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An accurate, specific, sensitive, high-throughput method based on a microsphere immunoassay for multiplex detection of three viruses and bacterial fruit blotch bacterium in cucurbits

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ABSTRACT

To employ a microsphere immunoassay (MIA) to simultaneously detect multiple plant pathogens (potyviruses, *Watermelon silver mottle virus*, Melon yellow spot virus, and *Acidovorax avenae* subsp. *citrulli*) in actual plant samples, several factors need to be optimized and rigorously validated. Here, a simple extraction method using a single extraction buffer was successfully selected to detect the four pathogens in various cucurbit samples (cucumber, cantaloupe, melon, and watermelon). The extraction method and assay performance were validated with inoculated and field cucurbit samples. The MIA showed 98–99% relative accuracy, 97–100% relative specificity and 92–100% relative sensitivity when compared to commercial ELISA kits and reverse transcription PCR. In addition, the MIA was also able to accurately detect multiple-infected field samples. The results demonstrate that one common extraction method for all tested cucurbit samples could be applied to detect multiple pathogens; avoiding the need for multiple protocols to be employed. This multiplex method can therefore be instrumental for high-throughput screening of multiple plant pathogens with many advantages such as a shorter assay time (2.5 h) with single assay format, a lower cost of detection ($5 vs $19.7 for 4 pathogens/sample) and less labor requirement. Its multiplex capacity can also be expanded to detect up to 50 different pathogens upon the availability of specific antibodies.

1. Introduction

The Cucurbitaceae or cucurbit family (cucumber, cantaloupe, pumpkin, melon, and watermelon) is an economically important crop with a production of 165 million tons worldwide in 2006 (Wang et al., 2007). In Thailand, cucurbit seed export accounted for 33 million USD or 22.9% of total seed export in 2015 (http://www.oae.go.th/download/FactorOfProduct/ValueExportSeed47-52.html). However, cucurbit cultivation suffers from a wide range of pathogens, including virus, fungi, bacteria and insects (Ali et al., 2011; Blandchard et al., 1994). In Thailand, potyviruses, tospoviruses group and *Acidovorax avenae* subsp. *citrulli* (Aac) bacterium were found to cause serious damage to cucurbit crops (Chiemsombat et al., 2008; Himananto et al., 2011); jeopardizing the quality and the quantity of the production and in turn, resulting in a devastating economic loss (Lecoq, 2003).

To address the plant disease problems in cucurbits, accurate, inexpensive and rapid detection methods are needed. Currently, molecular techniques and immunoassay have been developed to expedite and facilitate the plant pathogen detection. Several molecular methods based on polymerase chain reaction (PCR) were therefore developed to distinguish the presence of pathogens even in asymptomatic plants (Schaad and Schuenzel, 2010). For instance, reverse transcription (RT)-PCR was used to identify tospoviruses (Charoenvilaisiri et al., 2014; Chiemsombat et al., 2008; Seepiban et al., 2015; Seepiban et al., 2011). Even though these molecular techniques offer high sensitivity and accuracy, they require multiple-step protocols for RNA or DNA extraction which are time-consuming and require skilled workers to avoid potential contamination problems. Although multiplex PCR enables detection of multiple targets, the development of such assay can be challenging as it requires extensive

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optimization for amplification conditions.

On the other hand, immunoassays, such as an enzyme-linked immunosorbent assay (ELISA), are commonly used for plant disease screening because they require minimal sample preparation steps. ELISA utilizes the interaction between antigen and antibody and has been used to detect various pathogens since 1977 (Clark and Adams, 1977). For instance, ELISA was developed to detect Aac in cucurbit leaves and seed (Himananto et al., 2011). The other immunoassay techniques such as plate-trapped antigen ELISA (Seepiban et al., 2011; Venkataraman et al., 2014) tissue-blot (TBIA), and dot-blot immunoassays (DBIA) were successfully used to detect *Cucurbit yellow stunning disorder virus* (CYSDV) (Hourani and Abou-Jawdah, 2003). While these techniques were simple, robust and do not require sophisticated skills for routine testing, they can become tedious when dealing with a large amount of samples (Boonham et al., 2014).

With the large number of samples involved in plant disease testing, multiplex detection methods have successfully been developed. For multiplex methods based on molecular techniques, multiplex real-time PCR and multiplex RT-PCR were developed to detect multiple plant pathogens such as Aac and *Didymella bryoniae* detection in cucurbit seedlots (Ha et al., 2009), Cucurbit chlorotic yellows virus (CCYV) and CYSDV detection (Abrahamian et al., 2013), and the seven main tomato-infecting RNA viruses (Panno et al., 2012). These methods greatly reduce the time, and labor associated with conventional methods; however, they still require RNA/DNA extraction and purification steps, which added to the cost of testing.

Recently, several multiplex immunoassay methods have been established to minimize the sample preparation step allowing for rapid and accurate results to be obtained without specialized workers. For instance, our group has successfully developed an antibody array that could detect four crucial plant pathogens, namely, *Chilli vein-banding mottle virus* (CVbMV, potyvirus), *Watermelon silver mottle virus* (WSMoV, tospovirus serogroup IV), Melon yellow spot virus (MYSV, tospovirus) and bacterial fruit blotch bacterium Aac by using a recombinant coat protein or nucleocapsid proteins of viruses for assay development (Charlermroj et al., 2014). Nevertheless, when the antibody array system was employed to detect pathogens in field samples, the background signal from the plant matrices interfered with the detection signals (unpublished data).

To address the problem which arose from the testing of samples with complicated matrices, a microsphere immunoassay (MIA) based on the universal microsphere array (xMAP technology) was developed. These microspheres were paramagnetic, which could significantly reduce background problems in the detection of complex or difficult matrices. The MIA has been proven to be superior to others in different diagnostic applications such as in food pathogens (Charlermroj et al., 2016) and toxin (Kim et al., 2010) and several biomarkers for potential clinical diagnostics (den Reijer et al., 2013; Mushaben et al., 2013). In plant diseases, the microsphere immunoassay has been demonstrated to be able to simultaneously detect *Potato virus Y, Potato virus X* and *Potato leafroll virus* in naturally infected potato leaves (Bergervoet et al., 2008). For cucurbits, our group has previously optimized the microsphere immunoassay to detect CVbMV (potyvirus), WSMoV, MYSV and Aac (Charlermroj et al., 2013). However, the system has not been validated with the field samples to ensure that it can detect the pathogens in different cucurbit varieties. Therefore, this study aimed to find an effective sample extraction buffer for multiplexing and to validate the developed MIA method in field samples of different cucurbits (cucumber, cantaloupe, melon, and watermelon) to ensure its applicability to the actual industry. The results from the MIA were compared with commercial ELISA kits and RT-PCR.

2. Methods

2.1. Plant samples

Inoculated and field plant samples were used to validate the microsphere immunoassay (MIA). For the inoculated samples, three types of viruses (*Papaya ringspot virus* Type W (PRSV-W, potyvirus), *Watermelon silver mottle virus* (WSMoV, tospovirus group IV) and Melon yellow spot virus (MYSV)) were inoculated to *Datura stramonium*, *Physalis minima* and *Cucumis sativus* samples, respectively. The mechanical inoculation method of plant viruses was performed as previously described (Hull, 2005). Briefly, the virus-infected plant samples (2 g) were grounded in an inoculation buffer (10 mL, 0.05 M phosphate buffer, pH 7.0 containing 0.2% 2-mercaptoethanol). Celite powder (0.1 g) was added to the grounded virus-infected plant solution for 5 min before being applied onto both sides of the leaves of a 1-month-old healthy plant. Celite powder was subsequently washed off from the leaves with water. For the bacteria inoculated samples, *Acidovorax avenae* subsp. *citruli* (Aac) at 10^8 CFU/mL was inoculated to 2 week-old watermelon plants using a run-off spraying method as previously described (Himananto et al., 2011). The inoculated plant samples were grown in a greenhouse at 25–30 °C to prevent any insect infestation and to observe for any symptoms of disease for 4 weeks. The success of pathogen inoculation was confirmed using a PTA-ELISA and a sandwich ELISA before the leaves of the inoculated plants were harvested and kept at −80 °C for subsequent tests.

For the field samples, 146 samples exhibiting disease symptoms in cucurbits (cucumber, n = 31; cantaloupe, n = 20; melon, n = 64; watermelon, n = 31) were collected from ten fields from various provinces (Chachoengsao, Kanchanaburi, Maha Sarakham, Nakhonsawan, Phetchaburi, Ratchaburi, Sukhothai, and Supanburi) in Thailand. The samples were homogenized in liquid nitrogen, aliquoted into portions and kept in −80 °C for subsequent tests.

2.2. Antibodies

All antibodies used in this study were produced at the Monoclonal Antibody Production Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC, Thailand). The specificity of the antibodies were previously characterized as shown in Table 1 (Himananto et al., 2011; Kumpoosiri et al., 2007; Seepiban et al.,

Table 1
Summary of antibodies used in the microsphere immunoassay method and their specificity.

Plant pathogens detection	Antibody-coated microsphere	RPE-labeled antibody
Potyviruses	1G8[a]	1B4[a]
Papaya ringspot virus Type W (PRSV-W)		
Watermelon mosaic virus group 2 (WMV2)		
Zucchini yellow mosaic virus (ZYMV)		
Chilli vein-banding mottle virus (CVbMV)		
Tospovirus IV	TosIV[b]	2D6[a]
Watermelon silver mottle virus (WSMoV)		
Capsicum chlorosis virus (CaCV)		
Melon yellow spot virus (MYSV)	5E7[a]	GenTos[b]
Acidovorax avenae subsp. *citruli* (Aac)	MPC[b]	MPC[b]

[a] Mouse monoclonal antibody.
[b] Rabbit polyclonal antibody.

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