

## SEED TRANSMISSION OF *Acidovorax citrulli*: IMPLEMENTATION OF DETECTION IN WATERMELON SEEDS AND DEVELOPMENT OF DISINFECTION METHODS

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### ABSTRACT

*Acidovorax citrulli* is a seed-borne pathogen and the causal agent of bacterial fruit blotch of cucurbits. It is listed as an A1 quarantine pathogen by EPPO. Seed certification is based on the availability of a sensitive and specific pathogen detection in seed lots: this is a must for an effective disease management strategy. Therefore, an effective DNA extraction and purification procedure is a critical issue to ensure a robust PCR analysis. Pathogen detection in seed lots has been implemented by testing different known contamination levels by *Acidovorax citrulli*. Initially, two different sample preparation methods have been tested: a) Overnight soaking; b) Hammering of dry seeds, followed by three different manual DNA extraction. Each DNA sub-sample obtained has been analysed with two different primers sets, SEQID3/SEQID4 and WFB1/WFB2, to evaluate the capability to detect the pathogen. Results showed that a DNA extraction and purification procedure, based on soaking the seeds, followed by the use of the DNeasy Plant Mini kit (Qiagen) on the washing fluids gave the highest amount of DNA, sufficient to increase the detection threshold of the pathogen. This will allow the improvement of current detection procedures.

Furthermore, naturally contaminated watermelon seeds were treated through different methods, in order to achieve a possible sanitation or eradication of *Acidovorax citrulli*: a bacterial antagonist, a microbial consortium, a plant polyphenol. Our results showed that treated seeds were only partially disinfected, and the pathogen was not eradicated after any of the methods used.

**Key words:** *Acidovorax citrulli*, watermelon, pathogen detection, seed disinfection.

### INTRODUCTION

Bacterial fruit blotch of cucurbits (BFB) is a relatively new disease and became a severe problem in watermelon in the late 1980s. It was first reported to occur in Australia in 1988 ([Wall & Santos, 1988](#)) and observed in U.S. commercial watermelon fields in 1989 ([Latin & Rane, 1990](#)). Since then, BFB has locally spread worldwide. BFB can be devastating for growers, with fruit losses reaching 80-100%, in watermelon ([Latin & Hopkins, 1995](#); [Schaad \*et al.\*, 2003](#)), and in recent

years, in melon ([Burdman \*et al.\*, 2005](#)). The causal organism is the Gram negative, non-fluorescent, rod-shaped bacterium, *Acidovorax citrulli* (Acit) ([Schaad \*et al.\*, 2008](#)). Acit is a seed-borne pathogen and infects seeds, which represents the most important source of primary inoculum for BFB epidemics ([Latin & Hopkins, 1995](#); [Walcott & Gitaitis, 2000](#)).

Strategies, which exclude Acit from seeds, are the main issue to avoid further phytosanitary problems to the crop during the growing season. The need for an efficient, fast and reliable detection method is widely

required and several methods, mainly PCR-based, have been proposed (Walcott & Gitaitis, 2000; Schaad *et al.*, 2000; Song *et al.*, 2003). Melon seed was included in this study, in order to compare detection threshold obtained from watermelon seed to another cucurbit species, being BFB a serious threat for watermelon and, in the recent years, for melon (Burdman *et al.*, 2005).

The above-mentioned PCR-based protocols present some limitations: in particular, seed represents a far more difficult matrix to analyse, due to presence of several contaminants, coating chemicals, PCR inhibitors as (but not only) a high starch content in the cotyledons. Therefore, DNA extraction and purification are critical to ensure a reliable PCR analysis of seed lots for certification or other purposes. This study was aimed to implement a suitable and accurate seed sample preparation strategy, followed by a comparison of 3 different DNA extractions Kits to be used prior to a PCR assay.

Seed treatments with biomolecules or microorganisms have been reported to reduce disease severity and increase seed germination (Gupta *et al.*, 2002; Jensen *et al.*, 2004). Microbial consortia, plant polyphenols and an effective bacterial antagonist were assayed for their possible effect to reduce the seed-borne inoculum. This study aimed to implementing an effective BFB management, based on a highly sensitive molecular detection of the pathogen and developing a biological seed treatment, which might significantly reduce the seed-borne inoculum.

## MATERIAL AND METHODS

### Sample preparation for analysis: calibrated contamination with Acit

Experimentally infected watermelon seeds, cv. Charleston Gray and melon seeds, cv. Silver Star, were produced. Five hundred seeds for each cucurbit were dipped into an Acit bacterial suspension prepared at the concentration of  $1 \times 10^8$  CFU/ml, spectrophotometrically adjusted, and followed by vacuum infiltration (-60 cm/Hg) for 90 minutes. Seeds were then dried in an incubator at 25°C (with fan) overnight in the dark. Number of CFU per seed was determined by taking 10 seeds per crops and incubating them for 2 hours in 1 ml of PBS, added with 0.2% Tween 20 (PBST). Each seed was ground in a sterile mortar with a pestle, and 100 µl of the seed macerate were used

to prepare 10-fold dilution series, up to  $10^4$  dilution and plated onto nutrient sucrose agar (Crosse, 1959), supplemented with 250 ppm of cycloheximide and 200 ppm of ampicillin (NSA-250). Each dilution was represented by 6 drops of 10 µl. Agar plates were then incubated at 28°C for 4-8 days, followed by counting the colonies grown, so to precisely calculate the number of CFU per seed. Experimentally contaminated seeds were used to obtain different contaminated batches of seeds: 1 contaminated seed in 10; 1 in 100; 1 in 1000. Negative control (500 seeds proved to be Acit negative) and positive control (500 seeds into PBST spiked with Acit, to obtain a final concentration of  $10^6$  CFU/ml) were also assayed. Both overnight soaking and direct hammering of dry seeds sample preparation methods were tested.

### Sample preparation for analysis: soaking

Each contaminated batch was placed overnight in a PBST soaking buffer (2 ml of soaking buffer per gram of seed) and shaken at 90 rpm at room temperature on a rotary shaker. The seed washing fluids were centrifuged for 5 minutes at  $650 \times g$  to collect seed debris, followed by a centrifugation at high speed of the resulting supernatant for 20 minutes at  $12.000 \times g$ , to obtain a final pellet with the target bacteria. Pellets obtained were finally resuspended in 1 ml of sterile water prior to DNA extraction and purification.

### Sample preparation for analysis: hammering

Dry seeds of each contaminated batch were crushed by hammering and placed into PBST soaking buffer (3 ml of soaking buffer per g of seed). The crushed seed samples were then shaken for 3 hours at 90 rpm at room temperature on a rotary shaker; the extraction fluids were initially centrifuged at  $650 \times g$  for 5 minutes to collect seed debris. The supernatants were then centrifuged at  $12.000 \times g$  for 20 minutes, and each of resulting pellets was resuspended in 1 ml of sterile water, prior to DNA extraction and purification.

### DNA extraction

Three different extraction procedures were tested: DNeasy Plant Mini Kit (Qiagen), DNeasy Blood and Tissue (Qiagen) and Wizard Magnetic 96 DNA Plant

System (Promega). DNA extraction procedures were done according to the manufacturer's instructions.

### PCR assay

DNA, extracted and purified from each sample, was amplified in parallel with two different primers sets: SEQID3/SEQID4 (Schaad *et al.*, 2000) and WFB1/WFB2 (Walcott & Gitaitis, 2000). The primers of Schaad *et al.* (2000) were used in a protocol modified as follows: amplifications were carried out in a final volume of 25  $\mu$ l, containing 1x PCR Buffer (Promega), 1.5 mM of  $MgCl_2$  (Promega), 0.2  $\mu$ M of each primers, 1.0 U Go Taq® G2 Flexi DNA polymerase (Promega), 200  $\mu$ M each dNTP (Promega) and approximately 50 ng of target DNA. PCR reactions were performed using the following conditions: denaturation at 94°C for 10 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 45 seconds and elongation at 72°C for 1 minutes, with a final elongation step of 7 minutes at 72°C. DNA amplicons obtained from both amplifications were run in a 2% agarose gel, stained with ethidium bromide and observed with the BioDoc Analyze (Biometra, Göttingen, Germany).

### Biological seed treatments

Naturally contaminated watermelon seeds were subject to 3 different biological treatments, in order to test their efficacy in seed sanitation from Acit. Untreated seeds were used as a positive control.

The following compounds were tested: a commercial microbial consortium, plant polyphenols and a bacterial antagonist. The microbial consortium (Micosat F, CCS Aosta, Italy) was composed by: *Glomus* spp., *Trichoderma* spp., *Agrobacterium radiobacter*, *Bacillus subtilis*, *Streptomyces* spp. Treatment was done according to the manufacturer's indications: seed was dipped in a suspension of the microbial consortium, calculating 4.5 g/kg of seed. Commercial plant polyphenols based on tannins (AGRITAN Silvateam, San Michele di Mondovì, Italy) were used according to the manufacturer's suggestions at a concentration of 10 g/l in deionised water. A bacterial antagonist (*Pseudomonas synxantha*, strain DLS 65, from UNIMORE culture collection) was used to prepare a suspension of  $10^8$  CFU/ml. Treatment was done by dipping seeds in the bacterial suspension, keeping the seeds soaking for 90 minutes on a rotary shaker at 90 rpm. Seeds were then dried in an incubator at 25°C (with fan) overnight in the dark and stored in a seed storage room 1 month before sowing.

### Germination and disease incidence assay

Germination tests *in vitro* were done according to ISTA rules. Three replicates of 100 seeds were used for germination test. One hundred seeds were placed on top of two layers Whatman n° 5 filter paper, moistured with 5 ml of sterile distilled water in Petri dishes. Petri dishes were placed at 25°C in the dark. Germination counts were assessed every day, up to 14 days. Germination test on blotter was carried out in 3 replicates of 100 seeds for each treatment. Germination counts were assessed every day, up to 14 days. Greenhouse temperature was kept at 28 to 30°C and the relative humidity at 75%. Pot test was assayed on triplicates of 100 seeds for each treatment; seeds were sown into pots containing a steam sterilized peat for seedling production. Greenhouse condition was kept as above (germination test on blotters). Disease symptoms were daily evaluated up to 28 days. Symptomatic seedlings were collected, placed in a Stomacher Bag and homogenised in PBST buffer. The washing fluids were filtered with a sterile gauze, transferred in a centrifuge vial and centrifuged for 5 minutes at 1.250 x g to pellet soil and plant debris. The supernatants were then centrifuged at 12.000 x g for 20 minutes; the pellets were resuspended in 1 ml of sterile water. DNA was then extracted and purified using the DNeasy Plant Mini kit (Qiagen). The DNA isolated and purified was assayed, according to the modified protocol of Schaad *et al.* (2000) described above, in order to confirm the presence of Acit.

### Statistical analysis

All tests were performed in triplicates. Data were presented as mean  $\pm$  SD for each treatment. Univariate analysis of variance (ANOVA) with Tukey post-test was applied using GraphPad Prism 6.0 software (La Jolla, California, USA) when multiple comparisons were performed. The differences were considered significant when  $p \leq 0.05$ .

## RESULTS

### Detection assay of Acit

The level of contamination calculated for melon and watermelon seeds was consistently assessed about  $10^3$  CFU/seed. PCR detection threshold obtained using the DNeasy Plant Mini Kit (Qiagen) allowed detecting 1 artificially contaminated seed in 1000 for each species.

This was achieved with both soaking and hammering of seeds and using both primers pairs. Other procedures were not such sensitive (Table 1). Other combinations of seed treatment, DNA extraction and PCR detection were, in general, either less sensitive or less specific.

Biological seed treaments

Germination test *in vitro* showed a slight increase of germination rate by using the microbial consortium and the commercial plant polyphenols: 2 and 4% respectively, compared to untreated seeds. On the contrary, treatments using the bacterial antagonist significantly ( $p \leq 0.05$ ) reduced the germination rate by 9%. On blotter, effects of treatments on germination were not significant ( $p \geq 0.05$ ), and showed an increase of germination rate by using the microbial consortium and the commercial plant polyphenols (4 and 6% respectively), compared to untreated seeds. Finally, treatment with the bacterial antagonist had a similar value as untreated seeds. Regarding disease development on seedlings, assessed after all treatments, our data showed a significant reduction (27%,  $p \leq 0.05$ ) of symptomatic seedlings, when the bacterial antagonist was applied to seed. The application of the microbial consortium and the commercial plant polyphenols apparently reduced the percentage of diseased seedlings by 5 and 12% respectively, compared to the untreated control (Table 2).

DISCUSSION

Hammering of dry seeds might be a suitable method to allow bacteria present inside seeds to escape, since the thick seed coat is a strong barrier. This method proved to be very time consuming (~ 1 hour per 1000 seeds) and not feasible in routine seed analysis, although the matrix obtained by soaking was compatible with different DNA extraction methods. Overnight soaking is the easiest and fastest handling method. Detection threshold of Acit, as obtained by simplex-PCR, confirmed that DNA extraction with DNeasy Plant Mini Kit (Qiagen) gives the highest value (1 artificially contaminated seeds in 1000) in watermelon and melon seeds, assayed by means of soaking and hammering sample preparation. In order to further increase the detection sensitivity and the feasibility to analyse a sample size of 10.000 seeds (ISTA recommendation), an implementation and validation of a multiplex Real-Time TaqMan PCR assay is now in progress and is based on the results obtained from our studies on sample preparation and DNA extraction.

Biological seed treatments with plant/fungal extracts showed to reduce the percentage of symptomatic seedlings and slightly increasing the germination percentage. Bacterial antagonist DLS 65 used in this study significantly decreased the percentage of symptomatic seedlings by 27%, without affecting the germinability on blotter and in soil (data not shown). Seed treatments tested were not able to eradicate the bacteria, and this

**Table 1.** Detection threshold (1 contaminated seed in x seeds) by simplex PCR with SEQID3/SEQID4 (Schaad *et al.*, 2000) and WFB1/WFB2 (Walcott *et al.*, 2000) primers. DNA extraction methods: PMK= DNeasy Plant Mini kit (Qiagen); B&T= DNeasy Blood and Tissue (Qiagen); Wizard 96 = Wizard Magnetic 96 DNA Plant System (Promega).

		Melon		Watermelon	
		SEQID3/4	WFB 1/2	SEQID3/4	WFB 1/2
PMK	Soaking	1:1000	1:1000	1:1000	1:1000
	Hammering	1:1000	1:1000	1:1000	1:1000
B&T	Soaking	1:1000	1:1000	Negative	1:100
	Hammering	1:100	1:1000	Negative	1:1000
Wizard 96	Soaking	1:1000	1:100	Negative	1:1000
	Hammering	1:1000	1:100	Negative	1:100

**Table 2.** Germination percentage *in vitro* and on blotter and percentage of diseased watermelon seedlings grown after treatments. Data presented as mean ± SD for 3 replicates for each treatment. An asterisk indicates that data are significant ( $p \leq 0.05$ ).

Treatment	Germination %		Diseased seedlings %
	<i>in vitro</i>	blotter	
Microbial consortium (Micosat F, CCS Aosta, Italy)	92.33 ± 2.08	90.00 ± 1.00	56.00 ± 3.00
Plant polyphenols (AGRITAN, Silvateam, Italy)	94.00 ± 3.00	91.67 ± 2.52	59.67 ± 2.65
Bacterial antagonist strain DLS 65	*81.00 ± 1.53	84.67 ± 2.00	*46.67 ± 2.52
Untreated	90.33 ± 4.35	86.00 ± 6.02	63.33 ± 4.16



could be explained by the localization of Acit in the embryos of watermelon seeds (Dutta *et al.*, 2012); nevertheless such treatments can be inducers of resistance and can also enhance and boost the performance of seeds either in processing and planting equipment or mitigate environmental stress. Additional research is planned to improve the seed treatment protocol, with the application of a new formulation obtained by addition of methylcellulose to the bacterial antagonist suspension prior to treatment, in order to achieve a better adhesion of the seed coating.

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