Improved bio-PCR test for detection of *Acidovorax avenae* subsp *citrulli* in watermelon and cantaloupe seeds

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Abstract

Acidovorax aveane subsp *citrulli* (aac), is seed borne in watermelons and cantaloupes. Although a Bio-PCR test that utilizes primers developed by USDA is effective in detecting as low as one colony in the isolation plates, its successful application to detect seed borne infection depends on efficient extraction prior to plating. We compared sample sizes, buffer systems, extraction temperature levels and enrichment techniques to recover low levels of aac (less than 1000 cfu). Small sub samples (approximately 200 seeds) extracted in MOPS buffer for 4 hours at room temperature or 24 hours in refrigerator gave consistently positive recoveries. Agar plating was better than liquid enrichment. In testing 3 reps of 10,000 seeds of naturally infected watermelon and cantaloupe seeds (0.015% infection by grow out), the improved procedure (small 200 seed sub samples extracted in MOPS buffer) detected pathogen in all three replications compared to only one positive sample by the previous procedure (2000-seed subsamples extracted in phosphate buffer).

Materials and Methods

Extraction Buffer

Phosphate buffer is routinely used for bacterial extraction from seeds. We observed that survival of aac was inconsistent in buffer controls. Therefore we compared phosphate and MOPS buffers for bacterial survival. 100 mL buffer was added to 1000 watermelon seeds. 1 mL of bacterial suspension containing 100,000 cfu of aac was added. After 4 hours, suspensions were assayed on agar media. Bacterial population decreased in phosphate buffer (Table 1). On the other hand, bacterial population was not affected in the seed extracts regardless of the buffer used.

Buffer	% Recovery From Seed Extract	% Recovery From Buffer Alone
10 mM phosphate, pH 7.3	100 %	8 %
40 mM phosphate, pH 7.3	100 %	10 %
10 mM MOPS + 2 g $CaCl_2$ per liter, pH 7.3	100 %	100 %
40 mM MOPS + 2 g $CaCl_2$ per liter, pH 7.3	100 %	200 %

Table 1: Effect of buffer on pathogen survival.

Extraction Temperature

Aac is considered cold sensitive and thermo-tolerant. Extraction at 41 °C is desired as it can lead to reduction of saprophytes and aid in recovery of pathogen. To confirm this hypothesis, we took several samples of 1000 seeds and buffer added at 100 mL per sample. Pathogen was added to a final concentration of approximately 100 cfu/0.1 mL. The samples were incubated for 4 hours at 22 °C or 41 °C and 24 hours at 4 °C and plated on selective media. Pathogen survived well at 22 °C as well as at 4 °C but recovery was greatly reduced at 41 °C. Saprophytes were reduced at 41 °C also. We concluded that extraction should be done at room temperature for 4 hours or at 4 °C for 24 hours. This experiment was conducted on artificially inoculated seeds, therefore, results can not be compared with naturally infected seeds.

Figure 1: Visual reading of liquid plating on P-278 media. Small colonies (arrow) are of the pathogen.

Enrichment on Agar Media vs Liquid Media

Pathogen enrichment in liquid media is attractive as it allows for increasing the target cells using a larger volume of seed wash liquid. We investigated enrichment in 3 semi-selective broth media EBB, NAV and P-278. Watermelon seeds, artificially inoculated with 1000 cells of pathogen, were extracted in 50 mL buffer. 0.5 mL seed extract (theoretically containing 10 cells) was added to 100 mL liquid media and incubated on shaker for 24 hours. Likewise, 0.5 mL was plated onto 5 plates of P-278 agar and harvested in 15 mL after 72 hours (Bio-PCR). 5 µL samples of enriched suspensions were tested by PCR. After several experiments, we concluded that enrichment in liquid media is not satisfactory. Enrichment on agar media gave consistently positive results. On agar, 1 pin point colony contained 20,000–100,000 cells. Mathematically speaking, harvesting 1 colony from 5 plates in 15 mL would result in 6–30 cells per 5 µL and get detected by PCR.

Sample Size

Pathogen is usually present in a few seeds in a lot (in our experience 1–7 seeds per 10,000 seeds) and the amount of inoculum per seed varies. Accordingly, in a liquid plating/Bio-PCR test, an infected seed containing 300 cells will only be detected if amount of buffer in an extraction does not exceed 30 mL.

We tested 3 reps of 10,000 seeds of a cantaloupe seed lot naturally infected with an infection level of 0.015% as determined by grow out test. Two sample sizes (200 seeds and 2,000 seeds) were tested. For 200 seed sample, 30 mL buffer was used and plated at 0.1 mL. Five plates were pooled for

PCR reaction. For 2,000 seeds, 250 mL buffer was used and plated at 0.1 mL/plate in 5 plates and all plates combined for Bio-PCR. Results are shown in Table 2.

Subsample Size	Detection in 10K R1	Detection in 10K R2	Detection in 10K R3
200 seeds (30 mL buffer)	+	+	+
2,000 seeds (200 mL buffer)	+	—	—

Table 2: Effect of sample size on detection of pathogen by real time Bio-PCR.

Small Samples: Comparison of Bio-PCR (Real Time PCR) and Grow Out

Naturally infected seed lots of cantaloupe and watermelon were used for this study. 10K seeds were divided into 80 samples of 125 seeds each. For Bio-PCR, each subsample was extracted in 20 mL MOPS buffer for 4 hours and plated on M-153 media. After 72 hours, bacteria were harvested Figure 2) and and 5 subsamples pooled for real time PCR using Smart Cycler (Figure 3, 4).

The *A. avenae* subsp. *citrulli* specific PCR primers, AacF3 and AacR2 and FAM-labeled probe AacP2 (8), were used in real time PCR with the following cycling conditions with the Smart Cycler: 95 °C for 30 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. The assays were performed in a total volume of 25 µL containing the following reagents and volumes (µL): 10x PCR buffer (2.50), 25 mM MgCl2 (5.00), 1.25 mM dNTPs (4.00), 25 µM primer AacF3 (1.00), 25 µM primer AacR2 (1.00), 10 µM probe AacP2 (1.00), 5U/µL AmpliTaq® DNA polymerase (0.25), 5x additive reagent containing BSA at 1 mg/mL, Trehalose at 750 nM, and Tween-20 at 1% v/v (Cepheid, 5.00); sterile MBG water (4.25), sample (1.00). The cycle threshold (Ct) values were determined and samples with a Ct value of 38 or less were considered positive. Ct value is defined as the PCR cycle number at which time the signal (fluorescence) of the probe rises above background. The earlier the Ct value the better the PCR performance.

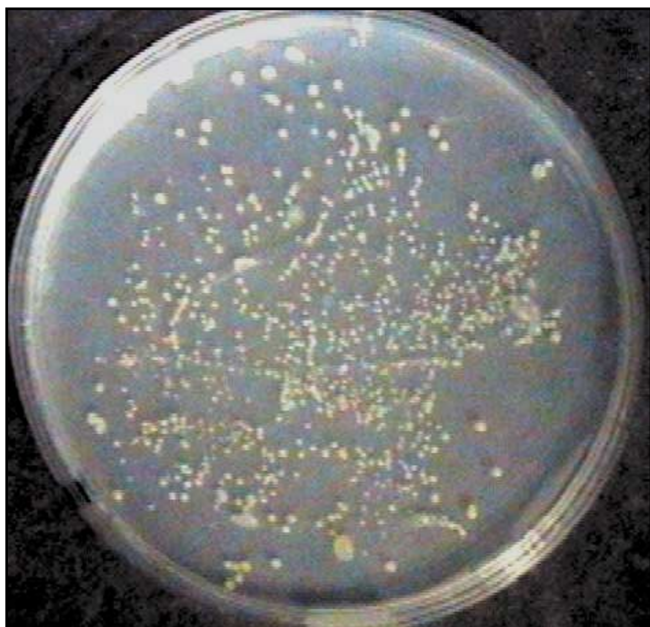

Figure 2: M-153 media plate ready to harvest for real time PCR. Saprophyte colonies are visible. Tiny colonies of pathogen are not distinguished.

Many samples gave a lower Ct value showing high population in the sample. For positive testing pooled samples, individual 5 samples were tested by real time PCR to get a total count of positive subsamples. Any positive samples were considered to contain 1 infected seed. For grow out 125 seeds were grown in each flat. Any flats that contained infected seedlings were considered to contain only 1 infected seed. Additional infected seedlings were considered secondary infection. Data (Table 3) shows good correlation between grow out and real time Bio-PCR. It appears that grow out overestimates the infection rate due to spread of disease as evident from 3 adjoining trays infected (trays 17, 18, 19) for one sample. For a surface sterilized lot only one seed was found positive. It may be coincident to have one infected seed in the lot for grow out and no infected seed in the lot for real time-PCR.

Figure 3: Smart Cycler for real time PCR. The system allows 16 samples run at the same time. The run is completed in 30 minutes.

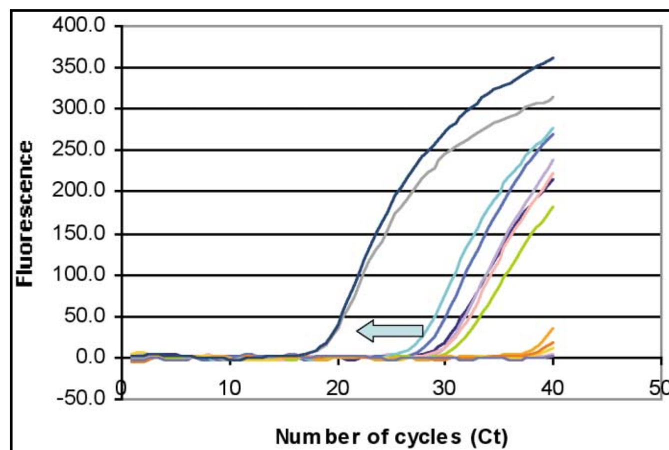

Figure 4: Smart Cycler results. Two samples with a Ct value of 20 (arrow) indicate higher population of aac in the test samples.

Sample ID	Grow Out	Real Time Bio-PCR
Cantaloupe 6574	17 (Tray no: 3,4,12,13, 21,24, 25,26,27,40 42,44, 49,50,51,54,73)	11
Watermelon triploid 6160	3 (Tray no 17, 18, 19)	1
Watermelon triploid 6397	3 (Tray no 1, 19, 2)	2
Watermelon triploid 6397 surface sterilized	1	0

Table 3: Number of infected seeds determined by grow out and real time Bio-PCR (N=10,000 seeds).

Conclusions

◆MOPS buffer is superior to phosphate buffer for extraction.

◆Extraction can be done for 4 hours at RT or 24 hours at 4 °C.

◆Agar based enrichment is superior to liquid enrichment.

◆Small sample is the key to detect the pathogen.