Table 1).

Table 1. Isolates of *F. oxysporum* f. sp. *cepae* used for virulence and DNA characteristics testing

Isolates code	Origin	Description
FCp1	Gondosuli	Endemic
FCp2	Gondosuli	Endemic
FCp3	Gondosuli	Endemic
FCp4	Gondosuli	Endemic
FCp5	Blumbang	Non-endemic
FCp6	Blumbang	Non-endemic
FCp7	Blumbang	Non-endemic
FCp8	Blumbang	Non-endemic

Virulence test results

Inoculation of garlic seeds on colonies of F. oxysporum *f. sp.* cepae *on PDA medium*

The inoculation of garlic seeds on colonies of *F. oxysporum* f. sp. *cepae* (Figure 1) showed that the intensity of disease attack between isolates on garlic seeds was less varied. All existing isolates caused disease intensity or were virulent, while for controls, there were no damage symptoms. In addition, all isolates caused a high average disease intensity, which was more than 75% (Figure 2).

Based on the intensity of disease in the PRC varieties, it can be concluded that the virulence between isolates of *F. oxysporum* f. sp. *cepae* varies. However, if it is associated with the area of origin, the grouping is not related to the disease status of the area of origin. Not all isolates from endemic areas showed high virulence; vice versa, not all isolates from non-endemic areas showed low virulence. For example, FCp1 isolates from endemic areas were not virulent, with a disease intensity of 0.00%. On the other hand, FCp5 isolates from non-endemic areas showed the highest virulence by showing the highest disease intensity, 100%.

Different results were shown by inoculation of garlic seeds on soil infested with *F. oxysporum* f. sp. *cepae* with local varieties of garlic Tawangmangu which results are less varied. All pathogen isolates can cause disease intensity.

Inoculation of garlic seeds on soil infested with F. oxysporum *f. sp.* cepae *with local varieties of garlic Tawangmangu*

Local varieties of garlic Tawangmangu is one type of onion widely cultivated in Tawangmangu. Still, this variety is only a local superior, so it is not widely known and cultivated in other areas. The testing of the Tawangmangu Local variety was also carried out in the same way as the PRC variety. It was used as a comparison to find out how far the virulence caused by the various isolates was. Results of inoculation of garlic seeds on soil infested with *F. oxysporum* f. sp. *cepae* with local varieties of garlic Tawangmangu showed less varied results. The highest disease intensity was caused by FCp4 isolates, which was 73.33% from endemic areas, but isolates from non-endemic areas, namely FCp5 and FCp7 isolates, also caused quite serious damage, reaching more than 60% (Figure 3).

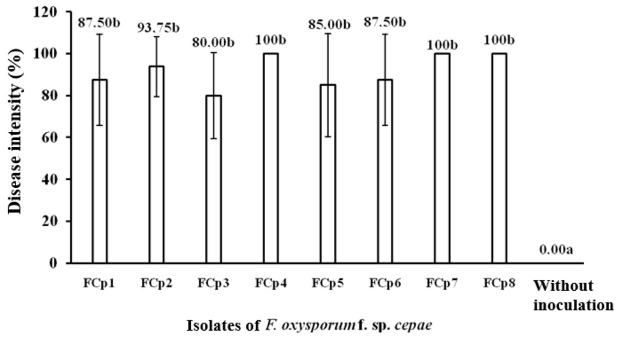

Figure 1. The average disease intensity of garlic seeds over a colony of 8 isolates of *F. oxysporum* f. sp. *cepae* on a PDA medium in a petri dish. Note: Numbers followed by the same letter show no significant difference based on DMRT 5% based on data transformed to Arc sin X

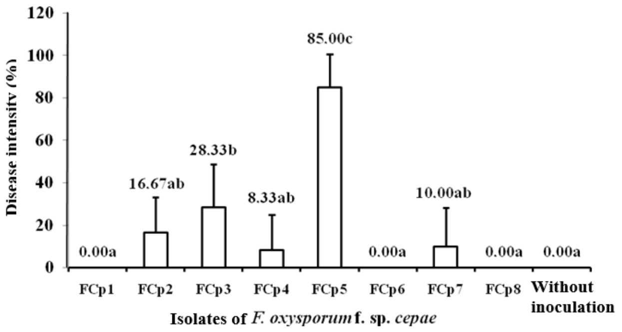

Figure 2. The average percentage of disease intensity in garlic seeds of PRC varieties grown on soil infested with *F. oxysporum* f. sp. *cepae.* Note: Numbers followed by the same letter show no significant difference based on DMRT 5% based on data transformed to Arc sin X

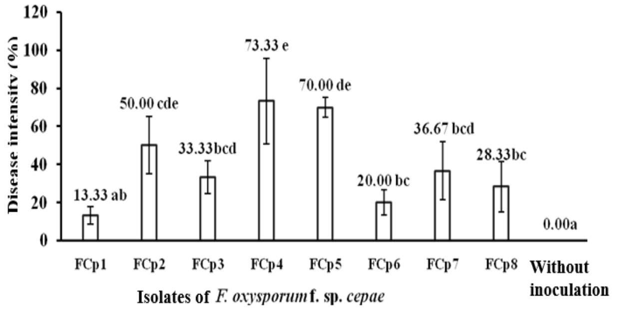

Figure 3. The average percentage of disease intensity in local varieties of garlic seeds Tawangmangu grown on soil infested with *F. oxysporum* f. sp. *Cepae.* Note: Numbers followed by the same letter show no significant difference based on DMRT 5% based on data transformed to Arc sin X

This result was different from inoculation using the PRC variety. It is thought to be caused by all the isolates that have long been associated with the Tawangmangu local variety so that the fungus is adaptive as a pathogen in that variety. The higher attack of *F. oxysporum* f sp *cepae* on the Tawangmangu local variety compared to the PRC variety was different from the results of field observations which showed the opposite, namely, the Tawangmangu local variety was more resistant (Hadiwiyono 2004). This difference in results is thought to be caused by different environmental conditions; namely, the virulence test was carried out in the lowlands, 110 m above sea level (asl), while the Tawangmangu field was above 600 m asl. Environmental differences, especially temperature due to altitude differences, can affect the ability of pathogen infection and plant resistance. In addition, plants that grow outside their ecological area can cause crop stress. Environmental stress in plants can predispose them to pathogen infection by weak parasites such as *F. oxysporum* f. sp. *cepae* (Agrios 2005).

Genetic diversity test of *F. oxysporum* f. sp. *cepae*

The results of the visualization of DNA fragments through PCR-RAPD are shown in Figure 4. The length of the fragments ranges from 80 to 200 base pairs (bp). The dendrogram (Figure 5) shows that the coefficient of genetic similarity between the sample isolates is between 0.25-1.00 (25%-100%). At the coefficient value of 25%, it was divided into 2 groups of strains, namely group 1 (FCp3, FCp5, FCp6, FCp8) and group 2 (FCp1, FCp4, FCp2, and FCp7), so that the overall isolate group was divided into 3 isolate strains, namely strain 1 (FCp1 and FCp4), strain 2 (FCp2 and FCp7), and strain 3 (FCp3, FCp5, FCp6, and FCp8). Of the three strains, only strain 1 (FCp1 and FCp4) and strain 2 (FCp2 and FCp7) had the closest relationship of 51%, while the highest relationship of 100% was found in each strain. The furthest kinship between strains 1, 2, and 3 was only 25%.

Analysis of the level of genetic diversity of 8 isolates based on RAPD analysis had a coefficient of genetic similarity of 25% or genetic variation of 75%. It proves that there has been a genetic change in *F. oxysporum* f. sp. *cepae* with high genetic variation due to the continuous cultivation of garlic. These results are also in line with studies on isolates of *F. oxysporum* f. sp. *cubense* with RAPD-PCR, which shows very high genetic diversity with a similarity level of 0.25-0.95 (25%-95%) and can be grouped into 4 groups (Anonymous 2008).

Based on the virulence test, there is no significant difference in the damage caused by various isolates between endemic and non-endemic areas. These results are also seen in the DNA pattern of PCR-RAPD. There is no difference in the pattern of DNA fragments in endemic and non-endemic areas, but from these results, 3 groups of DNA strains can be obtained (Figure 5). The grouping between lines does not include differences between endemic and non-endemic areas. In line 1, all isolates are isolates from endemic areas; in line 2, there are isolates from endemic areas (FCp2 isolates) and non-endemic (FCp7 isolates); while strain 3 is also a mixture of isolates from endemic areas (FCp3 isolates) and non-endemic areas. (FCp5, FCp6, and FCp8) isolates. It proves that there is genetic diversity in *F. oxysporum* f. sp. *cepae* isolated from Tawangmangu. However, the genetic diversity of these pathogens is not related to the disease status of the area of origin of the isolates.

The results of this study indicate that the virulence of the pathogen is not the only factor that causes endemic disease or the low intensity of base rot disease in certain fields. According to Hadiwiyono and Widono (2008) and Hadiwoyono et al. (2008; 2009), the root rot of garlic in Tawangmangu is determined by the physical, chemical, and biological characteristics of the soil. The high content of organic matter and N and low P and K causes an increase in the intensity of garlic root rot. In addition, non-endemic soils have a higher microbial population (fungi, bacteria, and actinomycetes) than endemic soils.

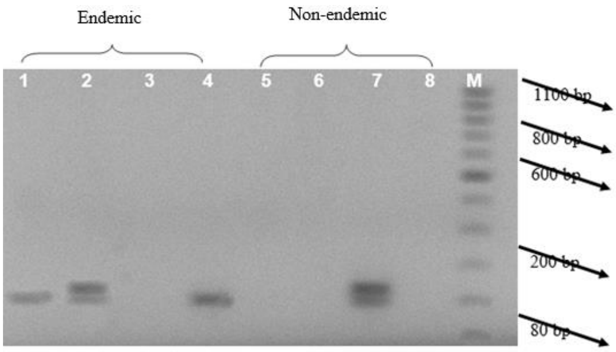

Figure 4. The pattern of DNA fragments of *F. oxysporum* f. sp. *cepae* via PCR-RAPD. Note: 1: FCp1 isolate, 2: FCp2 isolate, 3: FCp3 isolate, 4: FCp4 isolate, 5: FCp5 isolate, 6: FCp6 isolate, 7: FCp7 isolate, 8: FCp8 isolate, and M: Marker

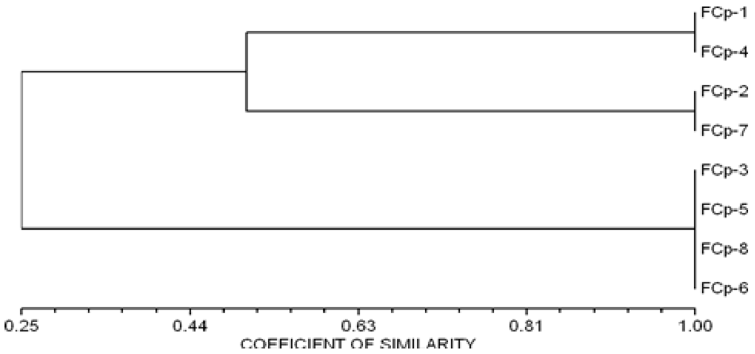

Figure 5. Dendrogram-UPGMA based on the pattern of DNA fragments from PCR-RAPD results in 8 isolates of *F. oxysporum* f. sp. *cepae* (Coefficient of similarity)

In conclusion, the PCR-RAPD analysis of *F. oxysporum* f. sp. *cepae* could be grouped into 3 strains. Still, the grouping is unrelated to the virulence and origin of endemic or non-endemic isolates.

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