Novel and Highly Specific Monoclonal Antibody to *Acidovorax citrulli* and Development of ELISA-Based Detection in Cucurbit Leaves and Seed

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Abstract

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A novel monoclonal antibody (MAb) specific to the seedborne bacterium *Acidovorax citrulli* was produced. MAb 11E5 reacted specifically with 19 strains of *A. citrulli* but not with three closely related bacteria in the family *Comamonadaceae* (i.e., *A. facilis*, *Comamonas acidovorans*, and *C. testosteroni*) and another seven phytopathogenic bacteria. Moreover, this MAb detected a strain of *A. citrulli* that was not detected by a commercial enzyme-linked immunosorbent assay (ELISA)-based kit and a commercial immunochromatographic strip test. In Western blot analysis, MAb 11E5 reacted with an *A. citrulli* protein of a molecular mass >170 kDa. MAb 11E5 was employed to

develop two sandwich ELISA systems: MAb captured-sandwich ELISA (MC-sELISA) and polyclonal antibody captured-sandwich ELISA (PC-sELISA). MC-sELISA was 10 times more sensitive than PC-sELISA for detection of *A. citrulli* in cucurbit leaf and seed extracts. The detection limit of the MC-sELISA was 5×10^4 CFU/ml. Detection of *A. citrulli* in naturally infected cucurbit leaves, fruit, and seed was also feasible using MC-sELISA. The newly established MC-sELISA provides another alternative for specific detection of *A. citrulli* in cucurbits and can be applied for routine field inspection.

Acidovorax citrulli (originally *Pseudomonas pseudoalcaligenes* subsp. *citrulli* and subsequently changed to *A. avenae* subsp. *citrulli*) (15,16,23) is a gram-negative bacterium that causes bacterial fruit blotch (BFB) of cucurbits, resulting in severe losses in cucurbit production worldwide. This bacterium causes serious concerns for the vegetable seed industry because it is naturally borne and transmitted by seed (2,7,13). BFB can be devastating for seed producers because it can result in 100% yield reduction (9). In Thailand, cucurbit seed, such as watermelon, cantaloupe, cucumber, gourd, squash, and pumpkin seed, account for 30% (approximately U.S.\$29 million) of the total seed exported (approximately U.S.\$98 million) in 2009 (The Office of Agricultural Regulation, Department of Agriculture Thailand, Retrieved from, http://www.oae.go.th/ewtadmin/ewt/oae_web/ewt_news.php?nid=8115&filename=index). Phytosanitary certification is required for cucurbit seed export. Field inspection prior to harvest or seed testing is required depending on regulation of each country. Standard methods for seed testing to detect *A. citrulli* are the seedling grow-out assays (SGO) and bacterial isolation on semiselective media, which are laborious and time consuming and require large areas of greenhouse space. To alleviate this problem, most of the previous studies have focused on developing new methods for seed testing such as immuno-

magnetic separation polymerase chain reaction (PCR) and immunocapture-PCR (19,21,22).

Nevertheless, a majority of importing countries, such as China, France, Japan, Netherlands, Israel, and the United States, requires field inspection prior to harvest (S. Kessank, Plant Protection Research and Development Office, Department of Agriculture, Thailand, *personal communication*). For field inspection, general methods for detection of suspicious plant samples are the enzyme-linked immunosorbent assay (ELISA) or PCR methods for initial screening. Positive samples are then confirmed with bacterial isolation on semiselective media (1). However, ELISA is the more widely used technique for routine field inspection because it is simple, cheap, and robust without a need of expensive equipment. Moreover, ELISA does not require pathogen isolation. For immunological assays, specificity and sensitivity depend on the characteristics of antibodies; therefore, highly specific antibodies are critical for this type of assay.

In the case of *A. citrulli*, available rabbit polyclonal antibodies (PABs) yield false-positive results because they also react with closely related bacteria such as other *Acidovorax* spp. and *Comamonas acidovorans* (18,21). Monoclonal antibodies (MAbs) offer several advantages over conventional polyclonal antiserum such as specificity for a single epitope, consistent quality, and unlimited quantity of antibodies. Although a MAb against *A. citrulli* is commercially employed in an ELISA test kit for *A. citrulli* detection (Agdia), its production and characterization has never been reported. Therefore, this study reports the first detailed production and characterization of a highly specific MAb against *A. citrulli* and the establishment of the MAb captured-sandwich (MC-s)ELISA for direct detection of this pathogen in cucurbit leaf, fruit and seed samples.

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Materials and Methods

Bacterial strains. Bacterial strains used in this study are shown in Table 1. Confirmation of *Acidovorax citrulli* strains was performed using carbon-source metabolic fingerprinting (Biolog system) according to manufacturer's instructions (Biolog Inc., Hayward, CA) and pathogenicity assays on various cucurbit seedlings such as watermelon (*Citrullus lanatus*), melon (*Cucumis melo*), and cucumber (*C. sativas*) (6). All strains were also tested by PCR using primers specific to the 16S rRNA gene of *A. citrulli* (20). All bacteria were preserved in Nutrient Broth (NB), (Difco, France) with 15% glycerol at -80°C .

To prepare the bacterial cell suspensions, bacteria were cultured on NB with 1.5% Bacto agar (Difco) and incubated at 28°C for 48 h. A single colony was selected and inoculated into 50 ml of NB. The cultures were incubated at 28°C for 18 h, with shaking at 200 rpm. The optical density at a wavelength of 600 nm (OD_{600}) was measured using a spectrophotometer (spectro UV-VIS RS; Labomed Inc.). The total numbers of bacteria were calculated based on a conventional plate count method (OD_{600} of 1.0 corresponding to approximately 1×10^9 CFU/ml). The bacteria were harvested after centrifugation at 5,000 rpm for 10 min (Sorvall). The cell pellets were washed twice with 5 ml of 0.1 M phosphate-buffered saline (PBS; 1.5 mM KH_2PO_4 , 137 mM NaCl, 20 mM Na_2HPO_4 , and 2.7 mM KCl [pH 7.2]) and resuspended in 5 ml of PBS to obtain a final concentration of 1×10^{10} CFU/ml.

Antigen preparation. The *A. citrulli* strain KK9 (Ac KK9) was isolated from symptomatic watermelon fruit collected in Khon

Kaen province (Thailand) and used as an antigen for MAb production. A cell suspension of Ac KK9 was cultured as described above and sonicated using ultrasonic sonicator (Sonic Vibra cell) at 35% amplitude for 5 min on ice with 10-s intervals. Protein concentration was determined by the Bradford method (3) and adjusted to 0.5 mg/ml. The antigens were stored at -20°C until use.

MAb production. The hybridoma production protocol used was modified from the method developed by Oi and Herzenberg (12). Six-week-old BALB/c mice were injected intraperitoneally (i.p.) with 100 μg of sonicated cell suspension of *A. citrulli* (approximately 1.8×10^8 CFU). Three immunizations were performed at 2-week intervals. Serum from each immunized mouse obtained 7 days after each immunization was tested for antibodies against cell suspension of *A. citrulli* using plate-trapped antigen (PTA)-ELISA as described below. The mouse with the highest antibody titer was challenged i.p. with 120 μg of the antigen and sacrificed 3 days later for hybridoma preparation. Isolated mouse splenocytes were fused with P3X63-Ag8.653 mouse myeloma cells (spleen cells/myeloma cells ratio of 5:1) in the presence of 50% (wt/vol) polyethylene glycol (1450 Hybri Max; Sigma-Aldrich, St. Louis). Hybridoma cells were selected by culturing in hypoxanthine aminopterin thymidine (HAT) medium, which composed of RPMI 1640 medium (Invitrogen), 20% (vol/vol) fetal bovine serum (PAA Laboratories, Austria), 1 $\times$ HAT supplement (Sigma-Aldrich), and 10% (vol/vol) BM-Condensed H1 (Hybridoma Cloning Supplement, Roche). Cultures were propagated and screened for production of antibodies to *A. citrulli* using PTA-ELISA. Cross reactivity of antibodies with *Comamonas acidovorans* and nonspecific bind-

Table 1. Specificity of monoclonal antibody (MAb) 11E5 and MAb 10B2 against *Acidovorax citrulli* and other bacterial strains using plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA), compared with those of commercial ELISA test kit and commercial immunochromatographic strip test^a

Bacterial strains	Host	Source ^b	PTA-ELISA activity (A_{405})		Commercial test kit	
			MAb 11E5	MAb 10B2	ELISA	QuickStix
<i>Acidovorax citrulli</i>						
Ac-KK9	Watermelon	Khon Kaen	1.68 (+)	2.61 (+)	3.19 (+)	+
Ac-WM-MK 5/2	Watermelon	Maharakham	2.78 (+)	3.19 (+)	3.20 (+)	+
Ac-WM-MK 4/2	Watermelon	Maharakham	2.57 (+)	3.31 (+)	3.32 (+)	+
Ac-WM-MK 4/1	Watermelon	Maharakham	2.82 (+)	3.25 (+)	3.24 (+)	+
Ac-WM-MK 5/1	Watermelon	Maharakham	2.56 (+)	3.39 (+)	3.24 (+)	+
Ac-WM-NB 4/2	Watermelon	Nongboulampoo	2.41 (+)	3.14 (+)	3.23 (+)	+
Ac-DOAC 4 - 1364	Melon	DOA	2.59 (+)	3.36 (+)	2.69 (+)	+
Ac-DOAC 4 - 1194	Watermelon	DOA	1.81 (+)	2.56 (+)	2.77 (+)	+
Ac-LS	Cantaloupe	DPAN	2.03 (+)	3.09 (+)	3.09 (+)	+
Ac-WS	Watermelon	DPAN	2.11 (+)	3.09 (+)	3.41 (+)	+
Ac-CU	Cucumber	DPAN	1.98 (+)	3.12 (+)	3.11 (+)	+
Ac-WM007/1 ^c	Bottle gourd	Imported seed	1.18 (+)	3.02 (+)	3.23 (+)	+
Ac-WM 015/2 ^c	Squash	Imported seed	2.66 (+)	3.35 (+)	3.30 (+)	+
Ac-WM006/1 ^c	Bottle gourd	Imported seed	2.76 (+)	3.34 (+)	3.04 (+)	+
Ac-WM006/2 ^c	Bottle gourd	Imported seed	1.34 (+)	3.09 (+)	3.23 (+)	+
Ac-WM 015/1 ^c	Squash	Imported seed	3.04 (+)	3.32 (+)	3.22 (+)	+
Ac-WM007/2 ^c	Bottle gourd	Imported seed	2.71 (+)	3.35 (+)	3.15 (+)	+
Ac-SQ type A ^c	Squash	Imported seed	2.11 (+)	0.10 (–)	3.23 (+)	+
Ac-SQ type B ^c	Squash	Imported seed	2.04 (+)	0.09 (–)	0.10 (–)	–
<i>A. facilis</i>	Watermelon	DPAK	0.11 (–)	0.11 (–)	0.12 (–)	–
<i>Burkholderia plantarii</i>	Watermelon	DPAK	0.11 (–)	0.09 (–)	0.08 (–)	–
<i>Comamonas acidovorans</i>	Watermelon	DPAK	0.16 (–)	3.47 (+)	0.10 (–)	–
<i>C. testosteroni</i>	Watermelon	DPAK	0.15 (–)	2.02 (+)	0.11 (–)	–
<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>	Chinese cabbage	DPAK	0.11 (–)	0.07 (–)	0.11 (–)	–
Fluorescent <i>Pseudomonas</i> spp.	Cucurbits	DPAK	0.09 (–)	0.08 (–)	0.10 (–)	–
<i>Pantoea ananur</i>	Tomato	DPAK	0.11 (–)	0.07 (–)	0.12 (–)	–
<i>Ralstonia solanacearum</i>	Tomato	DPAK	0.12 (–)	0.08 (–)	0.13 (–)	–
<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>	Tomato	DPAK	0.08 (–)	0.07 (–)	0.12 (–)	–
Carbonate buffer (negative control)	–	–	0.09 (–)	0.08 (–)	0.09 (–)	–

^a PTA-ELISA and commercial ELISA kit (Agdia) were performed in three replicates for each sample. A sample was considered positive when an absorbance at 405 nm (A_{405}) value was higher than twice the value of carbonate buffer (negative control). Bacteria were also tested with immunochromatographic strip test, QuickStix (Envirologix) following the manufacturer's instruction.

^b Sources from Thailand, including DOA: Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand; DPAK: Department of Plant Sciences and Agricultural Resources, Khon Kaen University, Khon Kaen 40002, Thailand; and DPAN: Department of Plant Pathology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand.

^c Bacterial isolate obtained from seedling grow-out test for bacterial fruit blotch pathogen. All of the tested seedlings were sterilized by autoclave after examination.

ing to the plate (no antigen) were also evaluated in this assay. Hybridoma cultures that produced antibodies to *A. citrulli* were cloned by the limiting dilution method to produce monoclonal cultures of antibody-producing hybridoma cells (14).

Characterization of MABs. Immunoglobulin types were identified with the Mouse MonoAb ID Kit (Zymed; Invitrogen). Specificity of MABs against *A. citrulli* and other bacteria was determined by PTA-ELISA and Western blot analysis as described below. To compare between our MAB and MABs in commercial tests, ELISA kit (Agdia) and immunochromatographic strip test (Envirologix) were also evaluated for specificity against these bacteria.

PTA-ELISA. Ninety-six-well microtiter plates (Corning Inc.) were coated overnight at 4°C with 100 µl of 10⁸ CFU/ml of a bacterial cell suspension diluted in carbonate buffer (14 mM Na₂CO₃ and 35 mM NaHCO₃, pH 9.6). The plates were washed four times with 400 µl of PBS + 0.05% Tween 20, pH 7.2 (PBST) before being blocked with 100 µl of 2% bovine serum albumin (BSA; Sigma-Aldrich) in PBST for 1 h at 25°C. Subsequently, plates were washed as described above. Hybridoma culture supernatant, cultured medium containing antibodies secreted from hybridoma cell (100 µl), was then added and incubated for 1 h at 25°C. Each plate was washed with PBST before 100 µl of 1/2,000 goat anti-mouse immunoglobulins conjugated with alkaline phosphatase (Sigma-Aldrich) diluted in 0.5% BSA in PBST were added and incubated for 1 h at 25°C. After washing, 100 µl of a substrate solution for alkaline phosphatase, *p*-nitrophenyl phosphate (pNPP, Zymed; Invitrogen), was added and incubated for 1 h at 25°C. The absorbance at 405 nm was measured using an automated microplate reader (VersaMax). A result was considered positive when the absorbance was at least twice that of the negative controls.

Western blot analysis. Proteins from a whole-cell suspension (1 × 10⁸ CFU) of *A. citrulli* and *C. acidovorans* were subjected to the discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis using a 5% stacking gel and 10% separating gel under reducing conditions (8). Protein molecular weight markers (number SM0671; Fermentas, UK) were included in each gel. Proteins were transferred to a nitrocellulose membrane (BioTrace; Pall Corporation) using a semidry blotting apparatus (Trans-Blot Semi-Dry Electrophoretic Transfer Cell; Bio-Rad). The membranes were soaked in 5 ml of Tris-buffered saline plus Tween 20 (TBST; 10 mM Tris [pH 8], 150 mM NaCl, and 0.05% Tween 20) for 10 min and blocked with 5 ml of 4% BSA in TBST for 1 h. The nitrocellulose membranes were then incubated with 5 ml of mouse MAB against *A. citrulli* for 1 h. After washing with TBST three times for 10 min each, detection was carried out using alkaline phosphatase conjugated goat anti-mouse immunoglobulin G (IgG) (H+L) (Sigma-Aldrich) for 1 h. The membranes were washed as above and incubated for 2 to 3 min in the substrate solution (NBT/BCIP Kit; Zymed). The color development was stopped by immersing the membrane in distilled water. The membrane was air dried. Molecular weight of each protein band was compared with standard molecular weight markers.

Development of sandwich ELISAs for detection of *A. citrulli* in plant samples. Two systems of sandwich (s)ELISAs were developed. First, MC-sELISA used MAB 11E5 as capture antibodies and rabbit PABs against a membrane protein complex of *A. citrulli* (anti-Ac MPC rPAB; purchased from the Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Kamphaeng Saen Campus, Thailand) as detecting antibodies. Second, PAB captured-sandwich ELISA (PC-sELISA) used anti-Ac MPC rPAB as capture antibodies and MAB 11E5 as detecting antibodies.

For MC-sELISA, 96-well microtiter plates were coated overnight at 4°C or for 2 h at 25°C with 100 µl of MAB 11E5 at 2 µg/ml diluted in carbonate buffer. The plates were washed four times with 400 µl of PBST. Bacteria (100 µl) with 10-fold serial dilution (0 to 10⁸ CFU/ml) in an extraction buffer (PVPBST⁺: 0.06 M Na₂SO₃, 2% [wt/vol] polyvinylpyrrolidone 40, egg albumin at 2

g/liter, and 2% Tween 20 in PBS) were added to the wells and incubated for 1 h at 25°C. Each plate was washed with PBST as described above before 100 µl of 1/4000 anti-Ac MPC rPAB diluted in 0.5% BSA in PBST was added and incubated for 1 h at 25°C. Each plate was then rewashed. Goat anti-rabbit immunoglobulins conjugated with alkaline phosphatase (Sigma-Aldrich) diluted 1/10,000 in 0.5% BSA in PBST (100 µl/well) were added and incubated for 1 h at 25°C. The chromogenic signal was obtained as described above. For PC-sELISA, the protocol was similar to MC-sELISA except that anti-Ac MPC rPAB was used as the capture antibody and MAB 11E5 was used as the secondary antibody. Goat anti-mouse immunoglobulin conjugated with alkaline phosphatase was used as the tertiary antibody.

To optimize these two sELISAs, three extraction buffers (PVPBST⁺, Tris-Na₂SO₃, and PBST) were compared in *A. citrulli* detection. Buffer PVPBST⁺ was composed of 0.06 M Na₂SO₃, 2% (wt/vol) polyvinylpyrrolidone 40, egg albumin at 2 g/liter, and 2% Tween 20 in PBS. Buffer Tris-Na₂SO₃ was composed of 0.05 M Tris-HCl and 0.06 M Na₂SO₃, pH 8.5. Buffer PBST was composed of PBS with 0.05% Tween 20, pH 7.4. *A. citrulli* and other bacteria (fluorescent *Pseudomonas* spp., *Xanthomonas campestris* pv. *vesicatoria*, *Pectobacterium carotovorum* subsp. *carotovorum*, and *C. acidovorans*) were individually diluted in an extraction buffer to obtain final concentration of approximately 10⁸ CFU/ml. The bacterial suspensions were assayed by both sELISA procedures as described above.

In order to compare the sensitivity of the two sELISAs, *A. citrulli* cells were spiked into PVPBST⁺ extraction buffer and leaf and seed extracts to obtain final concentrations of 0 to 10⁸ CFU/ml. Leaf extracts were prepared by grinding leaves from healthy plants (20%, wt/vol) in PVPBST⁺ extraction buffer. Seed wash was prepared by incubating noninfested watermelon seed (100 seeds) in 10 ml of PVPBST⁺ for 1 h at approximately 25°C with agitation at 200 rpm. For seed macerate, noninfested watermelon seed (100 seeds) were crushed in 10 ml of PVPBST⁺ using a homogenizer (Philips, The Netherlands). Both seed extracts were passed through two layers of cheesecloth to remove the seed debris and the filtrate was kept for further use. Ac KK9 was cultured and prepared as described above. The Ac KK9 suspension was adjusted to an OD₆₀₀ of 1.0 (approximately 10⁹ CFU/ml). Using this cell suspension, 10-fold serial dilutions were made in PVPBST⁺ buffer, leaf extracts, seed wash, and seed macerate. Each diluted sample was analyzed by the two ELISA assays along with the commercial ELISA kit (Agdia).

Detection of *A. citrulli* in naturally infected leaf and fruit. The MC-sELISA was applied to test naturally infected leaf and fruit samples. In total, 56 leaf and fruit samples of watermelon and melon showing BFB-like symptoms were collected from 10 production fields in six provinces in Thailand. Leaf extracts were prepared as described above. For the fruit extraction, approximately 5 g of fruit skin with lesion were ground (50%, wt/vol) in PVPBST⁺ extraction buffer. The samples were analyzed by MC-sELISA in parallel with a commercial *A. citrulli* ELISA test kit (catalog number SRA 14800; Agdia). Healthy leaf and fruit samples were included in all experiments as negative controls.

Detection of *A. citrulli* in artificially and naturally infested seed. Artificially infested seed were prepared by soaking noninfested watermelon seed (*n* = 100) in 20 ml of *A. citrulli* suspension (10⁸ CFU/ml). The seed suspension was then vacuum infiltrated for 20 min in a vacuum jar and the seed were allowed to air dry overnight at room temperature (21). Each infested seed contained approximately 10⁶ to 10⁷ CFU of *A. citrulli*, determined by bacterial plate count. The infested seed were mixed with noninfested seed to generate different levels of *A. citrulli* contamination (number of infested seed/total number of seed tested): 0% (0/1,000), 0.1% (1/1,000), 0.3% (3/1,000), 1% (10/1,000), and 5% (50/1,000). Each test was performed in three replicates. Seed extracts (seed wash and seed macerate) were prepared and tested by MC-sELISA as described above. Noninfested seed were used as negative control. For naturally infested seed lots, watermelon and cucumber seed

lots from 10 production fields in Khon Kaen province (Thailand) were screened for *A. citrulli* using MC-sELISA. Seed ($n = 400$) from each lot were ground in 40 ml of PVPBST⁺ and prepared as described above. Seed macerate samples were subjected to MC-sELISA and the commercial ELISA kit for comparison. The tests were performed in parallel with a modified SGO assay as follows. For SGO, 100 seeds per lot were placed on blotter paper (Hoffman Manufacturing Inc., Albany, OR), saturated with sterile water, and incubated in a closed, transparent plastic box (6 by 24 by 33.5 cm [height by width by length]) (10). Seed were incubated in a transparent plastic box for 14 days under conditions of 28 to 35°C and >90% relative humidity. During this time, the seedlings were visually evaluated every day for BFB symptoms.

Results

Production and characterization of MAbs. To produce MAbs specific to *A. citrulli*, the sonicated cell suspension of *A. citrulli* was used as an immunogen for MAb production. All 630 wells of the fused cells contained hybridoma cells. Culture supernatants from 10 of the 630 cultures strongly reacted with *A. citrulli* cells coated on the microtiter plates in PTA-ELISA and remained stable after cloning. However, 9 hybridoma clones (1D4, 2C2, 4C11, 6F3, 7F7, 7G10, 9D7, 10B2, and 10F6) of the 10 clones also cross-reacted with *C. acidovorans*. Only one MAb, 11E5, reacted specifically with *A. citrulli* and not with other closely related bacteria. Two hybridoma clones (MAb11E5 and MAb10B2) were selected to represent the two groups of MAbs (specific and nonspecific to *A. citrulli*) and further characterized.

The specificity of MAbs 11E5 and 10B2 was analyzed against a wide range of bacteria (Table 1) using PTA-ELISA. MAb 11E5 (isotype IgG₁, κ light chain) reacted specifically with all 19 strains of *A. citrulli* tested with no cross-reaction to closely related bacteria in the family *Comamonadaceae* (such as *A. facilis*, *C. acidovorans*, and *C. testosteronii*) and other phytopathogenic bacteria (Table 1). MAb 10B2 (isotype IgG₁, κ light chain) reacted with only 17 of the 19 strains of *A. citrulli* tested and also cross-reacted with closely related bacteria in the family *Comamonadaceae* such as *C. acidovorans* and *C. testosteronii*. However, MAb 10B2 did not cross-react with other unrelated bacteria (Table 1). Similar results were obtained by the Western blot analysis. MAb 11E5 specifically reacted with *A. citrulli* isolates, yielding a single protein band at molecular weight greater than 170 kDa, while MAb 10B2 bound to proteins from *A. citrulli* and *C. acidovorans* at approximately 43 and 34 kDa, respectively (Fig. 1). MAb 11E5 was chosen for further assay development due to its high specificity to *A. citrulli*. Moreover, MAb 11E5 reacted with all 19 *A. citrulli* strains tested while the commercial ELISA kit (Agdia) and immunochromatographic strip test (QuickStix; Envirologix) failed to detect one strain (Table 1).

Development of sELISA for detection of *A. citrulli* in plant samples. The MAb 11E5 was used to develop two sELISA proto-

cols (MC-sELISA and PC-sELISA) for detection of *A. citrulli* in plant samples. In order to optimize both ELISA systems, three different extraction buffers (PVPBST⁺, Tris-Na₂SO₃, and PBST) were compared. *A. citrulli* and other bacteria, including *X. campestris* pv. *vesicatoria*, *P. carotovorum* subsp. *carotovorum*, fluorescent *Pseudomonas* spp., and *C. acidovorans*, were spiked into each extraction buffer (10^8 CFU/ml) and analyzed by both sELISA systems. With the PC-sELISA system, all extraction buffer types gave comparable results without nonspecific reaction with other bacteria (Fig. 2A). For MC-sELISA, nonspecific reaction with other bacteria was found when Tris-Na₂SO₃ and PBST were used to dilute the bacteria (Fig. 2B). Specific detection of *A. citrulli* was obtained when PVPBST⁺ was used as an extraction buffer in MC-sELISA (Fig. 2B). Therefore, PVPBST⁺ was selected to use as an extraction buffer for further assay development.

After the suitable extraction buffer was selected, the two sELISA systems (MC-sELISA and PC-sELISA) were compared for their sensitivity in detecting *A. citrulli* spiked in buffer, leaf, and seed extracts. From the results, ELISA sensitivity was not affected by the type of sample (seed or leaf) or preparation method (grinding or washing; Fig. 3). MC-sELISA has the same sensitivity in detection of *A. citrulli* when compared with a commercial ELISA kit (Agdia) (Fig. 3). MC-sELISA was approximately 10-fold more sensitive than the PC-sELISA in detection of *A. citrulli* in all cases (Fig. 3). The detection limit of MC-sELISA was the same as that of the commercial ELISA kit (Agdia) (approximately 5×10^4 CFU/ml) while lower sensitivity was obtained in PC-sELISA (approximately 5×10^5 CFU/ml). In all subsequent experiments, the MC-sELISA protocol was used.

Detection of *A. citrulli* in naturally infected leaves and fruit.

In total, 56 leaf and fruit samples showing BFB-like symptoms were collected from 10 production fields in six provinces in Thailand. MC-sELISA and a commercial ELISA kit were used to assay for *A. citrulli* in these samples and both methods gave 100% agreement, with 31 positive and 25 negative results. Healthy leaves and fruit skins gave negative results using MC-sELISA and commercial ELISA kit.

Fig. 1. Western blot analysis of whole-cell suspensions of *Acidovorax citrulli* (lane 1) and *Comamonas acidovorans* (lane 2) probed with **A**, monoclonal antibody (MAb) 11E5 and **B**, MAb 10B2. Molecular-weight (MW) standards (Fermentas) are shown in kilodaltons on the left side.

Fig. 2. Comparison of extraction buffers. Bacteria were resuspended in three extraction buffers at the concentration of 10^8 CFU/ml and then were analyzed by **A**, polyclonal antibody captured-sandwich (PC-s) enzyme-linked immunosorbent assay (ELISA) and **B**, monoclonal antibody captured-sandwich (MC-s)ELISA. PBST = phosphate-buffered saline with Tween 20 and PVPBST⁺ = PBS with polyvinylpyrrolidone 40, Na₂SO₃, egg albumin, and Tween 20.

Detection of *A. citrulli* in artificially and naturally infested seed lots. To evaluate the sensitivity of MC-sELISA and commercial ELISA kit (Agdia) in detecting *A. citrulli*, watermelon seed lots with different percentages of artificially infested seed were tested using two different types of sample preparation: seed macerate and seed wash. Using seed macerate, both MC-sELISA and the commercial ELISA kit (Agdia) could detect *A. citrulli* in seed lots with 0.3% infestation. Compared with bacteria detection in seed macerate, the detection of *A. citrulli* in seed wash gave lower sensitivity and higher background using both methods; 5 and 1% infestation for MC-sELISA and commercial ELISA, respectively (Fig. 4). Therefore, seed macerate is recommended for preparation of seed extracts before applying MC-sELISA.

To determine the feasibility of this method for screening for *A. citrulli* in naturally infested seed, 10 commercial seed lots were tested using the MC-sELISA, a commercial ELISA kit (Agdia), and SGO. For MC-sELISA and the commercial ELISA test kit, three replicates were performed for each seed sample ($n = 400$). By both methods, *A. citrulli* was detected in three watermelon seed lots, numbers 1, 2, and 5, (Table 2). The results from MC-sELISA and the commercial ELISA test kit agreed with those from the standard SGO method. For the SGO method, BFB symptoms were observed on seedlings derived from three watermelon seed lots, numbers 1, 2, and 5, with 16, 4, and 8% of diseased seedlings, respectively (Table 2).

Discussion

In this study, we generated a specific MAb (MAb 11E5) against *A. citrulli* and used it to develop sELISA for detection of this pathogen in leaf, fruit, and seed samples. MAb 11E5 reacted with all *A. citrulli* strains tested with no cross-reactivity to other closely related bacteria in the family of *Comamonadaceae* such as *A. facilis*, *C. acidovorans*, and *C. testosteronii*. The MAb produced in this study has an advantage over the previously reported PABs (18,21) because of its higher specificity. Additionally, when this MAb is used in ELISA, it can detect a wider range of *A. citrulli* strains than the MAb-based commercial ELISA kit (Agdia) and MAb-based immunochromatographic strip test (QuickStix; Envirologix).

Generally, *A. citrulli* strains can be divided into at least two groups (groups I and II) based on pathogenicity assays, DNA-fingerprinting profiles, and whole-cell fatty-acid analyses (20). Group I includes strains mainly isolated from non-watermelon cucurbits, while group II includes the typical watermelon BFB-

causing strains (4,20). MAb 11E5 strongly reacted with Ac KK9, which is the same strain as Ac 202-69 that belongs to group II (20). However, because reference strains of group I are not available in Thailand because pathogen import is strictly prohibited, we used *A. citrulli* strains isolated from non-watermelon cucurbits (melon, cantaloupe, squash, bottle gourd, and cucumber) in Thailand and found that MAb 11E5 could also react to these strains.

MAb 11E5 could detect *A. citrulli* strains that were isolated from imported squash and bottle gourd seed. Although MAb 11E5 detected all strains of *A. citrulli* tested, the commercial ELISA kit (Agdia) and immunochromatographic strip test (QuickStix; Envirologix) failed to detect squash (SQ) type B strain, which was isolated from squash seed. Strain SQ type B was identified to be *A. citrulli* using carbon-source metabolic fingerprinting (Biolog system) and a PCR method using primers specific to the 16S rRNA gene of *A. citrulli* (21). *A. citrulli* strain SQ type B showed BFB symptoms on inoculated watermelon, melon, cucumber, bottle gourd, and squash seedlings (P. Thummabenjapone, unpublished data).

Because of its specificity to *A. citrulli*, MAb 11E5 was subsequently used for ELISA development. In the case of *A. citrulli*

Fig. 4. Detection of *Acidovorax citrulli* in artificially infested watermelon seed lots by monoclonal antibody captured-sandwich (MC-s) enzyme-linked immunosorbent assay (ELISA) and a commercial ELISA kit (Agdia). Two independent experiments were performed with three replicates per experiment. Data showed an average of the results with error bars representing standard deviation. Cut-off values were twice of values obtained from negative control (0% infested seed lot). For seed wash extraction, the dotted line designates cut-off value for MC-sELISA and the long dashed dot line designates cut-off value for the commercial ELISA kit (Agdia). For seed macerate extraction, the solid line designates cut-off value for MC-sELISA and the dashed line designates cut-off value for the commercial ELISA kit (Agdia).

Fig. 3. Comparison of the sensitivity of two sandwich enzyme-linked immunosorbent assay (ELISA) systems—monoclonal antibody captured-sandwich (MC-s)ELISA and polyclonal antibody captured-sandwich (PC-s)ELISA—as well as a commercial ELISA kit (Agdia) for detection of various concentrations of *Acidovorax citrulli* in extraction buffers, leaf sap, and seed extracts. Two independent experiments were performed with three replicates per experiment. Data showed an average of the results with error bars representing standard deviation. Cut-off values were twice of values obtained from negative control (*A. citrulli* at 0 CFU/ml in corresponding sample preparations). Horizontal line designates cut-off values for MC-sELISA, PC-sELISA, and the commercial ELISA kit (Agdia).

detection, a screening method for field inspection prior to harvest is crucial, and ELISA is the most practical method for this situation. Two sELISA systems were developed and compared for their sensitivity in detection of *A. citrulli* in this study. MC-sELISA was 10 times more sensitive than PC-sELISA in detection of *A. citrulli* in leaf and seed extracts. The increased sensitivity of MC-sELISA could be explained by the possibility that the PAb, when used as a detecting molecule, could bind to more epitopes on the antigen than the use of MAb that binds a single epitope.

Three extraction buffers were evaluated in this study to reduce nonspecific antibody binding and increase ELISA sensitivity (5). PVPBST⁺ yielded better results than the other two extraction buffers (Tris-Na₂SO₃ and PBST) when PAb was used as the detecting antibody in MC-sELISA, whereas Tris-Na₂SO₃ and PBST resulted in nonspecific reactions. Without an effective extraction buffer, it is possible that these bacteria were nonspecifically bound to the surface of microtiter plate and subsequently detected by the PABs. However, PVPBST⁺ extraction buffer contains Tween 20 and egg albumin that can more effectively block nonspecific binding of non-target bacteria to microtiter plate surfaces (17).

It was previously reported that substances in plant and seed extracts interfere with the PCR detection of *A. citrulli* (19,20). By using MC-sELISA, the effect of an interfering substance was negligible. Comparable sensitivity of detection was observed with the *A. citrulli*-spiked extraction buffer, and the *A. citrulli*-spiked leaf and seed extracts (*A. citrulli* at approximately 5×10^4 CFU/ml). It is important to note that seed preparation by grinding yielded higher reactivity than by washing. Previous reports showed that *A. citrulli* is localized on both the surface of the seed coat and the endosperm (11,19), which support our observation that grinding is better for extracting *A. citrulli* from seed. Therefore, the MC-sELISA is applicable for differentiation between healthy and the artificially infected plant samples. Moreover, MC-sELISA detected *A. citrulli* in naturally infested leaves and fruit with 100% correlation with the commercial ELISA kit.

For seed tests, the results obtained with MC-sELISA were completely in agreement with the commercial ELISA kit and SGO but the sensitivity was less than that reported for immunomagnetic separation and PCR (19,21). However, to achieve higher sensitivity, this new MAb against *A. citrulli* can be used with immunomagnetic separation and PCR to improve routine cucurbit seed detection.

In conclusion, the MAb specific to *A. citrulli* has been employed to develop an ELISA test that detected all tested *A. citrulli* strains. The ELISA system can be applied directly to leaf, fruit, and seed samples without pathogen isolation. This simple

and inexpensive method can be applicable for routine field disease inspection.

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Table 2. Detection of *Acidovorax citrulli* in commercial watermelon and cucumber seed lots using seedling grow-out method, monoclonal antibody captured-sandwich enzyme-linked immunosorbent assay (MC-sELISA), and a commercial ELISA kit (Agdia)^a

Seed samples	Seed (n)	Seedling grow-out					
		Germination		Diseased seedlings		Reactivity (A_{405})	
		Number	Percent	Number	Percent	MC-sELISA	ELISA
Watermelon Khonkaen number 1	100	87	87	14	16	2.00 ± 0.12 (+)	2.54 ± 0.51 (+)
Watermelon Khonkaen number 2	100	87	87	3	4	0.79 ± 0.07 (+)	0.97 ± 0.03 (+)
Watermelon Khonkaen number 3	100	76	76	0	0	0.12 ± 0.03 (–)	0.09 ± 0.04 (–)
Watermelon Khonkaen number 4	100	82	82	0	0	0.11 ± 0.02 (–)	0.08 ± 0.04 (–)
Watermelon Khonkaen number 5	100	79	79	6	8	1.39 ± 0.43 (+)	2.16 ± 0.44 (+)
Cucumber number 406 80101	32	31	97	0	0	0.17 ± 0.07 (–)	0.19 ± 0.02 (–)
Cucumber number 406 80201	32	27	84	0	0	0.21 ± 0.04 (–)	0.12 ± 0.02 (–)
Cucumber number 406 80202	32	30	94	0	0	0.16 ± 0.01 (–)	0.13 ± 0.02 (–)
Cucumber number 274 80301	32	32	100	0	0	0.14 ± 0.04 (–)	0.13 ± 0.03 (–)
Cucumber number 043 80201	32	26	81	0	0	0.22 ± 0.04 (–)	0.13 ± 0.03 (–)
Healthy watermelon seed	100	98	98	0	0	0.16 ± 0.01 (–)	0.12 ± 0.02 (–)
Healthy cucumber seed	32	31	97	0	0	0.13 ± 0.01 (–)	0.19 ± 0.02 (–)
Seed from <i>A. citrulli</i> -infected watermelon	200	176	88	23	13	0.85 ± 0.04 (+)	1.26 ± 0.12 (+)
Seed from <i>A. citrulli</i> -infected cucumber	32	30	94	6	20	0.58 ± 0.01 (+)	0.58 ± 0.06 (+)

^a Number of seed sampling for MC-sELISA and commercial ELISA, $n = 400$. The samplings were done in three replicates. Commercial ELISA kit was purchased from Agdia (ELISA kit, catalog number SRA 14800). Data shown represent the average of absorbance value (A_{405}) from three replicates. Samples with A_{405} greater than twice of those from the healthy seed were considered positive.

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