Incidence and severity of mottle disease in black pepper plants (*piper nigrum*) in Sukamulya Research Station, Sukabumi Regency, West Java

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Abstract. Mottle disease, caused by viral infection is one of the main disease in black pepper plants. *Piper yellow mottle virus* (PYMoV) and *Cucumber mosaic virus* (CMV) are recorded as the causal agent of mottle disease in several countries. Research was conducted at the Sukamulya Research Station, Sukabumi Regency, West Java, to observe disease incidence and identify the associated virus. Field observation and sampling was carried out on 50 black pepper plants, aged around 3-4 years. Virus detection was performed by PCR and reverse transcription (RT)-PCR using a primer pair that amplified the ORF III of PYMoV (AIB 104/AIB 105), and CMV coat protein (AIB 1/AIB 2), followed by sequencing and analysis of nucleotide sequences. Incidence and severity of mottle disease in Sukamulya reached 100% and 32.50%, respectively. Specific DNA target were successfully amplified, i.e. 400 bp and 650 bp for PYMoV and CMV, respectively. Phylogenetic analyses revealed that PYMoV isolate from Sukamulya is closely related with PYMoV isolates from India (DQ83623); while CMV isolate from Sukamulya is closely related with CMV isolates from Indonesian bananas (AB069971), and it is belonged to subgroup 1B, as did CMV from black pepper in India (AY545924).

Keywords: *Cucumber mosaic virus*, nucleotide sequence analysis, *Piper yellow mottle virus*, phylogenetic analyses, PCR

1. Introduction

Black pepper (family Piperaceae) is a perennial plant that starts to bear fruit at the age of 2 – 3 years. Crop yields increase every year until they are 8 years old, then it begins to decline. However, if plants are well maintained, they can still productive up to 15 years or more [1]. Black pepper is one of Indonesia’s important export commodities. Indonesia became the 4th largest exporter country of black pepper in the world in the 2009 – 2013 period, with a contribution of 4.3 – 5.73% of the world’s total needs [2]. As one of the main spice, black pepper is not only used as a cooking spice, but it also plays a significant role for medicine, including controlling blood pressure and fat, and as anticancer. In some countries, there have been diversified black pepper products such as various green black pepper products and its derivatives in the form of oil, oleoresin and microencapsulation of oleoresin [3].
Mottle disease caused by viruses is one of the black pepper main diseases and has been recorded in several countries i.e. India, Thailand, Malaysia, Sri Lanka, Brazil, Philippines and China [4, 5, 6]. This disease is associated with Piper yellow mottle virus (PYMoV) (Badnavirus : Caulimoviridae) and Cucumber mosaic virus (CMV) (Cucumovirus : Bromoviridae) [6, 7, 8, 9]. Both viruses have also been detected on black pepper plantations in Indonesia [10, 11]. The incidence of the disease reached 95% in Bangka and Lampung in 2005 [12]. The high incidence of this disease was also observed in Yogyakarta (86 – 93.75%) [10]. The molecular character of the viruses that cause mottle disease in black pepper in Indonesia has not been reported, although preliminary sequence analysis indicated the infection of PYMoV. BLAST analysis of partial ORF III showed high nucleotide sequence homology between PYMoV isolate from Bogor (West Java) and those of Vietnam (KF0568650) [13]. Earlier, phylogeny analysis of PYMoV isolate from Yogyakarta and Bangka showed their close relationship with those from India [10]. Meanwhile, molecular identity of CMV isolated from black pepper in Indonesia has not been reported.

Sukamulya Research Station is one of the black pepper nurseries of the Indonesian Spice and Medicinal Crops Research Institute (ISMECRI). The survey of mottle disease in 2005 showed that PYMoV and CMV were detected by serological method in several black pepper plants at Sukamulya [12]. Serological detection of seed from vegetative propagation in Sukamulya also showed a high incidence of CMV, i.e. 66.67% [14]. This study determined the current condition of mottle disease in black pepper plantation in Sukamulya Research Station and molecular identity of the viruses.

2. Materials and methods

Disease observation and leaf sampling were carried out randomly on 50 black pepper plants, aged around 3-4 years. Disease incidence (DI) and disease severity (DS) were determined based on the appearance of the symptoms on plants, and were calculated by the formula below; whereas scoring of symptom severity is based on the percentage of disease symptoms in plants as described before [10] with modification (Table 1).

\[
DI = \frac{n \times 100}{N}
\]

\[
DS = \frac{\sum (n \times v) \times 100}{N \times V}
\]

Table 1. Symptoms scoring for mottle disease of black pepper plants

| Score | Description, % symptom                          |
|-------|-------------------------------------------------|
| 0     | No symptoms                                     |
| 1     | Mild mottle, 1 – 10%                            |
| 2     | Mottle, 11 – 25%                                |
| 3     | Mottle and malformation, 26 – 50%               |
| 4     | Mottle and malformation, stunting, 51 – 75%    |
| 5     | Mottle and malformation, stunting, > 76%       |

n, number of symptomatic plants
N, sum of all plants observed
v, specific symptom score,
N, sum of all plants observed,
V, the highest score of disease symptom
Leaves showing obvious symptoms of mottle disease were collected as a sample for virus identification. In the laboratory, each leaf sample was weighed as much as 0.1 g, stored in thick plastic bags, then stored in a freezer of -80 °C until used. Virus detection was carried out by PCR and two-step reverse transcription-PCR (RT-PCR). Twenty-five leaf samples were subjected for virus detection. Total DNA isolation was performed according to the CTAB method with modification [5]. Total RNA isolation was conducted with the same buffer for DNA isolation, followed by incubation of solution at 55 °C for 10 minutes, and then the next step was done according to the previous protocol [15]. The quality of total DNA and RNA was measured by Nanophotometer Implen (Germany). Complementary DNA (cDNA) was synthesized by oligo dT primer with total RNA as template [16]. Specific primer for PYMoV was used for amplification of DNA, i.e. PYMoV3-F/-R (AIB 104/AIB 105), while primer for CMV was used for cDNA amplification, i.e. CMV-F/-R (AIB 1/AIB 2) (Table 2 and 3). For negative control, nuclease-free water was used as a template. The amplicon were loaded on to a agarose gel (1.5%) containing RedSafe™ nucleic acid (Intron Biotechnology) in TAE 0.5x buffer, and visualized using Geldoc Fire Reader V4 (Uvitec Cambridge).

DNA amplicon was then subjected for sequencing. Nucleotide sequence analysis was carried out using BioEdit Sequence Alignment Editor program, followed by construction of the phylogeny tree by MEGA X program.

**Table 2. Amplification program of CMV and PYMoV**

| Target viruses (primer code) | Program | References |
|-----------------------------|---------|------------|
| CMV (AIB 1/AIB 2)           | Pre denaturation (95 °C for 3 min); 40 cycles consists of denaturation (95 °C for 30 s), primer annealing (50 °C for 1 min) and elongation (72 °C for 1 min); final extension (72 °C for 10 min) | [15] |
| PYMoV (AIB 104/AIB 105)     | Pre denaturation (95 °C for 3 min); 34 cycles consists of denaturation (95 °C for 30 s), primer annealing (56 °C for 1 min) and elongation (72 °C for 1 min); final extension (72 °C for 10 min) | [5] |

**Table 3. Description of CMV and PYMoV primers**

| Primer code | Sequence (5’……3’) | Region amplified | Amplicon size (bp) | References |
|-------------|---------------------|-----------------|-------------------|------------|
| AIB 1       | ATGGACAATCTGAATCAAC | Coat protein    | 650               | [15]       |
| AIB 2       | TCAACTGGGAGCACCC    |                 |                   |            |
| AIB 104     | CTATATGAATGGCTAGTGATG | ORF III         | 400               | [5]        |
| AIB 105     | TTCTATGTTTTGGTATGTATG |                 |                   |            |

3. Result and discussion

3.1. Symptom, incidence and severity of the disease

General disease symptom found in the field was mottle on the leaves, with variations involving chlorotic, mosaic, malformation and thickening of the leaves. Imperfect formation of fruit set was observed in the black pepper fruit. Plants with a high disease severity showed stunting on the shoots, i.e. small leaves and longer stem segments (Fig 1). The symptoms observed were similar to those reported previously in Indonesia and other countries [10, 12, 17, 18]. Imperfect formation of fruit set is thought to cause yield loss of black pepper [17, 19].
3.2. Virus detection
Target DNA of 400 bp and 650 bp was successfully amplified using primer pairs AIB104/AIB105 and AIB1/AIB2, respectively (Fig 2a and 2b). Following sequencing of DNA amplicon it was confirmed that 400 bp and 650 bp bands was associated with PYMoV and CMV, respectively. A total of 24 out of 25 samples was positively infected by PYMoV; whereas CMV infection was only evidenced from 1 sample, i.e. plant showing stunting symptom. The same sample was also infected by PYMoV indicated multiple infection of CMV and PYMoV. The high frequency of PYMoV infection proved that PYMoV is the dominant virus causing mottle disease in Sukamulya. Multiplex RT-PCR detection with DNA and RNA template in the previous study also reported similar result, infection of PYMoV, CMV, and their combination was found from 24, 7, and 3 samples out of 49 samples tested, respectively [21]. More severe symptoms on plants infected by PYMoV and CMV in a mix infection situation had also been reported before [19].

3.3. Sequence Identities and Phylogenetic Analysis
Nucleotide and amino acids sequence of PYMoV isolate from Sukamulya shared high homologies with PYMoV isolates from India and China, i.e. 89.7 – 93.5% and 90.2 – 93.9%, respectively (Table 4). Furthermore, phylogenetic analysis showed that PYMoV isolate from Sukamulya is clustered with PYMoV isolates from India (DQ836237) (Fig 3). Previous study reported that two PYMoV isolates from Yogyakarta and one isolate from Bangka were closed to Indian isolates based on phylogeny tree analysis [10]. Unfortunately, the sequences could not been included in this study because it has not been submitted to Genbank. A close phylogenetic relationships between PYMoV isolate from Sukamulya and from India indicates that both viruses share a similar origin, and maybe migrated to Indonesia through infected plant materials or insect vectors.
Figure 2. Visualization of DNA fragments as PCR and RT-PCR products in 1.5% agarose gel. Amplification was carried out using AIB 1/AIB2 primer pairs (a) and AIB 104/AIB 105 primer pairs (b). M, Marker DNA 100 bp; sample 1, Negative control; samples 2 – 6, Black pepper samples infected with viruses.

Table 4 Sequence homology between partial ORF III of PYMoV isolate from Sukamulya with those of several isolates from GenBank

| Virus   | Genbank accession | Host         | Country | Homology (%) |             |             |
|---------|-------------------|--------------|---------|--------------|-------------|-------------|
|         |                   |              |         | Nucleotide   | Amino acid  |             |
| PYMoV   | DQ836237          | P. nigrum    | India   | 93.5         | 92.4        |
|         | MF996374          | P. nigrum    | China   | 92.0         | 93.9        |
|         | MF996373          | P. nigrum    | China   | 90.7         | 93.2        |
|         | KJ873043          | P. longum    | India   | 89.7         | 90.2        |
|         | KJ873042          | P. betle     | India   | 89.7         | 91.7        |
| CYMV*   | EU489744          | Citrus sp.   | India   | 65.2         | 63.1        |

Note: *CYMV, Citrus yellow mosaic virus (Caulimoviridae : Badnavirus) as outgroup

Sequence analysis revealed that CMV isolate from Sukamulya has high homologies with CMV isolates in subgroup IB, both in nucleotide and amino acid levels, i.e. 93.4 – 95.7% and 96.7 – 99.5%, respectively (Table 5). This result suggested that CMV isolate from Sukamulya was belonged to subgroup IB, like black pepper CMV isolate from India (AY545924) [8]. While CMV isolate from black pepper in China (JQ895557) [9] was indicated belongs to subgroup IA. Moreover, based on the phylogenetic tree, CMV isolate from Sukamulya was closely related to CMV isolate from Indonesia (AB069971) (Fig 4). This analysis confirmed that both isolates is Indonesian origin.
**Figure 3.** Phylogeny tree involving nucleotide sequence of partial ORF III of PYMoV isolate from Sukamulya and other PYMoV isolates from several countries. *Citrus yellow mosaic virus* (CYMV) is included as outgroup.

**Table 5.** Sequence homology between CP genes of CMV isolate from Sukamulya with those of several isolates from GenBank

| Virus   | Genbank accession | Host                     | Country     | Homology (%) |            |            |
|---------|-------------------|--------------------------|-------------|---------------|------------|------------|
|         |                   |                          |             | Nucleotide   | Amino acid |
| CMV IB  | AB069971          | *Musa sapientum*         | Indonesia   | 95.7          | 99.5       |
|         | KC122260          | *Solanum lycopersicum*   | Iran        | 95.2          | 97.7       |
|         | AY541691          | *Nicotiana tabacum*      | Greece      | 94.9          | 98.1       |
|         | LN813014          | *Lycopersicon esculentum*| Egypt       | 94.0          | 97.2       |
|         | HE999617          | *Nicotiana tabacum*      | Vietnam     | 94.0          | 96.7       |
|         | AY545924          | *P. nigrum*              | India       | 93.4          | 97.2       |
| CMV IA  | JQ895557          | *P. nigrum*              | China       | 91.9          | 96.3       |
|         | AY871069          | *Cucumis sativus*        | Iran        | 91.4          | 97.2       |
|         | JX570737          | *Canna indica*           | India       | 91.4          | 89.4       |
|         | AJ131623          | *Gladiolus sp*           | Netherland  | 90.7          | 95.4       |
| CMV II  | U10923            | *Spnacia oleracea*       | USA         | 76.2          | 80.3       |
|         | AB109908          | *Capsicum annum*         | Korea       | 76.5          | 81.7       |
|         | AJ1585086         | *Lilium sp*              | India       | 75.0          | 78.5       |
| PSV*    | KF800742          | *Medicago sp.*           | Iran        | 55.8          | 47.9       |

*PSV, Peanut stunt virus* (Bromoviridae, Cucumovirus) as outgroup.
4. Conclusions
Mottle disease of black pepper in Sukamulya Research Station was associated with PYMoV and CMV subgroup IB. The disease has spread widely in the field and probably no mother plants is virus free. Disease spread should be controlled by reducing inoculum source in the field. This can be done among others by using virus-free plant materials and removal of infected vines from the field. The PCR and RT-PCR method in this study might be applied for identifying infected black pepper plants, such as in the certification program of PYMoV-free mother plants.

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