Identification of the main virus infecting impatiens (Impatiens balsamina L.)

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Abstract. One of the most important obstacles of growing ornamental plants, such as impatiens is disease infections, including those caused by viruses. Identification of viruses infecting impatiens was carried out in the IOCRI Virology laboratory using serological techniques and RT-PCR. Infected plants showed different symptoms involving mosaic, mottle, malformation, and wrinkles (frizz) on the leaves. The results of serological test using 5 antisera, i.e. Impatiens necrotic spot virus (INSV), Tomato spotted wilt virus (TSWV), Cucumber mosaic virus (CMV), Turnip mosaic virus (TuMV), and Tobacco mosaic virus (TMV) on leaf samples showed negative reaction. Bioassay tests carried out using 5 different symptomatic viral inoculums showed that only 2 inoculums (no.1 and 3), i.e. those with mosaic symptoms was successfully transmitted by mechanical inoculation to Chenopodium quinoa. Virus confirmation from infected C. quinoa was carried out by RT-PCR using specific primer for INSV, TSWV, and CMV. Amplification of viral DNA target was obtained only using specific primer for CMV. Therefore, we concluded that the causal agent of mosaic symptom found on impatiens plants in Cianjur is CMV.

1. Introduction

Impatiens is one of the most important ornamental plants with high economic value and very popular to be developed as bedding plants and pot plants. Indonesia is known as one of diversity centers of impatiens in the world, some wild types are tolerant to high temperature and drought. In the last decade, exploration of wild type of impatiens has been carried out in various regions in Indonesia, including West Java, East Java, Bali, East Nusa Tenggara, South Sulawesi and North Maluku; as many as 100 accessions has been successfully collected.

Disease problems always become a constraint during cultivation of impatiens. Viruses infecting impatiens plants have been reported by many researchers from Netherlands [1], US [2], Brazil [3], New Zealand [4], Serbia [5], Bangladesh [6], and Korea [7]. Several types of viruses that have been reported infecting impatiens are Tomato spotted wilt virus (TSWV) [6], Impatiens necrotic spot virus (INSV) [8], Cucumber mosaic virus (CMV) [2], Turnip mosaic virus (TuMV), Tobacco mosaic virus (TMV), Helenium virus S, Tobacco streak virus (TSV), and Tobacco ringspot virus (TRV). INSV and TSWV are the 2 most commonly reported viruses in impatiens.

Common symptom on viruses-infected impatiens is the formation of ringspot and discoloration of the leaves which causes mottled leaves [9]. INSV infection in impatiens New Guinea hybrid plants causes stuntng and discoloration at the base of the leaves or brownish spots on the leaves [8]. The
appearance of necrotic symptoms in the leaves is also mentioned as the symptoms of TSWV in impatiens. These necrotic symptoms are usually followed by the formation of a concentric necrotic ring on young leaves [1]. In Impatiens walleriana, leaf malformation occurs in plants infected with the TSWV. In addition, another symptoms were also found, i.e. necrotic spots that form lines on the leaves, necrotic on sepals, petals and plant stems [10]. Infection of CMV infection causes dwarfing, leaf malformation, and abnormal on petal shape [7].

The level of viral infection in impatiens varies from mild with only a few infected plants to severe with high disease rates [9]. Previous study reported that the prevalence of viral infections on I. balsamine in Bangladesh reached 85% with chlorotic spots symptom [6]. Viral infection on impatiens also occurred in several European countries, for instance England, Netherlands, East Germany, Switzerland, Denmark, West Germany and Sweden [11]. The symptom intensity of viral infection depends on the susceptibility of cultivar, time of infection, nutrient availability, environment condition, and aggressiveness of isolate [12].

Viral infection in ornamental plants causes decreasing on aesthetic and market value. Infection of INSV in New Zealand had a significant impact on flower exports [4]. Another study also reported that infection of TSWV on I. hawkeri in Serbia caused this plant to be unacceptable in the market [5].

Currently, similar virus symptoms were observed on impatiens in West Java. A survey has been carried out in 2017 at various impatiens production centers in West Java to observe symptom types and assess disease intensity. Identification of virus associated with disease on impatiens had been conducted in the IOCRI Virology laboratory using serology and reverse transcription polymerase chain reaction (RT-PCR) technique. This paper will discuss the result of virus identification.

2. Methods

2.1. Survey and observation of symptom types
The activities was carried out in 2017 by collecting impatiens plants from the green house in the experimental garden of Ornamental Crops Research Institute (OCRI) and one of the ornamental plant nurseries in Cianjur regency. Disease incidence and intensity were recorded and assessed based on symptom types.

2.2. Virus identification

2.2.1 Sample collection. Leaf samples showing mosaic, mottle, malformation, and curly leaf shoots symptoms were taken randomly from several infectious accessions (more than 10). Symptom were also photographed and recorded. A total of 100 mg shoot leaves from each plant sample were weighed and then stored in the storage room (-20 °C) for further ELISA and RT-PCR test.

2.2.2. Serological Test. Serological test was conducted following modified enzyme linked immunosor bent assay (ELISA) method [13] using specific antisera to INSV, TSWV, CMV, TuMV, and TMV (Agdia, US). Samples for positive and negative controls was taken out from the same ELISA kit. Assessment of the result of ELISA was conducted by measuring absorbance value of the reaction using ELISA reader (biasan) at 405 nm wavelength.

2.2.3. RT-PCR. The protocol for RT-PCR consisted of total RNA extraction, cDNA synthesis, and cDNA amplification. Total RNA extraction was conducted was carried out using commercial kit from GeneJet Plant RNA Purification Mini Kit and following its manufacturer procedure (Thermoscientific US). Synthesis of complement DNA strands (cDNA) was carried out by the reverse transcription (RT) method with a total volume of 10 μL using Moloney Murine Leukemia Virus (MMuLV) / Reverse Revert Aid (200 U/μL) (Thermoscientific) at 25 °C for 5 min, 42 °C for 60 min. Amplification of virus target was only carried out for 3 viruses, i.e. INSV, TSWV, and CMV. Specific primers for each virus target is as followed: S1 INSVF (5’AAA TCA ATA GTA TTA3’)/ S2 INSVR (5’CTT CCT CAA
GAA TAG GCA3'); L2 TSWVF (5'ATC AGT CGA AAT GGT CGG CA3'): L1TSWVR (5'AATTGCCCTT GCAACCATTC3'); CMVF (5'GCCACC AAA AAT CCG3')/CMVR (5'ATCTGC GTTTTCTTGG3') [14, 15]. The amplification program for INSV and TSWV is as followed: pre-denaturation at 94 °C for 3 min, 35 cycles consisted of denaturation at 94 °C for 30 sec, annealing at 50 °C for 1 min, and extension at 72 °C for 70 sec. The amplification program for CMV is as followed: pre-denaturation at 93.5 °C for 3 min, 35 cycles consisted of denaturation at 93.5 °C for 45 sec, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. Post extension at 72 °C for 5 min was added at the end of the reaction. DNA visualization was performed on 1% agarose gel containing DNA staining day (SmoBio) 3 μL/30 mL. Observation of DNA bands was done under UV transiluminator.

2.3. Bioassay test
Bioassay was conducted by mechanical inoculation test on 4 indicator plants, i.e. tomato, Chenopodium quinoa, C. amaranticolor, and cucumber. Plant extract (sap) was prepared by grinding leaf samples on 0.05% phosphate buffer containing 1% 2-mercaptoethanol (2-ME).

3. Results and discussion

3.1. Inventory of symptoms type
During surveys on several locations, samples were successfully collected involving several species/clones/accessions of impatiens with various viral symptom types (Fig. 1 and 2). The types of disease symptoms included mosaic, mottle, leaf malformations, and wrinkles/curls of the leaves. These symptoms also found on impatiens plants which were cultivated in nurseries around Cianjur regency. Malformation symptoms were often found together with the mosaics or mottles symptoms. Symptom severity varies between individual plants.

3.2. Identification of viruses by ELISA and RT-PCR Test
Serological test of symptomatic samples by ELISA using 5 antiserum (INSV, TSWV, CMV, TuMV, and TMV) gave negative reaction, but gave positive reaction to the positive controls from the ELISA kit. The antiserum used was unable to recognize the presence of antigens from the target virus in samples of impatiens, so that there was no change in the color of the substrate. The change in the color of the substrate to yellow was only found in positive control samples. Similarly, the measurements using ELISA reader showed a high absorbance value for the positive control, but low for the test samples. Negative results were also obtained when the same sample was tested by RT-PCR. There was no DNA band found in the visualization on agarose gel. This indicated that the 3 primers used (INSV, TSWV, and CMV) were unable to amplify the target viral DNA in the impatient samples (Fig. 3).

3.3. Bioassay test
The symptomatic leaves of impatiens was further subjected for bioassay. Saps from 5 symptomatic impatiens accessions (Fig. 4) were mechanically inoculated to indicator plants. The result showed that C. quinoa that inoculated with sample 1 and 3 developed local lesion on the leaves and systematic mosaic symptoms, respectively.

Re-inoculation of symptomatic C. quinoa to other indicator plants, i.e. tomatoes and C. amaranticolor was successfully induced local symptoms. Symptom on tomato showed line pattern of chlorosis while chlorosis spot was observed on inoculated C. amaranticolor (Fig. 5). Local lesion were also reported on C. quinoa leaves inoculated with CMV from impatiens (African isolate) at 5 d post-inoculation [7].
Figure 1. Various symptom types typical of viral infection on some species of impatiens. Mottle (a, b, c), mosaic (d, f), and malformation (c, d, e, g, h, i).
Figure 2. Various symptom types typical of viral infection on some clones of impatiens. Malformation (a, b, c), Mottle (d, e, f, h), wrinkles/curls of the leaves (g).
Figure 3. Visualization of amplified DNA products from impatiens and inoculated indicator plants using CMV primer. M1, DNA marker 1 kb; M2, DNA marker 100 bp; K(-), negative control; (1), (2), (5), inoculum no. 1 on impatiens from the field, inoculated C. quinoa, and non-inoculated C. quinoa, respectively; (3), (4), (6), (7), inoculum no. 3 on impatiens from field, inoculated C. quinoa, inoculated tomato plants, and inoculated C. amaranticolor, respectively.

Figure 4. Symptomatic impatiens accessions used for bioassay; (1) Mosaic; (2) Mottle; (3, 5) wrinkles/curls of the leaves; (4) Necrotic local lesion.

Figure 5. Symptoms of CMV infection in indicator plants (from left to right: C. quinoa, C. amaranticolor, and tomato) which were inoculated by inoculum 1.

3.4. Virus identification from indicator plants using RT-PCR
The results of this preliminary study indicated that typical symptom of viral infection was found on impatiens plants, but only 1 symptom type was successfully detected associated with virus. By using RT-PCR technique, this symptom was identified associated with CMV.

Early virus identification by ELISA and RT PCR methods was unable to give positive reaction, probably due to inhibition reaction. The presence of mucus that produced during leaves extraction was suspected as the cause. Mucus on impatiens supposedly has high phenolic content. Calcium, collagen,
haematin, tannic acid are reported as inhibitor of polymerase activity. Melanin forms a reversible complex with DNA polymerase and polysaccharides that can interfere with the enzymatic process by mimicking the structure of nucleic acids. In addition, humic acid interacts with the DNA of templates and polymerases so as to prevent enzymatic reactions even at low concentrations. Various compounds, including polysaccharides and phenolic compounds in plant tissue can inhibit PCR amplification [16]. Phenolic compounds from the sample or carried over from DNA purification can inhibit PCR by binding to or denaturing the polymerase [17]. The presence of phenolic compound was thought to inhibit PCR amplification. Extract from various impatiens species contains significant amount of phenolic acid, flavonoid and ethanol [18, 19].

4. Conclusion
Many impatiens plants in the field showed typical virus infections, involving mosaic, mottle, malformation, frizz of the leaves. Preliminary identification indicated CMV as the causal agent of disease on impatiens plants.

Acknowledgment
This research was supported by Agricultural Research and Development Agencies Ministry of Agriculture, The Republik of Indonesia.

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