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Factors influencing the detection of *Acidovorax citrulli* in naturally contaminated cucurbitaceous seeds by PCR-based assays

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Abstract

The success of *Acidovorax citrulli* detection by seed health testing of commercial cucurbitaceous seeds depends on the efficiency of pathogen extraction. In the present study, different extraction techniques were compared to identify factors that influence *A. citrulli* detection in naturally contaminated cucurbit seeds. Embryo-infected watermelon seeds, produced by pistil inoculation, were mixed with non-infected seeds to generate samples (n = 1000 seeds) with low infection levels (approximately 10^4 cells per sample). Additionally, two naturally infested melon seed lots were tested. *A. citrulli* was extracted from seeds by soaking or crushing, followed by one or two centrifugation steps. Samples extracted by soaking seeds yielded better amplification efficiency (103%) compared with crushing (93%), as determined by quantitative real-time PCR analysis. This was most likely due to a reduction of the concentration of inhibitors present in the DNA samples. PCR assays using three different *A. citrulli*-specific primer sets highlighted that soaking followed by two centrifugation steps enhanced pathogen detection (100% of the samples) and the mean cycle threshold (Ct) value was significantly lower than those observed for the other pathogen extraction techniques. These results indicate that the optimised extraction protocol combined with PCR analysis can improve routine seed health testing for *A. citrulli.*

Keywords: watermelon, melon, bacterial fruit blotch, seed health testing, pathogen extraction techniques, real-time PCR assays

Introduction

Acidovorax citrulli (syn. *Acidovorax avenae* subsp. *citrulli*) (Schaad *et al.*, 1978, 2008; Willems *et al.*, 1992) is the causal agent of bacterial fruit blotch (BFB), a serious threat to cucurbitaceous crop production worldwide (Schaad *et al.*, 2003). BFB can cause up to 100% fruit loss under environmental conditions that are conducive for disease development (Burdman and Walcott, 2012). The first BFB outbreaks in commercial watermelon fields were reported in 1989 in Indiana (Latin and Rane, 1990), Florida (Somodi *et al.*, 1991),

Delaware, Iowa and Maryland (Latin and Hopkins, 1995). Subsequently, the disease has occurred worldwide (EPPO, 2017a). Since BFB can be a devastating disease of cucurbit crops (in particular melon and watermelon) and has been rarely reported in the European Plant Protection Organization (EPPO) region, *A. citrulli* was recently added to the EPPO quarantine A1 List (EPPO, 2017b).

At least two genetically and pathogenically distinct groups have been differentiated among *A. citrulli* strains (Walcott *et al.*, 2004). Of these, group I strains have mainly been isolated from non-watermelon cucurbit hosts, particularly from cantaloupe melon (*Cucumis melo* var. *cantalupensis*), whereas group II strains have mainly been recovered from watermelon (*Citrullus lanatus*) (Walcott *et al.*, 2000, 2004; Burdman *et al*., 2005). Interestingly, group I strains are moderately to highly aggressive on a range of cucurbit hosts, while group II strains are highly aggressive on watermelon, but mildly aggressive on non-watermelon hosts (Walcott *et al.*, 2000, 2004).

Acidovorax citrulli is seedborne and seed-transmitted, and infected/infested seeds are the most important source of primary inoculum for BFB epidemics in transplant and fruit production systems (Dutta *et al.*, 2012a). Under conducive environmental conditions, the pathogen can spread rapidly among seedlings in transplant houses and in fruit production fields causing severe and costly outbreaks. The risk of BFB epidemic development can be high even when seed lots have low levels of *A. citrulli* contamination (Dutta *et al.*, 2012b). Hence, exclusion of *A. citrulli* from cucurbit production systems is a challenge for effective BFB management (Walcott, 2008). To date, no seed treatments are completely effective against BFB and resistant cucurbit cultivars are not commercially available. Hence, the most common management strategies are: (i) pathogen exclusion in seed production areas; (ii) certification of transplants; and (iii) seed health testing (Gitaitis and Walcott, 2007).

Currently, polymerase chain reaction (PCR)-based methods and seedling grow-out (SGO) assays are most widely employed for *A. citrulli* detection in cucurbit seed lots (Gitaitis and Walcott, 2007; Feng *et al.*, 2013). Seedling grow-out assays have several limitations, including that they: (i) are time consuming, requiring up to three weeks for seedling germination and symptom development; (ii) require large areas in greenhouses (or incubators for sweatbox SGO assays); and (iii) have relatively low detection sensitivity (Walcott *et al.*, 2006; Bahar *et al.*, 2008). PCR-based assays provide an alternative approach for *A. citrulli* detection that enhance sensitivity and specificity, while minimising the time for assay completion and optimising costs (Feng *et al.*, 2013).

Ideally, effective seed health assays should consider pathogen location on/in seeds and be able to detect low levels of inoculum (Dutta *et al.*, 2012c). In watermelon, *A. citrulli* can gain access to seeds by two pathways, penetration of the stomata on the fruit pericarp or invasion of the gynoecium of the female flower. The latter can occur in the absence of BFB fruit symptom development (Frankle *et al.*, 1993; Walcott *et al.*, 2003). Depending on the invasion pathway, *A. citrulli* cells can be localised to the testa and perisperm-endosperm layers (with pericarp invasion) or the seed embryos (in the case of flower invasion through the gynoecium) (Dutta *et al.*, 2012a).

In previous studies, PCR-based detection assays were developed and evaluated using naturally contaminated seeds with high *A. citrulli* population densities (approximately

10^5-10^6 CFU per seed). Such seeds were then mixed with non-infested seeds to generate samples with varying percentages of contamination (Bahar *et al.*, 2008; Dutta *et al.*, 2012b). Currently, most real-time PCR-based seed health assays fail to detect *A. citrulli* in seed samples (n = 1000 seeds) containing $\leq 10^3$ CFU ml^{-1} of seed extract (Feng *et al.*, 2013). This is likely due to the effects of co-extracted seed wash compounds that can inhibit PCR amplification of DNA (De Boer *et al.*, 1995; Walcott *et al.*, 2000). Based on these observations and on results reported by Dutta *et al.* (2012c), the efficiency of PCR-based seed health assays is highly influenced by the technique by which pathogen propagules are extracted from seeds.

Among published PCR-based seed health assays, the extraction techniques for *A. citrulli* vary. Some pathogen extraction techniques are based on seed maceration/crushing, that can increase the yield of pathogen cells from seed embryo tissues (Dutta *et al.*, 2012c). Other assays employ seed washing/soaking (Bahar *et al.*, 2008) that is less time consuming and logistically easier to conduct than seed maceration/crushing, especially when processing large cucurbit seed samples (Dutta *et al.*, 2012c). Other extraction techniques include the use of one (Seminis Inc. PCR-Wash method) or two centrifugation steps (Syngenta SYBR Green PCR Method) (National Seed Health System) to concentrate target bacterial cells in the seed extract. The single centrifugation approach is attractive, because it is faster and technically easier than two-step centrifugation. However, the latter approach, which includes an initial low-speed centrifugation step, can remove coarse seed debris that can interfere with subsequent processing and analysis steps (Bahar *et al*., 2008). Indeed, different PCR reactions may vary in their sensitivity to inhibition by compounds co-extracted with nucleic acids that can interfere with PCR detection (Hugget *et al.*, 2008). Hence, the objective of this study was to compare different techniques for extracting *A. citrulli* cells from naturally infected cucurbit seeds. In addition, the efficiency of three *A. citrulli*-specific real-time PCR assays was evaluated using the DNA samples generated by various extraction techniques to improve the robustness, reliability and efficiency of PCR-based seed health testing.

Materials and methods

Sources of infected seeds

Infected watermelon seeds cv. 'Crimson Sweet' were generated in-house as described below. Additionally, two naturally infested commercial melon seed samples, hereafter referred to as melon A and B, were obtained from an anonymous source for this study.

Production of embryo-infected watermelon seeds

Infected watermelon seeds were generated under greenhouse conditions by inoculating the pollinated stigmas of female flowers with *A. citrulli* strain AAC00-1 (Walcott and Gitaitis, 2000). The pathogen was routinely grown on Lysogeny Broth agar (Bertani, 1951) for 48 hours at 28°C. Watermelon cv. 'Crimson Sweet' plants were grown in pots under greenhouse as previously described and at anthesis, open female flowers were manually pollinated and stigmas were inoculated with *A. citrulli* (Lessl *et al.*, 2007; Dutta *et al*.,

2012a). Specifically, female watermelon flowers were inoculated with 10 µl of a suspension containing approximately 1×10^8 CFU ml^{-1} (approximately 1×10^6 CFU $blossom^{-1}$). The concentration of *A. citrulli* cells was determined using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY) (optical density at 600 nm = 0.3; approximately 1×10^8 CFU ml^{-1}) and adjusted to the desired concentration. One fruit per plant was allowed to develop to harvest maturity (approximately 35 days after pollination). Fruits were then harvested and seeds were extracted manually, air-dried for 24 hours at room temperature and stored at room temperature until they were used (Dutta *et al.*, 2012a).

Amplification efficiency of TaqMan PCR assay for soaking and crushing extraction techniques

To determine the amplification efficiency of the TaqMan PCR assay using the BOX primer/probe set for soaking and crushing extraction techniques, calibration curves were generated by qPCR assay. Five non-infected seed samples were processed by soaking samples (n = 20 seeds) for 16 hours in 9 ml of buffer (NaPBS [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH = 7.2] plus 0.2% Tween 20 [NaPBST]) at 4°C on a rotary shaker at 250 rpm (Innova; New Brunswick Scientific, Edison, NJ) or by cracking dry seed samples (n = 20 seeds) with a hammer, followed by incubation for 16 hours in 9 ml of NaPBST at 4°C on a rotary shaker at 250 rpm. After incubation, seed extracts were passed through sterilised gauze (Dynarex, Orangeburg, NY) to remove seed debris, and were then spiked with *A. citrulli* cells. Briefly, 1 ml of ten-fold serial dilutions of bacterial suspension were generated, ranging from 10^7 to 10^3 CFU ml^{-1} and added to watermelon seed extracts (9 ml) just prior to the centrifugation steps. Thus, each sample contained *A. citrulli* cells ranging from 10^6 to 10^2 CFU ml^{-1} (final concentrations). The initial bacterial cell suspension was adjusted using a spectrophotometer. Non-infected seed samples were spiked with 1 ml of sterilised distilled water and used as a negative control. Bacterial cells in seed extracts were then subjected to two sequential centrifugation steps as follows. Seed extracts (10 ml) were centrifuged at 1,300 ×g for five minutes at 4°C (slow speed centrifugation) (Allegra 25, Beckman Coulter, Fullerton, CA) and the supernatant was transferred to a new tube and centrifuged at 10,000 ×g for 20 minutes at 4°C (high speed centrifugation). Each resulting pellet was resuspended in 1 ml of sterilised distilled water and divided into two aliquots of 900 and 100 µl. The 900 µl aliquot was used for DNA extraction using the UltraClean Microbial DNA kit (MO BIO, Carlsbad, CA) according to the manufacturer's instructions. 5 µl of DNA was then subjected to TaqMan real-time PCR assay using the *A. citrulli*-specific BOX primer/probe oligonucleotides (Ha *et al*., 2009) according to Dutta *et al*. (2012c). Three independent replicates were conducted for each extraction technique. Real-time PCR data were used to calculate mean cycle threshold (Ct) values and standard deviations of the means (SDs). Linear regression curves of the mean Ct values versus the logarithmic concentration (CFU ml^{-1}) of *A. citrulli* were generated and for both extraction techniques; amplification efficiency (E) (%) was calculated from the slopes of the calibration curves using the equation: $E = (10^{-1/slope} - 1) \times 100$ (Ramakers *et al.*, 2003). The second 100 µl aliquot was mixed with 20% glycerol and stored at -20°C for reference.

Characteristics of embryo-infected watermelon seeds

To confirm the location of *A. citrulli* cells in the embryo, pistil-inoculated watermelon seed samples (n = 20 seeds) were dissected into testa, perisperm-endosperm (PE) layer and embryo. Each embryo seed samples (n = 20 seeds) was then processed by crushing, as described above. Non-infected embryo seed samples (n = 20 seeds) were used as negative controls. Each seed extract (1 ml) was used for microbial DNA extraction followed by TaqMan PCR assay using the BOX primer/probe set. This test was repeated three times. Additionally, to estimate the total *A. citrulli* populations in embryo-infected watermelon seeds, seed samples (n = 20 seeds) were processed by soaking and crushing, as described above. Non-infected seed samples (n = 20 seeds) were used as negative controls. For each seed extract, the 900 µl aliquot was used for microbial DNA extraction followed by TaqMan PCR assay using the BOX primer/probe set. According to the standard curves generated for this study, mean Ct values obtained were used to calculate the *A. citrulli* concentration (cells seed^{-1}) in seed extracts. A paired Student's t-test was used to determine the statistical significance of differences between the bacterial populations extracted from seeds by soaking and crushing ($P \leq 0.05$). To confirm the presence of viable *A. citrulli* cells in seed samples, the 100 µl aliquot of seed extract was diluted ten-fold with NaPBS and spread onto TWZ semi-selective medium (Tian *et al.*, 2013). Cultures were incubated for 48-72 hours at 28°C and putative *A. citrulli* colonies were identified by TaqMan PCR using the BOX primer/probe set. This experiment was repeated three times.

Acidovorax citrulli extraction from embryo-infected watermelon seeds

We investigated if *A. citrulli* cells that are localised in embryo tissues are extracted from watermelon seeds by soaking and crushing, as described above. Embryo-infected seed samples (n = 20 seeds) were surface-disinfected according to Fatmi *et al.* (1991). Briefly, seeds were incubated in 2% sodium hypochlorite (NaOCl) for 20 minutes at 25°C on a rotary shaker at 250 rpm (Innova; New Brunswick Scientific, Edison, NJ) and then rinsed under running tap water for three minutes. To ensure that NaOCl treatments did not affect PCR amplification, extracts from surface-disinfected watermelon seeds samples were spiked with *A. citrulli* cells to obtain a final concentration of approximately 5×10^3 CFU ml^{-1}. The spiked seed extracts were used as positive amplification controls (PAC) for seed extracts generated by soaking and crushing. Non-infected seeds treated with NaOCl served as negative controls. Seed samples were processed according to the soaking and crushing techniques as described above. Each seed extract was divided into aliquots of 900 and 100 µl. DNA was extracted from the 900 µl aliquot and 5 µl were used as template for real-time PCR assay with the BOX primer/probe set. The 100 µl aliquot was diluted ten-fold and plated on TWZ media. After incubation for 24-48 hours at 28°C, putative *A. citrulli* colonies were selected for identification by PCR with the TaqMan BOX PCR assay. This experiment was repeated three times.

Comparison of techniques for extraction A. citrulli from cucurbit seeds

To improve the efficiency of *A. citrulli* extraction from cucurbit seeds, we compared soaking versus crushing seed samples and one-step (high speed) centrifugation versus a two-step (low speed to remove coarse debris followed by high speed to concentrate

bacterial cells) centrifugation. Infested watermelon seed samples were generated by mixing 990 non-infected seeds with ten embryo-infected seeds to give seed lots with 1% infection (approximately 10^4 cells sample^{-1}) and two naturally infested commercial melon seed lots were also used. Four different pathogen extraction techniques were evaluated: (1) soaking seeds for 16 hours, followed by two centrifugation steps (low speed then high speed, as described above); (2) soaking seeds for 16 hours, followed by one centrifugation step (high speed); (3) crushing (cracking) seeds followed by soaking for 16 hours followed by two centrifugation steps (low speed then high speed, as described above); and (4) crushing seed followed by soaking for 16 hours followed by one centrifugation step (high speed). To crack seeds, samples were placed in extraction bags (Bioreba, Reinach, Switzerland) and hammered for approximately three minutes. The weight of each 1000-seed sample was determined and used to calculate the appropriate extraction buffer volume. Seed samples were incubated at 4°C in Erlenmeyer flasks with a ratio of 2 ml of NaPBST per gram of seed on a rotary shaker at 250 rpm. Finally, to remove seed debris, the extracts were filtered through sterilised gauze and divided into 10 ml aliquots. Both extraction techniques (soaking and crushing) were followed by one or two centrifugation steps, as described above. The resulting pellets were resuspended in 1 ml of sterilised distilled water and used for DNA extraction, as described above. To assess the sensitivity of the four extraction techniques, DNA samples and their 10-fold dilutions were analysed by real-time PCR assay. For each sample, the DNA was subjected to real-time PCR amplification using TaqMan-based PCR with the BOX (Ha *et al.*, 2009) assay; TaqMan-based PCR with the UGA 3937 (R.R. Walcott, *unpublished*) assay and, SYBR-green-based PCR with the BX-S primer set (Bahar *et al.*, 2008). Samples with cycle threshold values (Ct) ≤ 35 were considered positive for all assays. Each experiment was conducted three times and mean Ct values were used to compare the sensitivity of the four pathogen extraction techniques. The results were subject to ANOVA and chi-squared tests at $P \leq 0.05$ using SPSS 15.0 for Windows® (SPSS Inc., Chicago, IL).

Results

Amplification efficiency of TaqMan PCR assay for soaking and crushing extraction techniques

To determine amplification efficiency of the TaqMan BOX PCR assay, two calibration curves were generated using ten-fold serial dilutions of *A. citrulli* cells in watermelon seed extracts generated by soaking and crushing seeds (figure 1). For both extraction techniques, there was a strong relationship ($R^2 \geq 0.99$) between *A. citrulli* concentration and mean Ct values generated with the real-time PCR assay of infected seed extracts. As expected, the standard deviation of mean Ct values increased as *A. citrulli* concentration decreased. The slopes of the linear regression lines for the relationships between Ct values and pathogen concentrations were calculated as -3.25 and -3.49 for soaking and crushing extraction, respectively. Based on these slopes, amplification efficiency (E) was determined to be 103% for soaking and 93% for crushing.

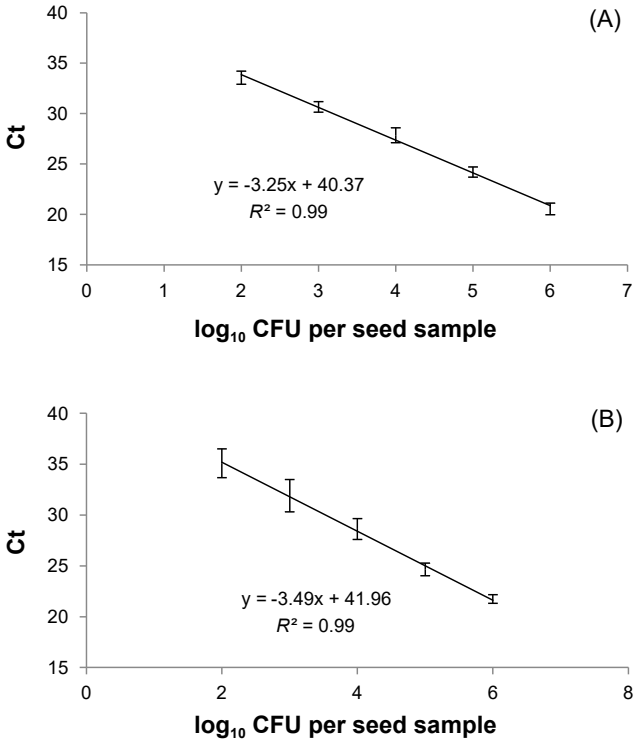

Figure 1. Calibration curves obtained for *Acidovorax citrulli* serial dilutions performed in watermelon seed extracts generated by soaking (**A**) and crushing (**B**) seeds and using the BOX primer set to quantify *A. citrulli* by qPCR. Each data point represents the mean Ct value for the pathogen at each concentration for three independent replicates, and the bars represent the standard error of the means. Lines represent the linear regression analysis of the relationship between pathogen concentration ($\log_{10}$ CFU per seed sample) and Ct values generated by real-time PCR.

Characteristics of embryo infected watermelon seeds

Acidovorax citrulli was detected in 100% of the seed embryo extracts from seed samples ($n = 20$ seeds), displaying a mean Ct value of 30.76 (SD = 0.28). *A. citrulli* was not recovered from non-infected embryo seed samples with regards to the total population density of the embryo-infected seeds, the mean Ct values for infected seed samples ($n = 20$ seeds) extracted by seed soaking and crushing were 29.31 (SD = 0.71) and 26.90 (SD = 1.13), respectively. Based on the calibration curves, these Ct values corresponded to bacterial loads of approximately 2.5×10^2 (SD = 33.5) cells per seed for soaking extraction and 1.3×10^3 (SD = 83.3) cells per seed for extraction by crushing. Paired *t*-test showed no significant difference ($P = 0.70$) between mean *A. citrulli* populations extracted by soaking and crushing seeds. The presence of viable *A. citrulli* cells in seed extracts was confirmed by isolation on TWZ agar.

Table 1. Mean cycle threshold (Ct) values obtained by real-time PCR with the BOX primer set to investigate the extraction of *Acidovorax citrulli* from surface-disinfected embryo-infected watermelon seeds and processed by soaking and crushing techniques.

Replicate	NIC[A]	Crushing	Soaking	PAC[B] (crushing)	PAC[C] (soaking)
1	Negative	31.34	Negative	28.71	26.32
2	Negative	27.93	Negative	30.48	27.66
3	Negative	29.57	Negative	26.89	25.11
Mean Ct	ND	30.95 (SD = 2.95)	ND	28.69 (SD = 1.80)	26.36 (SD = 1.28)

A = non-infected watermelon seeds; B = surface-disinfected watermelon seeds, processed by crushing and spiked with *A. citrulli*; C = surface-disinfected watermelon seeds, processed by soaking and spiked with *A. citrulli*; ND = not determined.

Acidovorax citrulli extraction from embryo-infected watermelon seeds

Acidovorax citrulli was consistently detected in extracts (generated by soaking and crushing surface-disinfested seeds), to which cells of the pathogen were added. Mean Ct value of 28.69 and 26.36, were observed for seed extracts generated by soaking and crushing seeds, respectively (table 1), confirming that seed disinfection with NaOCl did not inhibit PCR amplification. In embryo-contaminated seeds disinfected with NaOCl, *A. citrulli* was not detected by soaking followed TaqMan PCR assay with the BOX primers/probe set. In contrast, when surface-disinfected seeds were crushed, the pathogen was consistently detected by TaqMan PCR assay with a mean Ct value of 30.95. *A. citrulli* detection was confirmed by pathogen recovery on TWZ medium for both PACs and seed samples processed by crushing (data not shown). *A. citrulli* was not recovered from surface-disinfected watermelon seeds extracted by soaking (data not shown).

Comparison of techniques for extraction of A. citrulli from cucurbit seeds

The BOX TaqMan PCR assay detected *A. citrulli* in 100% of melon A seed lot samples processed by techniques 1 (soaking followed by low speed then fast speed centrifugation), 2 (soaking followed by high speed centrifugation) and 3 (crushing followed by low speed then high speed centrifugation). Technique 1 displayed a mean Ct value of 26.41 (table 2), which was significantly lower ($P \leq 0.05$) than the mean Ct values obtained for the other extraction techniques. With technique 4 (crushing followed by high speed centrifugation), the pathogen was detected in one replicate out of three, with a Ct value of 34.65. Similar to tests with melon seeds from lot A, seeds from melon seed lot B displayed a consistent number of positive assays (100% of samples) for techniques 1, 2 and 3. The mean Ct value obtained by technique 1 was 29.78, which was significantly lower ($P \leq 0.05$) than those obtained from techniques 2 and 3. Technique 4 failed to detect the pathogen in all replicates. In the watermelon seed sample analysed with technique 1, *A. citrulli* was detected in 100% of the attempts with a mean Ct value of 31.93. Using technique 2, the detection frequency decreased to one replicate out of three (33% of samples), with a Ct value of 34.19. Conversely, *A. citrulli* was not detected by techniques 3 and 4. No real-time PCR signals were observed in negative control samples by the techniques applied.

Table 2. Results of four seed extraction techniques, in combination with three different real-time assays for the detection of *Acidovorax citrulli* in naturally infected melon and watermelon seed lots. Techniques applied for pathogen extraction were: (1) soaking followed by two centrifugation steps (low speed followed by high speed); (2) soaking followed by one centrifugation step (high speed); (3) crushing followed by two centrifugation steps; and (4) crushing followed by one centrifugation step. Different letters within columns indicate significant differences according to the chi-squared tests ($P \leq 0.05$).

Primer set	Technique	Melon A			Melon B			Watermelon 1%		
		Frequency[1]	Mean Ct[2]	SD[3]	Frequency[1]	Mean Ct[2]	SD[3]	Frequency[1]	Mean Ct[2]	SD[3]
BOX	1	3/3	26.41 ^{A}	± 1.23	3/3	29.78 ^{A}	± 0.42	3/3	31.93	± 1.90
	2	3/3	30.59 ^{B}	± 1.45	3/3	31.83 ^{B}	± 0.20	1/3	34.19	-
	3	3/3	32.40 ^{BC}	± 1.80	3/3	32.42 ^{B}	± 0.86	0/3	ND	-
	4	2/3	34.65 ^{C}	± 0.73	0/3	ND	-	0/3	ND	-
UGA 3937	1	3/3	22.51^{A}	± 0.48	3/3	25.60 ^{A}	± 0.32	3/3	32.57	± 1.37
	2	3/3	24.43^{B}	± 1.08	3/3	29.58 ^{B}	± 2.27	1/3	33.76	-
	3	3/3	27.36^{C}	± 0.46	2/3	31.20 ^{B}	± 1.41	1/3	34.13	-
	4	3/3	28.86^{D}	± 0.38	2/3	31.69 ^{B}	± 0.35	0/3	ND	-
BX-S	1	3/3^{E}	30.87 ^{A}	± 2.14	3/3 ^{E}	33.38 ^{A}	± 1.25	0/3	ND	-
	2	3/3^{E}	31.05 ^{A}	± 0.77	3/3 ^{E}	34.25 ^{A}	± 0.71	0/3	ND	/
	3	3/3 ^{E}	32.48 ^{A}	± 0.66	0/3	ND	/	0/3	ND	/
	4	1/3 ^{E}	34.00	/	0/3	ND	/	0/3	ND	/

[1] detection frequency, the number of positive assays divided by the number of samples tested; [2] mean cycle threshold value (Ct); [3] standard deviation; ND = not detected.

The UGA 3937 primer set consistently detected *A. citrulli* in samples from melon seed lot A. Technique 1 displayed a mean Ct value of 22.51, which was significantly lower ($P \leq 0.05$) than those obtained with other techniques. For samples from melon seed lot B, techniques 1 and 2 detected the pathogen in all replicates, whereas for techniques 3 and 4, amplification was observed in only two replicates out of three. Technique 1 yielded a mean Ct value of 25.60, which was significantly lower ($P \leq 0.05$) than the mean Ct values obtained for other techniques. In infected watermelon seeds assayed by technique 1, *A. citrulli* was detected in 100% of samples, yielding a mean Ct value of 32.57. With techniques 2 and 3, the detection frequency decreased to one replicate out of three. *A. citrulli* was not detected using technique 4. As expected, amplification was not observed in negative controls, regardless of the extraction procedure used.

The BX-S primer set detected *A. citrulli* in 100% of the samples from melon seed lot A tested by techniques 1, 2 and 3. The frequency of *A. citrulli* detection was reduced to 33% for technique 4. Interestingly, the real-time PCR assay amplified the 10-fold DNA dilution, but not the undiluted DNA. Moreover, there was no significant difference between the mean Ct values obtained for techniques 1, 2 and 3 for seeds from the melon seed lot A. In samples from melon seed lot B, the detection frequency was 100% for samples processed by techniques 1 and 2, with mean Ct values of 33.38 and 34.25, respectively. However, these differences were not statistically significant. Conversely, with techniques 3 and 4, *A. citrulli* was not detected. Using the BX-S assay, *A. citrulli* was not detected in watermelon seed samples with low levels of contamination (approximately 10^4 cells per sample) regardless of the pathogen extraction technique. As expected, amplification was not observed in negative controls.

Discussion

Infected seeds are the most important source of primary inoculum for BFB epidemics, and facilitate the global dissemination of *A. citrulli.* Hence, *A. citrulli*-infected seeds represent a serious threat for commercial cucurbits fruit and seed producers worldwide (Bahar and Burdman, 2010). Currently, seed health testing is the first tool used by national plant protection organisations, inspection services and seed companies to prevent the introduction of *A. citrulli* into greenhouses or fields. Therefore, sensitive and robust methods for pathogen detection are essential for *A. citrulli* exclusion and efficient pathogen extraction from seed samples are critical for detection

In this study, we compared different techniques for extracting *A. citrulli* from cucurbit seeds that may influence detection by PCR-based assays. Assessment of pathogen contamination levels in commercial seed lots is difficult, since there is a log-normal distribution of infected seeds in lots, i.e., most seeds in a commercial lot are non-contaminated (Dutta *et al.*, 2014). Hence, to compare the efficiency of the soaking and crushing extraction techniques, we generated watermelon seed samples with low levels of contamination (approximately 10^4 cells per sample) by mixing embryo-infected with non-infected watermelon seeds.

For extracting *A. citrulli* from cucurbit seeds, soaking and crushing techniques were conducted by overnight incubation in sterilised saline phosphate buffer with a continuous *shaking*. Long incubation times (16 hours) are necessary for bacteria to be released from seed tissues to facilitate detection (He *et al*., 2012). Additionally, a low incubation temperature is recommended to limit growth of saprophytic microorganisms that can interfere with the amplification of target DNA and thereby prevent false negative results (Wilson *et al.*, 1997).

Amplification efficiency values generated by qPCR analysis for soaking and crushing extractions were also used to assess the quality of the nucleic acid extracts (Bustin *et al.*, 2009). An optimal calibration curve for qPCR assay should have a R^2 value > 0.98 and a slope between -3.1 and -3.6 corresponding to PCR efficiencies of 90-110% (Roche Diagnostics, 2000; Applied Biosystems, 2004; Bolha *et al.*, 2012). According to our results, calibration curves generated by soaking and crushing watermelon seeds followed by spiking with ten-fold serial dilutions of *A. citrulli* showed a strong correlation ($R^2 \geq 0.99$) between pathogen concentration (CFU ml^{-1}) and mean Ct values. However, seed extract generated by soaking (E = 103%) yielded a value closer to the optimal amplification efficiency (E = 100%) than that generated by crushing seed (E = 93%). Although this experiment was conducted with a small seed sample ($n = 20$ seeds), the reduced amplification efficiency observed with crushed seed extract may have been due to the release of excessive inhibitory compounds (Svec *et al*., 2015), such as starch granules (Bickley and Hopkins, 1999).

According to qPCR results with embryo-infected seeds, there was no significant difference ($P = 0.70$) between the mean *A. citrulli* population assessed by soaking and crushing techniques. Hence, we assumed that *A. citrulli* cells located in the embryo could be extracted by soaking seeds for 16 hours at 4°C. However, this assumption was not supported when embryo-infected seed samples were surface-sterilised before extraction by soaking. In this case, all extracts were negative by TaqMan BOX assay. In contrast, when surface-sterilised embryo-infected seeds were crushed, the extract tested positive. This observation suggests that soaking can extract *A. citrulli* cells located on seed surfaces and *A. citrulli* cannot be detected from seed embryos by soaking.

In the present study, four seed extraction techniques were compared using large samples ($n = 1000$ seeds) of naturally infected melon and watermelon seeds. Based on real-time PCR results obtained for melon seed lots A and B, soaking for 16 hours followed by two centrifugation steps increased *A. citrulli* detection frequency and lowered mean cycle threshold (Ct) values, compared with the other extraction techniques. Technique 1 was also more effective for detecting *A. citrulli* in watermelon seed samples (3⁄3 positive replicates compared with 1⁄3 for technique 2 and 0/3 for technique 3 and 4, respectively). The observation that soaking for 16 hours at 4°C is more effective for detecting *A. citrulli* in seeds than crushing, even in embryo-infected seeds, can be explained by the fact that low levels of contamination of testae might occur in seeds from pistil-inoculated lots (Dutta *et al.*, 2012a, c). Walcott *et al*. (2003) observed that fruit pulp tissues also became contaminated with *A. citrulli* by pistil inoculation. Hence, *A. citrulli* cells present in watermelon pulp could subsequently become associated with testae, similar to seeds in pericarp-inoculated fruits. Conversely, when crushing was used to extract bacteria

from embryo-infected seeds (Dutta *et al.*, 2012b), it also releases particulate seed debris and constituents that can interfere with the real-time PCR assay and pathogen detection. Therefore, inhibition of PCR-based assays by compounds released by crushing seeds is an important factor influencing the sensitivity and robustness of the detection assay, and should be considered when developing PCR-based seed health tests.

Regarding the centrifugation methods, significant differences in pathogen detection were observed with low- and high-speed centrifugation steps. Although low-speed centrifugation represents an additional step in the procedure, it removes coarse seed debris and yields better detection sensitivity. Interestingly, when melon samples were tested by real-time PCR assay with the BS-X primer set, *A. citrulli* was detected in samples of melon seed lot A and B, only after diluting the DNA samples ten-fold. It is likely that DNA sample dilution may increase detection sensitivity by reducing the concentration of inhibitors present in the DNA samples. Based on these observations, one major limitation of PCR-based seed health testing is sensitivity to inhibitory substances that are co-extracted with DNA and result in false-negative results. Hence, highly pure nucleic acids are required for PCR-based seed health assays, and optimal extraction techniques are critical for a reliable PCR analysis of large seed samples.

This study compared the performance of different seed extraction techniques and three *A. citrulli*-specific real-time PCR assays using cucurbit seed lots that were naturally infected with *A. citrulli*. The comparison was of special interest because co-extracted PCR inhibitors did not affect all PCR reactions equally. For example, embryo-infected watermelon seed lots were consistently detected with extraction technique 1 (soaking for 16 hours followed by two centrifugation steps) with BOX and UGA 3937 TaqMan PCR assays, whereas all amplifications were negative with the SYBR-green-based BS-X PCR assay.

Based on our results, an overnight soaking extraction method, followed by sequential low- and high-speed centrifugation resulted in the most consistent PCR based detection of *A. citrulli* from naturally infected watermelon and melon seeds. However, these results should be confirmed by testing healthy seed lots from various sources spiked at determined infection levels, because different levels of saprophytes residing on/in the seeds might influence detection efficiency of seed health testing for *A. citrulli*.

This study should inform the current validation of *A. citrulli* detection assays in large commercial seed samples (e.g. 5.000 to 10.000 seeds; Walcott *et al.*, 2003), as envisioned by the European EUPHRESCO network (Anonymous, 2016).

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