International Seed Federation
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Detection of *Acidovorax citrulli* in Melon Seed by Sweat Box Grow-out

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Developed by ISHI-Veg

Detection of *Acidovorax citrulli* in Melon Seed by Sweat Box Grow-out

Crop: Melon (*Cucumis melo*)
Pathogen: *Acidovorax citrulli*
Version: 1.0 (May 2021)

PRINCIPLE

Detection of infectious *Acidovorax citrulli* bacteria on melon seeds is done by growing out seeds under environmental conditions highly conducive to producing disease symptoms in a sweat box followed by a bioassay on suspected seedlings to confirm the presence of infectious *A. citrulli*.

A seed extract qPCR assay (SE-qPCR) may be used as a pre-screen. The test is complete if no *A. citrulli* is detected and the seed lot is considered healthy. However, as qPCR detects both viable and non-viable bacterial DNA, a positive pre-screen SE-qPCR is followed by the grow-out to determine the presence of viable *A. citrulli*.

The complete method process workflow is presented in Figure 1.

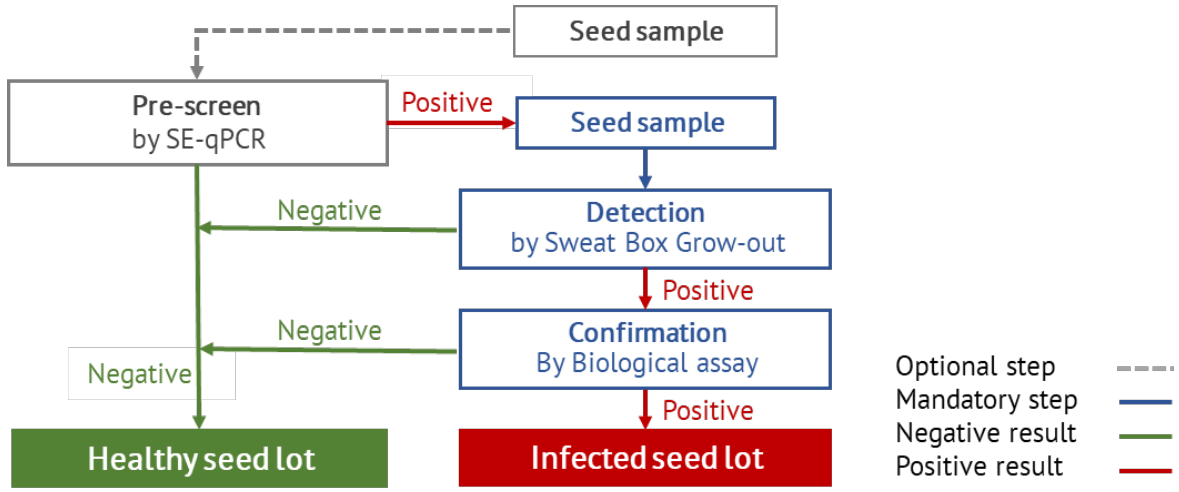

Figure 1. Method process workflow

METHOD VALIDATION

The sweat box grow-out has been validated according to the ISHI-Veg Guidelines for the Validation of Seed Health Test (Lybeert *et al.*, 2020).

RESTRICTIONS ON USE

Technical details on the reagents / material used in the validation study (e.g. supplier information) are provided in the protocol and the validation report. If material and consumables from different suppliers are used, it is necessary to verify their performance.

This test method is suitable for untreated seed and for seed that has been treated using chemicals or physical processes with the aim of disinfestation / disinfection, as well as seed treated with protective chemicals or biological substances.

METHOD EXECUTION

To ensure process standardization and valid results, it is strongly recommended to follow the best practices described by ISHI-Veg.

SAMPLE AND SUB-SAMPLE SIZE

The minimum recommended sample size is 10,000 seeds and can be up to 30,000 seeds per lot.

REVISION HISTORY

Version	Date	Changes (minor editorial changes not indicated)
1.0	May 2021	First version of the protocol.

Protocol for detecting *Acidovorax citrulli* in Melon Seed by Sweat Box Grow-out

I. PRE-SCREEN BY SEED-EXTRACT qPCR (OPTIONAL STEP)

See the protocol for the Detection of *Acidovorax citrulli* in cucurbit seeds by SE-qPCR on the ISF website.

II. DETECTION BY SWEAT BOX GROW-OUT ASSAY AND CONFIRMATION BY BIOASSAY

Materials

- Plastic sweat box with a lid (35 x 25 x 20 cm ± 5 cm difference) (e.g. Rotho: Ref 4045, 10 L)
- 70% alcohol
- Potting soil
- Vermiculite
- Thiram fungicide solution (40 mg/L)*
- Growth chamber 25 – 28 °C
- Saline solution 0.85% (Table II.1)
- Controls (Table II.2)
- Small grinding plastic bags and a press grinder (or equivalent)
- De-ionized water
- Laminar airflow cabinet
- Microliter pipettes (e.g. Gilson or Finn)
- Lab disposables

* If a different fungicide is used, it is necessary to verify it does not affect the performances of the validated protocol.

Table II.1. Saline solution.

Compound	g/L
Sodium chloride (NaCl)	8.5
De-ionised water to a final volume of	1000 mL

Note: Autoclave at 121 °C, 115 psi for 15 min.

Table II.2. Types of controls used

Control type	Description	Assay
Positive Process Control (PPC)	Known *A. citrulli*-positive seed sample	Sweat box
Negative Process Control (NPC)	Known *A. citrulli*-negative seed sample	
Positive Control (PC)	Reference *A. citrulli* strain	Bioassay
Negative Control (NC)	Saline solution	

1. Sweat box grow-out

1.1. Clean, disinfect (with 70% alcohol) and label the plastic sweat boxes with the appropriate code number.

1.2. Add 1000 mL of potting soil to each sweat box.

1.3. Spread 800 seeds of each subsample and the PPC and NPC (Table II.2) evenly in the sweat boxes.

1.4. Carefully add 2 L of clean and fresh vermiculite in the sweat boxes and spread evenly.

1.5. Add 1 L of the irrigation solution of Thiram into each sweat box.

Notes: prepare the Thiram solution extemporaneously. Treating the seed to replace the Thiram irrigation solution by water is possible (1.4 g Thiram per kg seeds).

1.6. Close the sweat boxes and incubate at 25 – 28 °C with minimal 14 hours light / day.

Note: These are the regulation temperatures. A comparison made earlier showed no difference in the expression of disease symptoms between 25 °C and 28 °C in the box.

1.7. Fourteen days after sowing, inspect seedlings for typical disease symptoms (water soaked lesions on cotyledons and leaves: see Figure II.1 for suspect seedlings) or showing necrosis (doubtful symptoms).

Figure II.1. Symptoms of *Acidovorax citrulli* on leaves of melon seedlings.

1.8. Pick and place suspect seedlings (cotyledons only and not the stems or roots) in small grinding plastic bags. Each small grinding plastic bag should contain no more than 5 seedlings. Seedlings with doubtful symptoms must be treated in the same manner. Do not mix suspect and doubtful seedlings in a bag. Pick no more than a maximum of 20 (to form a maximum of 4 pools) suspect and doubtful seedlings per sweat box favouring suspect ones.

Note: When no suspected or doubtful seedlings are found, the test result can be considered negative when the NPC and PPC give the expected result. The PPC should give suspected seedlings, and the NPC should give no symptoms.

Example of sample pools:

If in a sweat box there are 15 suspect and 7 doubtful seedlings, they would make
- 3 pools of 5 suspect seedlings + 1 pool of 5 doubtful seedlings

If in a sweat box there are 24 suspect and 15 doubtful seedlings, they would make
- 4 pools of 5 suspect seedlings

If in a sweat box there are 13 suspect and 3 doubtful seedlings, they would make
- 2 pools of 5 suspect, 1 pool of 3 suspect, 1 pool of 3 doubtful seedlings

1.9. Take only 2 small grinding plastic bags of 5 plants each for the NPC and PPC (suspect seedlings).

1.10. Add 5 mL of 0.85% sterile saline to each small grinding plastic bag containing the suspected cotyledons from the test and control samples. Grind them with a press grinder or equivalent.

1.11. Proceed with the bioassay for confirmation.

Note: Samples can be stored at 4 °C for a maximum of 48 hours before the confirmation step.

2. Bioassay confirmation

2.1. Sow seeds from the Negative Process Control (NPC) 7 days after the sowing date of the sweat box assay and incubate at 23 ºC with 16-20 hours light / day. Sow ten seeds per pot and keep only five seedlings that are at the right physiological stage for the biological assay.

Note: The right physiological age of the seedlings is important; keep only those seedlings where just the tip of the developing first true leave can be observed (7 to 10 days, depending on the speed of development, see Figure II.2).

Figure II.2. Five healthy melon seedlings at the optimum stage for a biological assay.

In case the plants are not at the right physiological age for the biological assay keep the extracts in the refrigerator at 4 °C but no longer than 2 days.

2.2. Place a droplet of 10 µL from each sample extract obtained at point 1.10 between the cotyledon and the stem of five seedlings in the same pot. Stab the spot on the seedlings where the droplets have been placed with a toothpick. Label the pot with the sample-extract number.

2.3. Follow the same procedure to inoculate five seedlings with the Negative Control (NC), and five more with 10^5-10^8 CFU of the Positive Control (PC), see Table II.2.

2.4. Place the pots with inoculated seedlings in trays and put the trays in a bigger container / sweat box with some water on the bottom and close firmly with a lid. Place the containers / sweat boxes in the climate chamber at 28 °C; with 16 hours light / day and high humidity.

2.5. Evaluate the plants after 7 days. Compare the symptoms of the test plants to the PC and NC plants (see Figure II.3). A biological assay is considered as positive if at least one plantlet gives symptoms as the PC.

Note: Test results are only valid when all included controls presented in Table II.2 give the expected result. The test plant inoculated with the PC and PPC should develop clear symptoms, the test plants inoculated with the NC and NPC should develop no symptoms.

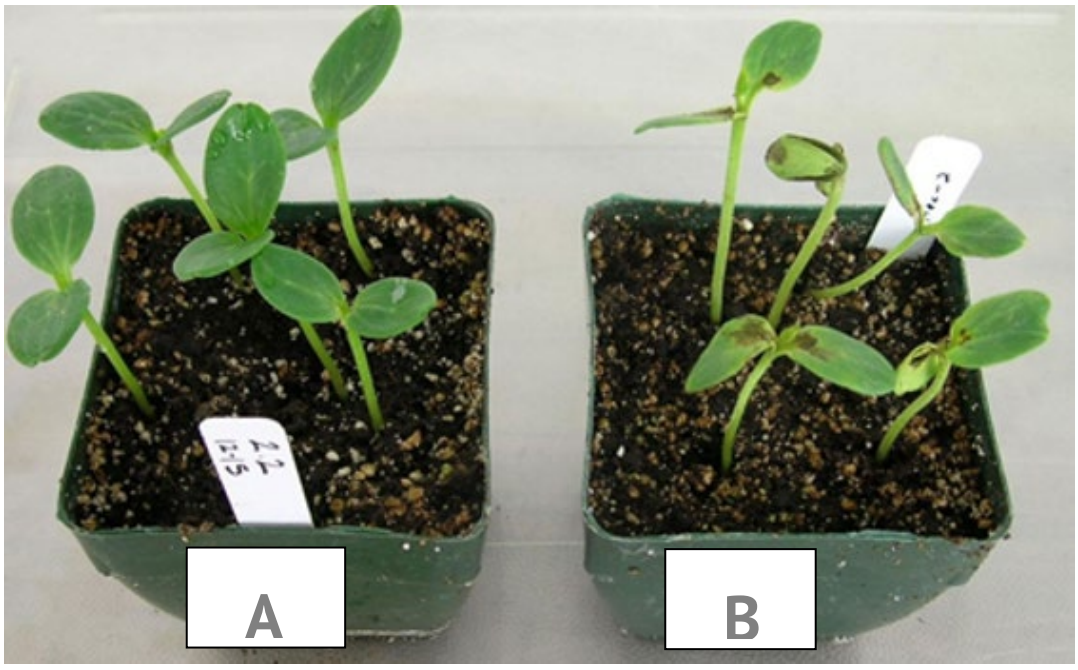

Figure II.3. (A) Negative control plants at 7 days post inoculation with sterile saline. No developed symptoms. (B) Positive control plants at 7 days post inoculation. Development of clear symptoms.

REFERENCES

Lybeert, H., Sahuguede, C., Orgeur, G., Ponzio, C. & Woudenberg, J. H. C. (2021). Detection of *Acidovorax citrulli* associated with melon seeds by grow-out in sweat boxes. Validation report, International Seed Federation (ISF), Nyon, Switzerland. https://worldseed.org/our-work/seed-health/ishi-method-development-and-validation/