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Detection and Practical Differentiation of Phytoplasmas from Several Host Plants Using PCR-RFLP

Deteksi dan Pembedaan Praktis Fitoplasma dari Beberapa Tanaman Inang Menggunakan PCR-RFLP

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ABSTRACT

Phytoplasma as a phytopathogenic prokaryote with a wide host range is a pathogen that needs more attention in Indonesia. This pathogen is relatively difficult to detect and identify due to its complicated biological properties. This study involved detection of phytoplasmas by polymerase chain reaction (PCR) technique with P1/P7 primers from seven symptomatic plants, i.e. Bermuda grass white leaf, bamboo yellows, witches' broom of peanut, soybean, yard long bean, and cactus, and sweet potato little leaf. The phytoplasma DNA of the 16S rRNA gene resulting from PCR amplification was examined by digestion reaction using three endonuclease enzymes *Alu*I, *RSa*I, and *MSe*I to generate restriction fragment length polymorphism (RFLP) profile. The seven diseased plants were confirmed positive to be associated with phytoplasma as indicated by the PCR product of 1800 bp. Based on the RFLP profiles of the three enzymes, the phytoplasmas were divided into two groups, namely group I (Bermuda grass and bamboo) and group II (peanuts, soybeans, yard long beans, cactus, and sweet potatoes). Cactus phytoplasma is a sub-group (strain) because it has a slightly different fragment of *MSe*I RFLP profile.

Keywords: 16SrRNA gene, fastidious prokaryotes, restriction endonucleases, yellows disease, witches' broom

ABSTRAK

Fitoplasma adalah prokariota fitopatogenik dengan kisaran inang yang luas dan merupakan patogen yang perlu mendapatkan perhatian lebih banyak di Indonesia. Patogen ini relatif sulit untuk dideteksi dan diidentifikasi mengingat sifat biologinya yang rumit. Penelitian ini meliputi deteksi fitoplasma menggunakan teknik *polymerase chain reaction* (PCR) dengan primer P1/P7 dari tujuh tanaman bergejala, yaitu daun putih rumput Bermuda, kuning bambu, sapu kacang tanah, kedelai, kacang panjang dan kaktus, serta daun kecil ubi jalar. DNA gen 16S rRNA fitoplasma hasil amplifikasi PCR selanjutnya direaksikan dengan masing-masing enzim endonuklease restriksi *Alu*I, *RSa*I dan *MSe*I untuk menghasilkan pola *restriction fragment length polymorphism* (RFLP). Tujuh tanaman sakit tersebut terkonfirmasi berasosiasi dengan fitoplasma yang ditunjukkan oleh produk PCR sebesar 1800 pb.

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Berdasarkan profil RFLP tiga enzim, fitoplasma dibedakan ke dalam dua kelompok, yaitu kelompok I (rumput Bermuda dan bambu) dan kelompok II (kacang tanah, kedelai, kacang panjang, kaktus dan ubi jalar). Fitoplasma kaktus merupakan sub-kelompok (galur) karena memiliki sedikit perbedaan fragmen dari profil RFLP dengan enzim *MSe*I.

Kata kunci: endonuklease restriksi, gen 16SrRNA, penyakit kuning, penyakit sapu, prokariota fastidius

INTRODUCTION

Phytoplasma formerly known as mycoplasma-like organism (MLO) is a prokaryotic organism that lacks of rigid cell wall from the class Mollicute in the taxonomy of Bacteria. Phytoplasmas are known to cause disease in hundreds of host plant species (Bertaccini and Lee 2018). This pathogen causes types of diseases such as yellows, proliferation or witches' broom and decline. In Indonesia, diseases caused by phytoplasmas have been reported in various important crops, including peanut, soybean, yard long bean, yam bean, and crotalaria (Leguminosae); rice, sugarcane, bamboo and grasses (Graminae); sweet potato (Convolvulaceae); ornamental cactus opuntia (Cactaceae); coconut (Palmae); and carrots (Apiaceae) (Prasetya *et al.* 2018; Wulandari *et al.* 2021)

Disease caused by phytoplasma is relatively difficult to detect and identify because the symptoms are very similar to disease caused by viruses. Besides, phytoplasma is an obligate pathogen that cannot be cultured on artificial media, infects limited tissues in the phloem and is naturally transmitted through specific insect vectors. A reliable method for the detection and identification of phytoplasmas in plants or their vectors is polymerase chain reaction (PCR) using specific primers for amplification of the phytoplasma 16S rRNA gene region (Smart *et al.* 1996; IRPCM 2004; Wei and Zhao 2022). However, to determine down to the group (Sr16 group) or species (*Candidatus* phytoplasma sp.) further testing must be carried out with PCR amplicon nucleotide sequencing and nucleotide analysis (Duduk and Bertaccini *et al.* 2011) which can take time. The differentiation of the various phytoplasma 16S rRNA genes can be demonstrated by their so-called restriction fragment length polymorphism (RFLP) profiles (Lee *et al.* 1998; Duduk *et al.* 2013;). The RFLP profile can be developed based on DNA pattern after digestion of the DNA using endonuclease enzyme. Each endonuclease enzyme can cut the DNA at its specific restriction site. This enzymatic reaction can also be used to cut the phytoplasma DNA as the PCR product mentioned above to generate a restriction profile.

The objective of this study was to detect the phytoplasmas from several host plants showing disease symptoms, i.e. Bermuda grass, bamboo, peanut, soybean, yard long bean, sweet potato, and ornamental cactus using the PCR technique and further differentiated based on RFLP profiles generated from DNA pattern after digestion using restriction endonuclease enzymes *Alu*I*, RSa*I, and *MSe*I.

MATERIALS AND METHODS

Diseased plants suspected of being infected by phytoplasma with symptoms of white, yellow, broom, and small leaves were found in Bogor, Cianjur, and Manokwari (Table 1). Samples of young and fresh shoots, leaves, and petioles without necrosis were taken from all plants, except for the cactus in which young stems were taken. As much as 0.25 g of each sample was used in total DNA extraction following the Dellaporta *et al.* (1983) method.

PCR amplification using forward primer P1 (5'-AAG AGT TTG ATC CTG GCT CAG GAT T-3') (Deng and Hiruki 1991) and reverse primer P7 (5'-CGT CCT TCA TCG GCT CTT-3') (Schneider *et al.* 1996) to produce an amplicon of ±1800 bp were performed in a total volume of 50 µL consisted of reagents as follows: 5 µL buffer containing 1.5 mM $MgCl_2$, 5 µL dNTPs 0.1 mmol, 1–2 µL of 5 DNA template, each primer P1 and P7 0.4 µmol,

Table 1 Samples of diseased plants obtained in the field for laboratory testing

Plant host (species)	Family	Symptom	Location
Bermuda grass (*Cynodon dactylon*)	Graminae	White leaf	Bogor, West Java
Bamboo (*Bambusa vulgaris*)	Graminae	Yellows	Cianjur, West Java
Peanut (*Arachis hypogea*)	Leguminosae	Witches' broom	Bogor, West Java
Soybean (*Glycine max*)	Leguminosae	Witches' broom	Bogor, West Java
Yard long beans (*Vigna unguiculata*)	Leguminosae	Witches' broom	Bogor, West Java
Ornamental cactus (*Opuntia* sp.)	Cactaceae	Witches' broom	Cianjur, West Java
Sweet potato (*Ipomoea batatas*)	Convolvulaceae	Little leaf	Manokwari, West Papua

1–2 µL extracted DNA, 1 µL and *Taq* polymerase 1.5 U. The reaction was carried out in a thermocycler (Corbett FTS-320, USA) with the following program: initial denaturation at 92 °C, for 1 min; 35 times denaturation cycle (95 °C, 1 min), annealing (55 °C, 1 min), extension (72 °C, 1.5 min); elongation (72 °C, 10 minutes). The PCR amplicon DNA is stored in the freezer for next use.

Reaction of each restriction enzyme *Alu*I, *RSa*I, and *MSe*I (New England Biolabs®) to digest DNA PCR amplicons of each plant was carried out in a volume of 10 µL in a 0.5 mL tube consisting of: 1.5 µL buffer, 3 µL H_2O, 0.5 mL restriction endonuclease enzyme and 5 µL DNA amplicon PCR. The reaction was performed under incubation conditions of 37 °C for 2 hours using a thermocycler. The reaction was stopped by adding 2 µL dye (bromophenol blue 0.25%, xylene cyanol 0.25%, and 30% glycerol in H_2O).

As much as 2–3 µL of DNA PCR amplicon was separated by electrophoresis of 1% agarose gel in 0.5% TBE buffer, at 80 volts DC for 60 minutes; while for DNA resulting from restriction enzymes as much as 5–10 µL was electrophoresed with 2% agarose at 80 volts DC for 120 minutes. PCR DNA products or restriction profile fragments were visualized with a UV transilluminator and photographed.

RESULTS

Visual Symptoms of Diseased Plants from the Field

Bermuda grass with symptoms of white leaves begins with pale yellow chlorosis on young leaves, which then turn white. The size of the leaves does not change much. The white leaves dry out easily (Figure 1A). The symptoms on Bamboo are indicated by yellowing of the leaves in a group of twigs, the leaves appear to cluster like a broom symptom because the leaves are small but there are more in number than normal (Figure 1B). Symptoms of broom are shown by peanuts, soybeans and yard long beans: leaves that are very small in size yet in excessive quantities, but the color is still green; the overall crown was stunted (Figures 1 C, D, and E). The witches' broom symptom is also shown by the cactus in the form of the growth of small stem shoots but in large quantities. Diseased shoots can still grow larger with a flat shape and accompanied by a green mosaic between the red-green (Figure 1F). Diseased sweet potato has small leaves, reduced leaf size so that the plant becomes stunted, with a slightly pale color (Figure 1 G).

The Association of Phytoplasma on Several Host Plants

PCR method succeeded in proving that the seven symptomatic plant species were associated with phytoplasma infection as indicated by the amplification of bands measuring around 1800 bp (Figure 2). Phytoplasma specific P1/P7 primers amplify regions spanning the DNA of the whole 16S rRNA gene plus spacer region and the base of the 23S rRNA gene. The quantity of DNA as estimated by the band thickness appears to be slightly diverse although it was sufficient for further use in restriction enzyme digestion. The volume of PCR reaction as much as 50 µL for each positive sample is sufficient to guarantee the availability of material for the test as well.

Figure 1 Symptoms of diseased plants from the field confirmed to be infected by phytoplasma through PCR method.

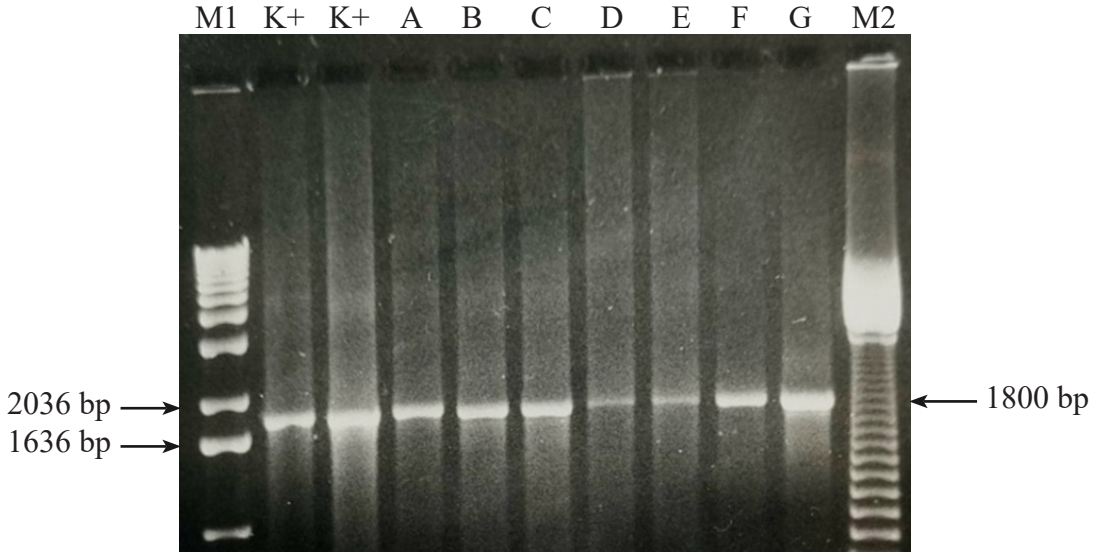

Figure 2 Phytoplasma specific products corresponded to 1800 bp 16S rRNA gene amplified using P1/P7 primers from Bermuda grass (A), bamboo (B), peanut (C), soybean (D), yard long bean (E), cactus (F), and sweet potato (G). K+, Positive control; M1, 1 Kb Ladder; and M2, 100 bp Ladder.

PCR-RFLP Profiles of Phytoplasmas from Several Host Plants

The enzyme digests of PCR products using *Alu*I, *RSa*I, and *MSe*I produced RFLP profiles which can be differentiated based on numbers and sizes of DNA fragments (Figure 3 and Table 2). The use of *Alu*I enzyme which has a restriction site AG↓CT resulted in two RFLP profiles, i.e. profile I shown by the phytoplasmas from Bermuda grass and bamboo samples consisting of four fragments measuring approximately 750, 400, 290, and 200 bp respectively. Profile II is shown by the phytoplasma from peanut, soybean, yard long bean, cactus, and sweet potato plants which also have four DNA fragments around 800, 380, 290, and 200 bp in size, respectively. Several non-specific bands appeared, for example in phytoplasma from yard long bean (approx. 1080, 980, and 580 bp), but were ignored from the RFLP profile because they have resulted from incomplete digestion. The use of *RSa*I enzyme which has a restriction site GT↓AC also produced two RFLP patterns, i.e. profile I was shown by the phytoplasma from Bermuda grass and bamboo consisting of three fragments measuring 870, 500, and 430 bp; while profile II was shown by the phytoplasma from peanut, soybean, yard long bean, cactus, and sweet potato which had four DNA fragments each measuring around 500, 450, 410, and 330 bp. The use of *MSe*I enzyme which has a restriction site T↓TAA resulted in three RFLP profiles with more fragments, i.e. profile I which was shown by the phytoplasma from Bermuda grass and bamboo which consisted of seven fragments measuring 410, 390, 250, 140, 130, 85, and 75 bp; while profile II was shown by the phytoplasma from peanut, soybean, yard long bean, and sweet potato which had eight DNA fragments measuring around 430, 350, 290, 180, 140, 100, 85, and 75 pb. Profile III shown by cactus is actually similar to pattern II which also has eight fragments, but the difference is that the first fragment is 550 in size, while the next seven fragments are the same as in profile II above.

DISCUSSION

Diagnosis of the disease caused by phytoplasma based on symptomatology is quite helpful because of the specificity of the symptoms. However, diseases caused by phytoplasmas has similarity with diseases caused by viruses for their symptoms and insect vectors (Wei and Zhao 2022). More accurate and sensitive detection method, such as using PCR-based method is required. Specific primers for phytoplasma, i.e. P1/P7 are commonly used in PCR. Seven diseased plants from fields are confirmed positively associated with phytoplasmas by PCR using P1/P7 primers.

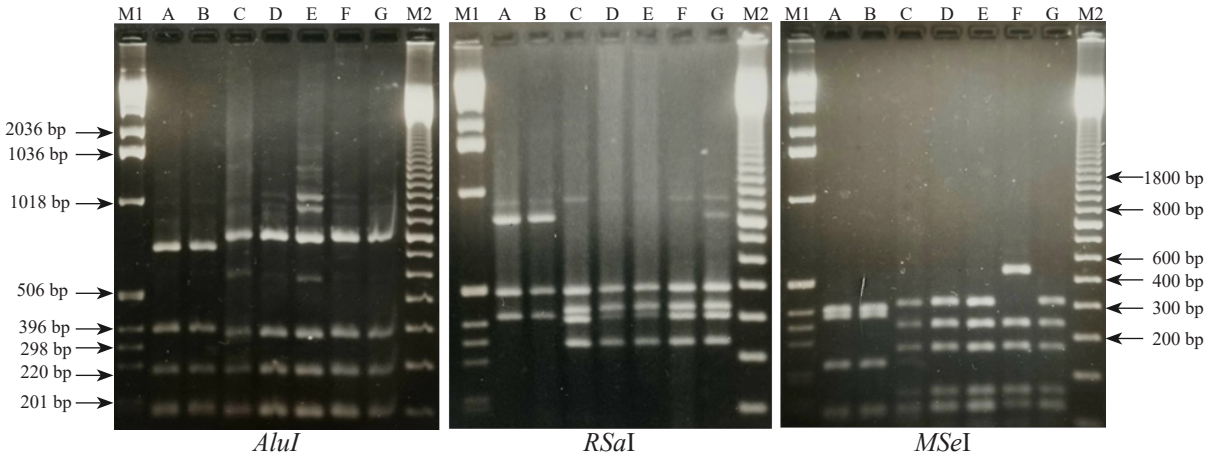

Figure 3 Fingerprint profile of *AluI*, *RSa*I and *MSe*I enzyme digestions for 16S rRNA gene PCR product of phytoplasma from Bermuda grass (A), bamboo (B), peanut (C), soybean (D), yard long bean (E), cactus (F) and sweet potato (G). M1, 1 Kb Ladder; and M2, 100 bp Ladder.

Table 2 Profile of the RFLP fragments of the phytoplasma 16S rRNA gene with enzymes *AluI, RSa*I and *MSe*I

Enzyme	Host plants	RFLP fragments of the phytoplasma16SrRNA gene (bp)											
*Alu*I	Bermuda grass		750	400		290	200						
	Bamboo		750	400		290	200						
	Peanut	800			380	290	200						
	Soybean	800			380	290	200						
	Yard long bean	800			380	290	200						
	Cactus	800			380	290	200						
	Sweet potato	800			380	290	200						
*RSa*I	Bermuda grass	870	500		430								
	Bamboo	870	500		430								
	Peanut		500	450	410	330							
	Soybean		500	450	410	330							
	Yard long bean		500	450	410	330							
	Cactus		500	450	410	330							
	Sweet potato		500	450	410	330							
*MSe*I	Bermuda grass			410	390			250		140	130	85	75
	Bamboo			410	390			250		140	130	85	75
	Peanut		430			350	290		180	140	100	85	75
	Soybean		430			350	290		180	140	100	85	75
	Yard long bean		430			350	290		180	140	100	85	75
	Cactus	550				350	290		180	140	100	85	75
	Sweet potato		430			350	290		180	140	100	85	75

For further identification and classification at lower levels of the genus (*Candidatus* phytoplasma), the nucleotide sequencing of the phytoplasmic ribosomal gene and nucleotide analysis became the standard method (Wei and Zhao 2022; IRPCM 2004). In Indonesia, it is very rare for laboratory testing to have nucleotide sequencing machine facilities, so nucleotide sequencing samples are usually sent to commercial companies abroad, which take several weeks to several months to obtain the results.

The use of dozens of selected restriction endonuclease enzymes, including *AluI, RSa*I, and *MSe*I to produce the RFLP profiles of the 16S rRNA gene as well as the complete nucleotide sequence of the gene (Prasetyo *et al.* 2017) has been established as the basis for the identification and classification of formal phytoplasmas at the species and even strain level (Lee *et al.* 1998; IRPCM 2004; Wei 2007). The use of *AluI, RSa*I, and *MSe*I for practical laboratory testing and immediate interest in this research has demonstrated its ability to discriminate between the phytoplasma of different plant groups. Based on RFLP profiles using these three enzyme restrictions, the phytoplasmas in this study can be differentiated into two groups and the groups can be correlated with disease symptoms and plant family taxon. Group I consisted of phytoplasmas infecting bamboo and Bermuda grass which both belong to Graminae family and causing yellow symptom. Group II showed different RFLP profile than those of group I and consisted of phytoplasma infecting peanuts, soybeans, yard long beans which all belong to Leguminosae family, cactus (Cactaceae) and sweet potato (Convolvulaceae). The symptom type on peanut, soybean, yard long bean and cactus is witches' broom, while on sweet potato is little leaf which are actually witches' broom types as well. There is a slight difference on RFLP pattern of phytoplasma infecting cactus compared to the pattern of the other group II members.

The pattern of *AluI* and *RSa*I is identical among all members, but there is one fragment different of *MSe*I pattern from cactus samples. Thus, phytoplasma infecting cactus is thought to be a different strain in group II.

This series of PCR-RFLP examinations including DNA extraction, PCR amplification, restriction enzyme reactions, and gel electrophoresis visualization can be carried out in one or two full days. Visualization of DNA profiles for the best PCR-RFLP results should be done using polyacrylamide gel electrophoresis. However, using agarose gel which is much simpler and faster was able to give sufficient profiles for the analysis. This whole method can be said to be a quick and practical method for the detection and identification of phytoplasmas as causative agents in the diagnosis of plant diseases.

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