Sensitivity of serological and polymerase chain reaction methods for detection of viruses in Allium spp.

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Abstract. Infection of Onion yellow dwarf virus (OYDV), Shallot yellow stripe virus (SYSV), and Garlic common latent virus (GCLV) has been reported on Allium spp. in Indonesia. Serology methods using enzyme-linked immunosorbent assay (ELISA), and dot immunobinding assay (DIBA) is a common method to detect viruses, while polymerase chain reaction (PCR) is popular as a molecular method to detect plant viruses nowadays. This research was conducted to assess the sensitivity of 3 detection methods, i.e. ELISA, DIBA and PCR for detecting viruses in Allium spp. Sensitivity level of each method was evaluated by diluting plant extract and antibody for ELISA and DIBA, and cDNA template for PCR. Result of this research showed that DIBA was able to detect OYDV, GCLV, and SYSV with plant extract dilution in the range of 10⁻¹ to 10⁻², and antibody dilution from 1:300 to 1:1000. ELISA seems to be more sensitive than DIBA because it was able to detect the same virus with plant extract dilution in the range of 10⁻¹, 10⁻³, and 10⁻⁵, and antibody dilution from 1:300 to 1:1000. RT-PCR method was the most sensitive method compared to ELISA and DIBA because it has the highest accuracy, specification and sensitivity level. Specific DNA fragment was amplified using universal primers for Potyvirus and Carlavirus up to 10⁻³ and 10⁻⁴ dilution factor of cDNA respectively. Moreover, amplification using specific primers of OYDV, SYSV, and GCLV is more sensitive than using universal primers, because up to 10⁻⁵ dilution factor of cDNA was able to amplify virus target.

1. Introduction

Shallot and garlic from the Allium group are important agricultural commodities in Indonesia. Based on data [1], the productivity of shallot in Indonesia in 2014, 2015 and 2016 has decreased, i.e. 10.22, 10.06 and 9.66 tons/ha, respectively; while garlic production in the same year was fluctuated, i.e. 8.83, 7.92, and 8.79 tons/ha, respectively. Shallot and garlic is commonly propagated through bulbs. Therefore, the quality of seed bulbs is an important factor in the production of shallot and garlic. In addition to the physical properties, pathogen-free bulbs play an important factor for increasing Allium production. Onion yellow dwarf virus (OYDV), Garlic common latent virus (GCLV), and Shallot yellow stripe virus (SYSV) infections have been found in shallots and garlic in Indonesia [2-6].

Virus infection in shallot and garlic are generally detected using serological and molecular methods. The most common used serological methods are enzyme-linked immunosorbent assay (ELISA), and dot immuno-binding assay (DIBA); while the molecular detection method
is polymerase chain reaction (PCR). ELISA and DIBA are suitable methods for rapid analysis with large numbers of samples [7], but DIBA is considered to have a higher sensitivity than ELISA. PCR as a molecular method is a very promising method for detecting viruses in very low concentrations, but this method is very expensive and limited in the number of samples that can be tested [8].

Some viruses have low concentrations in plant tissues, therefore the development of more sensitive methods for detecting viruses in plants is needed [9]. This study aimed to evaluate the sensitivity of ELISA, DIBA and RT-PCR as a method of virus detection in shallot and garlic. The sensitivity level of each method was tested in several levels of dilution of plant sap and antibodies for ELISA and DIBA, and dilution of cDNA for the RT-PCR method.

2. Methods
The experiment was conducted from March to Jul 2017 at Plant Virology Laboratory, Department of Plant Protection, Faculty of Agriculture, IPB University and Centre for Herbs and Medicinal Plants, Ministry of Agriculture.

2.1. Research samples
Leaf samples of shallot and garlic were obtained from Plant Virology Laboratory and has been confirmed infected by OYDV, SYSV and GCLV from previous research. Shallot leaf samples were collected from Brebes and Cirebon regencies, while garlic leaf samples were from Malang and East Lombok regencies. Each sample was weighed as much as 0.1 g, placed in a plastic bag, then stored in the freezer (-80 °C). All samples was then used for DIBA, ELISA, and reverse transcription polymerase chain reaction (RT-PCR).

2.1.1. Dot immunobinding assay (DIBA). Leaf extraction and DIBA protocol was carried out following [10]. Sensitivity of DIBA was evaluated by diluting leaf extract (sap) and antibodies in series $10^0$ to $10^{-5}$. DIBA reaction is differentiated based on the intensity of the color that appears on the membrane, i.e. very strong (+++), strong (++), weak (+), and negative (-).

2.1.2. Enzyme-linked immunosorbent assay (ELISA). Leaf extraction and ELISA protocol was carried out based on the guideline of ELISA kit (DSMZ). ELISA for OYDV and GCLV was carried out using Double Antibody Sandwich method (DAS-ELISA), and for SYSV using Triple Antibody Sandwich method (TAS-ELISA). Sensitivity of DIBA was evaluated by diluting leaf extract (sap) and antibodies in series $10^0$ to $10^{-5}$. Absorbance value of ELISA reaction was measured quantitatively using spectrophotometry (ELISA reader) at 405 nm wavelength. Positive reaction was determined when absorbance value of sample was 2 times higher than absorbance value of control negative.

2.1.3. Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA extraction was carried out by Cetyl triethylammonium bromide (CTAB) method, followed by reverse transcription (RT) reaction as described in [10]. cDNA obtained from RT will be used as template DNA and amplification was conducted using specific primers (Table 1) and amplification program according to the virus target (Table 2). DNA amplicons was visualized on 1.5% agarose gel in 0.5x TBE buffer (Tris-borate EDTA). Sensitivity of RT-PCR was evaluated by diluting cDNA in series $10^0$ to $10^{-5}$. 
Table 1. The primer used in polymerase chain reaction to amplify target viruses.

| Virus target | Primer | Product (bp) | References |
|---------------|--------|--------------|------------|
| Potyvirus     | F: 5'-AATTCATGRTITGGTGYATIGAAAYGG-3’  
R: 5'-ATCCCCGGGTTTTTTTTTTTTTTTTTTTTTTTTT-3’ | 690 | [11] |
| Carlaviruses  | F: 5'-TGCTGCYTTTGATACYTTGCAT-3’  
R: 5'-ATCCCCGGGTTTTTTTTTTTTTTTTTTTTTTTTT-3’ | 715 | [12] |
| OYDV          | F: 5'-GAAGCCGACATGCAAATGAG-3’  
R: 5'-CGCACACAATGTTGACAC-3’ | 290 | [13] |
| SYSV          | F: 5'-ACACGAGCCACACACGCAC-3’  
R: 5'-TCCCTAACAACCGTGAACACTCA-3’ | 749 | [14] |
| GCLV          | F: 5'-ATGTCAGTGAGTGAAACAGAG G-3’  
R: 5'-CTAGTCTGCATTGTTGGATCC-3’ | 960 | [15] |

Table 2. DNA amplification cycle for each virus target.

| No | Steps | Virus target |
|----|-------|--------------|
|    |       | Potyvirus    | Carlaviruses | OYDV | SYSV | GCLV |
| 1. | First Denaturation | 94 °C (5 min) | 95 °C (1 min) | 94 °C (5 min) | 95 °C (5 min) | 94 °C (3 min) |
| 2. | Denaturation      | 94 °C (20 sec) | 94 °C (20 sec) | 94 °C (1 mnt) | 95 °C (1 mnt) | 94 °C (30 sec) |
| 3. | Annealing        | 55 °C (1 min) | 56 °C (1 min) | 52 °C (1 min) | 57 °C (45 sec) | 56 °C (1 min) |
| 4. | Extension        | 72 °C (3 min) | 72 °C (1 min) | 72 °C (1 min) | 72 °C (1 min) | 72 °C (1 min) |
| 5. | Final Extension  | 72 °C (1 min) | 72 °C (3 min) | 72 °C (10 min) | 72 °C (7 min) | 72 °C (7 min) |

Steps no. 2-4 are repeated 35 times, except OYDV repeated 30 times.

3. Results and discussion

3.1. Virus detection by DIBA and ELISA

In general, DIBA was able to detect virus target up to 10^1 dilution of plant extract and varying dilutions for antibody (Tables 3, 4, and 5). This variation is caused by the quality of antibody for each virus target; which in turn affect their sensitivity. The sensitivity level of antibodies to GCLV and SYSV was up to 1: 1,000, whereas those to OYDV was up to 1: 300. Detection by ELISA showed similar result for the sensitivity of each antibody. A noticeable reaction between DIBA and ELISA
was shown in plant extract dilution limit. ELISA was able to detect up to $10^{1}$, $10^{-3}$, and $10^{-5}$ dilution of plant extract for OYDV, GCLV, and SYSV, respectively (Tables 6, 7, and 8).

3.2. Virus detection by RT-PCR Method.
Specific DNA fragments of 690 bp was successfully amplified from samples infected with OYDV and SYSV using general primers for *Potyvirus* and DNA fragments can be amplified to dilution $10^{-3}$ of cDNA template (Fig. 1 and 2). The amplification sensitivity was found better when using general primers for *Carlavirus*; specific DNA fragments of 715 bp was successfully amplified from samples infected with GCLV and DNA fragments can be amplified to dilution $10^{-4}$ of cDNA template (Fig. 3). The amplification sensitivity using specific primers for OYDV, SYSV and GCLV differed slightly in the detection of each target viruses; specific DNA fragments of OYDV and SYSV can be amplified to dilution $10^{-3}$ of cDNA template (Fig. 4 and 5) and those of GCLV can be amplified to dilution $10^{-5}$ of cDNA template (Fig. 6).

ELISA and DIBA is serological method based on specific reaction between antibodies and antigens (target viruses). These 2 serological methods are preferable for virus detection due to high sensitivity, capability of detecting viruses at low concentrations, ability to test large quantities of samples quickly, easy test procedures [16]. Positive reaction is indicated by colour change in the microtiter plate or nitrocellulose membrane for ELISA or DIBA, respectively. ELISA reaction is also commonly measured quantitatively using a spectrophotometer (ELISA reader) with a wavelength of 405 nm. Comparison of the 2 methods for detecting viruses on shallot and garlic showed that ELISA is more sensitive than DIBA, demonstrated by its ability to detect viruses to more diluted plant extract. However, DIBA is considered more efficient. Samples that have been spotted on to nitrocellulose membranes can be stored for a long time. Similarly, antibody solutions can be stored for up to 6 mo and can be reused without reducing their sensitivity [17].

PCR is a method used to multiply a specific DNA fragment and modification of PCR method, i.e. RT-PCR is also one of the methods of choice in detecting mRNA. In this study 2 universal pairs, namely U341/Poty1, and AlcarF/Poty1 were proven to be able to amplify viral fragments in the genus *Potyvirus* and *Carlavirus*, respectively. Amplification of target DNAs was better achieved using specific primers for each virus species. One disadvantage of using a specific primer is that it requires a separate primary reaction for each target; it also requires precision in its implementation and requires optimization of reaction conditions to get accurate target results [18].

| Table 3 Reaction of OYDV antibody to samples from Brebes and Cirebon by DIBA in serial dilutiona. |
|---|---|---|---|---|---|---|---|
| Antibody Dilutions | 10⁰ | 10⁻¹ | 10⁻² | 10⁻³ | 10⁻⁴ | 10⁻⁵ | Negative control | Buffer |
| Sample from Brebes | | | | | | | | |
| 1:100 | ++ | + | – | – | – | – | – | – |
| 1:300 | + | – | – | – | – | – | – | – |
| 1:500 | – | – | – | – | – | – | – | – |
| 1:1000 | – | – | – | – | – | – | – | – |
| Sample from Cirebon | | | | | | | | |
| 1:100 | ++ | + | – | – | – | – | – | – |
| 1:300 | + | – | – | – | – | – | – | – |
| 1:500 | – | – | – | – | – | – | – | – |
| 1:1000 | – | – | – | – | – | – | – | – |

a Reaction on nitrocellulose membranes: ++++, very strong; ++, strong; +, weak; -, negative.
Table 4. Reaction of GCLV antibody to samples from East Lombok and Malang by DIBA in serial dilution.

| Antibody Dilutions | 10^0 | 10^-1 | 10^-2 | 10^-3 | 10^-4 | 10^-5 | Negative control | Buffer |
|--------------------|------|-------|-------|-------|-------|-------|-----------------|--------|
| Sample from East Lombok |      |       |       |       |       |       |                 |        |
| 1:100              | +++  | ++    | -     | -     | -     | -     | -               | -      |
| 1:300              | ++   | +     | -     | -     | -     | -     | -               | -      |
| 1:500              | +    | +     | -     | -     | -     | -     | -               | -      |
| 1:1000             | +    | +     | -     | -     | -     | -     | -               | -      |
| 1:10000            | -    | -     | -     | -     | -     | -     | -               | -      |
| Sample from Malang |      |       |       |       |       |       |                 |        |
| 1:100              | +++  | ++    | -     | -     | -     | -     | -               | -      |
| 1:300              | ++   | +     | -     | -     | -     | -     | -               | -      |
| 1:500              | +    | +     | -     | -     | -     | -     | -               | -      |
| 1:1000             | +    | +     | -     | -     | -     | -     | -               | -      |
| 1:10000            | -    | -     | -     | -     | -     | -     | -               | -      |

a Reaction on nitrocellulose membranes: +++ very strong; ++ strong; + weak; -, negative.

Table 5. Reaction of SYSV antibody to samples from Brebes and Cirebon by DIBA in serial dilution.

| Antibody Dilutions | 10^0 | 10^-1 | 10^-2 | 10^-3 | 10^-4 | 10^-5 | Negative control | Buffer |
|--------------------|------|-------|-------|-------|-------|-------|-----------------|--------|
| Sample from Brebes |      |       |       |       |       |       |                 |        |
| 1:100              | +++  | +++  | +     | -     | -     | -     | -               | -      |
| 1:300              | ++   | +     | -     | -     | -     | -     | -               | -      |
| 1:500              | +    | +     | -     | -     | -     | -     | -               | -      |
| 1:1000             | +    | +     | -     | -     | -     | -     | -               | -      |
| 1:10000            | -    | -     | -     | -     | -     | -     | -               | -      |
| Sample from Cirebon |      |       |       |       |       |       |                 |        |
| 1:100              | +++  | +++  | +     | -     | -     | -     | -               | -      |
| 1:300              | ++   | +     | -     | -     | -     | -     | -               | -      |
| 1:500              | +    | +     | -     | -     | -     | -     | -               | -      |
| 1:1000             | +    | +     | -     | -     | -     | -     | -               | -      |
| 1:10000            | -    | -     | -     | -     | -     | -     | -               | -      |

a Reaction on nitrocellulose membranes: +++ very strong; ++ strong; + weak; -, negative.
Table 6. Reaction of OYDV antibody to samples from Brebes and Cirebon by DAS-ELISA in serial dilutiona.

| Antibody Dilutions | Dilution of plant extract | Negative control | Buffer |
|---------------------|---------------------------|------------------|--------|
|                     | 10^0 | 10^-1 | 10^-2 | 10^-3 | 10^-4 | 10^-5           |
| Sample from Brebes  |      |       |       |       |       |                  |
| 1:100               | +    | +     | -     | -     | -     | -                |
| 1:300               | +    | -     | -     | -     | -     | -                |
| 1:500               | -    | -     | -     | -     | -     | -                |
| 1:1000              | -    | -     | -     | -     | -     | -                |
| Sample from Cirebon |      |       |       |       |       |                  |
| 1:100               | +    | +     | -     | -     | -     | -                |
| 1:300               | +    | -     | -     | -     | -     | -                |
| 1:500               | -    | -     | -     | -     | -     | -                |
| 1:1000              | -    | -     | -     | -     | -     | -                |

The ELISA reaction is based on the absorbance value of ELISA (NAE): ++++, strong (NAE≥ 6 NAEK); ++, medium (4 <NAE <6 NAEK); +, weak (NAE 2-4 ≥ NAEK); -, negative (NAE <2 NAEK).

Table 7. Reaction of GCLV antibody to samples from East Lombok and Malang by DAS-ELISA in serial dilutiona.

| Antibody Dilutions | Dilution of plant extract | Negative control | Buffer |
|---------------------|---------------------------|------------------|--------|
|                     | 10^0 | 10^-1 | 10^-2 | 10^-3 | 10^-4 | 10^-5           |
| Sample from East Lombok |      |       |       |       |       |                  |
| 1:100               | +++  | +++   | ++    | +     | -     | -                |
| 1:300               | +++  | ++    | +     | -     | -     | -                |
| 1:500               | ++   | +     | -     | -     | -     | -                |
| 1:1000              | +    | -     | -     | -     | -     | -                |
| 1:10000             | -    | -     | -     | -     | -     | -                |
| Sample from Malang  |      |       |       |       |       |                  |
| 1:100               | +++  | +++   | ++    | +     | -     | -                |
| 1:300               | ++   | ++    | +     | -     | -     | -                |
| 1:500               | +    | +     | -     | -     | -     | -                |
| 1:1000              | +    | -     | -     | -     | -     | -                |
| 1:10000             | -    | -     | -     | -     | -     | -                |

The ELISA reaction is based on the absorbance value of ELISA (NAE): ++++, strong (NAE≥ 6 NAEK); ++, medium (4 <NAE <6 NAEK); +, weak (NAE 2-4 ≥ NAEK); -, negative (NAE <2 NAEK).
Table 8. Reaction of SYSV antibody to samples from Brebes and Cirebon by TAS-ELISA method.

| Antibody Dilutions | Dilution of plant extract | Negative control | Buffer |
|--------------------|---------------------------|------------------|--------|
|                    | 10^0 | 10^{-1} | 10^{-2} | 10^{-3} | 10^{-4} | 10^{-5} |          |        |
| Brebes             |      |         |         |         |         |         |          |        |
| 1:100              | +++  | +++     | +++     | +++     | +++     | +++     | –        | –       |
| 1:300              | +++  | +++     | +++     | ++      | +       | +       | –        | –       |
| 1:500              | +++  | ++      | +       | –       | –       | –       | –        | –       |
| 1:1000             | +    | +       | –       | –       | –       | –       | –        | –       |
| 1:10000            | –    | –       | –       | –       | –       | –       | –        | –       |
| Cirebon            |      |         |         |         |         |         |          |        |
| 1:100              | +++  | +++     | +++     | +++     | +++     | +++     | –        | –       |
| 1:300              | +++  | +++     | +++     | ++      | +       | +       | –        | –       |
| 1:500              | +++  | ++      | +       | –       | –       | –       | –        | –       |
| 1:1000             | +    | +       | –       | –       | –       | –       | –        | –       |
| 1:10000            | –    | –       | –       | –       | –       | –       | –        | –       |
|                   | 1:100000 | –  | –       | –       | –       | –       | –        | –       |

a The ELISA reaction is based on the absorbance value of ELISA (NAE): ++++, strong (NAE≥ 6 NAEK); ++, medium (4 <NAE <6 NAEK); +, weak (NAE 2-4 ≥ NAEK); -, negative (NAE <2 NAEK).

Figure 1. Visualization of DNA amplification from OYDV-infected shallots using general primers for Potyvirus (U341 / Poty1) in agarose gel. K-, negative control; M, 100 bp DNA marker; 0-5, cDNA samples with dilution factor of 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}, respectively.

Figure 2. Visualization of DNA amplification from SYSV-infected shallots using general primers for Potyvirus (U341 / Poty1) in agarose gel. K-, negative control; M, 100 bp DNA marker; 0-5, cDNA samples with dilution factor of 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}, respectively.
Figure 3. Visualization of DNA amplification from GCLV-infected shallots using general primers for *Carlavirus* primer (AlcarF / Poty1) in agarose gel. K-, negative control; M, 100 bp DNA marker; 0-5, cDNA samples with dilution factor of $10^0$, $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$, respectively.

Figure 4. Visualization of DNA amplification from OYDV-infected shallots using specific primers for OYDV (06-RT1/06-RT2) in agarose gel. K-, negative control; M, 100 bp DNA marker; 0-5, cDNA samples with dilution factor of $10^0$, $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$, respectively.

Figure 5. Visualization of DNA amplification from SYSV-infected shallots using specific primers for SYSV (SYSV-F/SYSV-R) in agarose gel. K-, negative control; M, 100 pb DNA marker; 0-5, cDNA samples with dilution factor of $10^0$, $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$, respectively.

Figure 6. Visualization of DNA amplification from GCLV-infected shallots using specific primers for GCLV (GarCLV-F/GarCLV-R) in agarose gel. K-, negative control; M, 100 bp DNA marker; 0-5, cDNA samples with dilution factor of $10^0$, $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$, respectively.
4. Conclusion
Comparison of 3 detection methods for Allium viruses indicated that RT-PCR had better capability to detect viruses from plant tissues with a low viral concentration. This method is more sensitive, accurate, and efficient in the use of time and energy.

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