International Seed Federation
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Detection of *Stagonosporopsis valerianellae* in Corn Salad Seed

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Developed by ISHI

Detection of *Stagonosporopsis valerianellae* in Corn Salad Seed

Crop: Corn salad (*Valerianella locusta*)

Pathogen(s): *Stagonosporopsis valerianellae (*previously called *Phoma valerianellae)*

Version: 1 (October 2024)

PRINCIPLE

Detection of viable *Stagonosporopsis valerianellae* (Sv) in seed of corn salad is done by plating on malt-agar medium. After malt-agar plating, a PCR is used for identification of the suspect fungal colonies. A positive PCR must be followed with a pathogenicity assay to determine if the suspect *S. valerianellae* isolate is pathogenic or not.

The full method process workflow is presented in Figure 1.

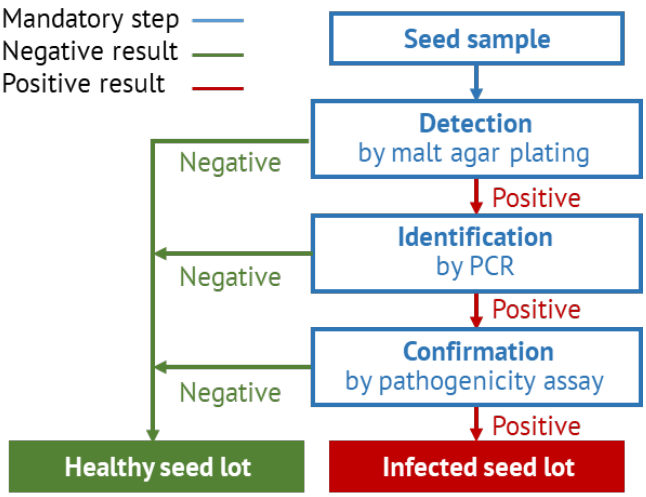

Figure 1. Method process workflow.

METHOD VALIDATION

The validation report for the detection of *S. valerianellae* in corn salad seed (Delisle and Orgeur, 2024) can be found at https://www.worldseed.org/our-work/seed-health/ishi-method-development-and-validation/.

RESTRICTIONS ON USE

Before using this protocol routinely, it is necessary to verify its performance, especially when material and consumables from different suppliers are used. Technical details on the reagents/material used in the validation study (e.g., supplier's information) are provided in the protocol and the validation report.

This test method is suitable for untreated seed and seed that has been treated using physical (e.g., hot water) or chemical (acid extraction, calcium or sodium hypochlorite, tri-sodium phosphate, etc.) processes with the aim of disinfestation/disinfection, provided that any residue, if present, does not influence the assay (longer incubation period might be needed). It is the responsibility of the user to check for inhibition by experimental comparisons or other means.

This test method has not been validated for seed treated with protective chemicals or biological substances. If treated seed is tested using this method, it is the responsibility of the user to determine empirically (through analysis, sample spiking, or experimental comparisons) whether the protective chemicals or biological substances have an effect on method results.

METHOD EXECUTION

To ensure process standardization and valid results, it is strongly recommended to follow the Best Practices for Seed Health Tests developed by ISHI.

SAMPLE AND SUBSAMPLE SIZE

The recommended minimum sample size is 400 seeds.

REVISION HISTORY

Version	Date	Changes (minor editorial changes not indicated)
1	October 2024	First version of the protocol

Protocol for detecting *S. valerianellae* in corn salad seed

I. DETECTION BY MALT AGAR PLATING

Materials

- Malt agar medium (Table I.1)
- Controls (Table I.2)
- Incubator at 20 ± 2 °C without light

Table I.1. Malt agar medium, pH 5.5 ± 0.1[a].

Compound	Amount/L
Bacto™ malt extract - Thermo Scientific 218630[b]	10 g
Agar	17 g
Streptomycin sulphate (10 mg/mL in de-ionized water)[c]	50 mg (5 mL)

[a] Recommended pH before adding the agar.

[b] If another provider of malt is used, it should be validated by the individual laboratory.

[c] Add after autoclaving (15 psi for 15 min at 121 °C). *Stagonosporopsis valerianellae* detection has also been validated without streptomycin in the malt agar. Removing streptomycin might induce the development of bacterial growth. Antibiotics stock solutions and other supplements prepared in distilled/de-ionized water must be sterilized using a 0.2 µm bacterial filter. Alternatively, add the antibiotic powder to autoclaved distilled/de-ionized water. Solutions prepared in ethanol need no sterilization.

Table I.2. Types of controls used.

Control type	Description
Positive control (PC)[a]	*Stagonosporopsis valerianellae* isolate
Positive process control (PPC)[a]	Known *S. valerianellae* infected seed sample with minimum 10% of infection rate
Negative process control (NPC)	Known healthy seed sample

[a] For the positive controls, include at least either the PC or PPC.

1. Plating

1.1. Prepare malt agar medium plates (Table I.1).

1.2. Place 10 seeds per plate, evenly spaced. Use 40 plates for a 400 seed sample. Use five plates with 10 seeds each for NPC and PPC. For the PC, transfer a small portion of malt agar medium containing the Sv isolate (around 0.5 cm^3 square) (in culture for 7 to 28 days) on a malt agar medium plate.

1.3. Incubate at 20 ± 2 °C for 14 days in the dark.

2. Evaluation of plating

2.1. Observe the plates and individually each seed for the development of characteristic mycelium. Check control plates first. Seeds infected by *S. valerianellae* are surrounded by a characteristic mycelium (Figure I.1). The mycelium is flat and dark olive green with white

ring on the edge. Sometimes, the mycelium becomes fluffy white on the top. Compare the morphology of suspect mycelium to the PPC and/or PC.

Note: Test results are only valid when all included controls presented in Table I.2. give the expected results. The PC and/or PPC should display typical morphology, as described above for *S. valerianellae*. The NPC should display no *S. valerianellae*.

2.2. The suspect mycelium should be identified by PCR (Section II) and confirmed by pathogenicity assay (Section III). If present, six suspect isolates should be tested per sample. Plates with suspect mycelium should be kept until PCR and pathogenicity results are obtained.

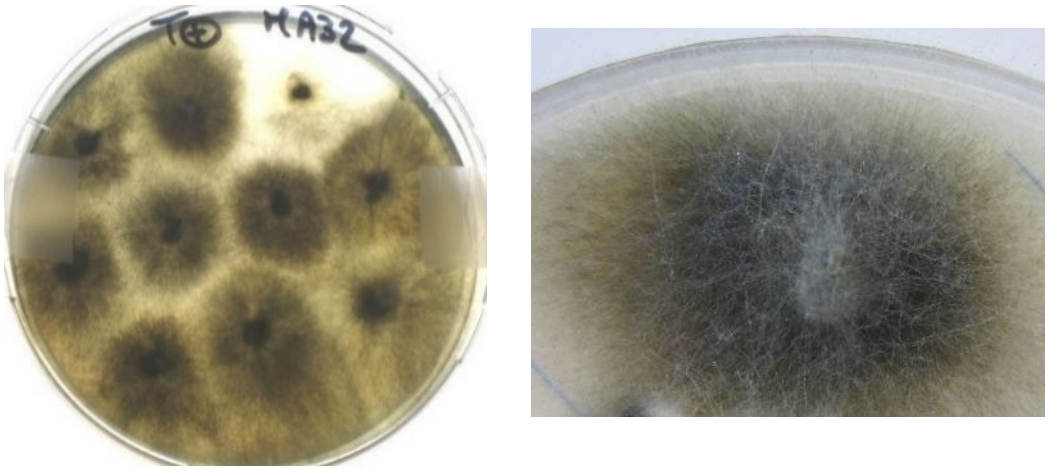

Figure I.1. Morphology of *S. valerianellae* on corn salad seeds grown on malt-agar medium.

II. IDENTIFICATION BY PCR

For PCR methods, in-house method optimization is often necessary, see Best Practices for PCR Assays in Seed Health Tests.

Materials

- Tris-borate-EDTA (TBE) buffer (Table II.1)
- PCR mix, primers (Table II.2) and equipment
- Controls (Table II.3)
- Gel-electrophoresis equipment
- Agarose
- Lab consumables

Table II.1. Tris-borate-EDTA (TBE) buffer 10×, pH 8.3.

Compound	Amount/L
Tris-base	108 g
Boric acid	55 g
EDTA 0.5M (pH 8.0)	40 mL

Table II.2. Primer sequences and references.

Name	Sequence (5' – 3')	Source
Pv-F	GGC TTT GCC TGC CAT CTC	Pellegrino *et al.*, 2010
Pv-R	GAC GTC GTC GTC TGG TTG	
18S uni-F	GCA AGG CTG AAA CTT AAA GGAA	Ioos *et al.*, 2009
18S uni-R	CCA CCA CCC ATA GAA TCA AGA	

Table II.3. Types of controls used.

Control type	Description
Positive process control (PPC)	*Stagonosporopsis valerianellae* isolate mycelium from the PPC or PC of the malt-agar plating assay (Table I.2)
Positive amplification control (PAC)	A known positive DNA extract from *S. valerianellae*
Negative process control (NPC)	Distilled water used for the mycelium DNA extraction
Negative template control (NTC)	PCR-grade water used to prepare the PCR (no DNA sample)
Internal amplification control (IAC)[a]	General conserved fungal sequences (18S rRNA gene)

[a] Note that the amplification control is tested in a separate PCR assay.

1. Sample preparation

1.1. Extract DNA from the suspect mycelium.

Option 1: Add 2 mL of distilled autoclaved water in the plate of a 7 day-old cultured from MA suspect mycelium. Scrape off the mycelium, and pipet 1 mL of the mycelium suspension in a microcentrifuge tube.

Option 2: Place the suspected infected seed in a microcentrifuge tube, add 300 µL of distilled autoclaved water and vortex well. Transfer the suspension without the seed to a fresh microcentrifuge tube.

1.2. Put the mycelium suspension at 100 °C for 5 min.

2. PCR

2.1. Perform the PCR on the extracted DNA with the two PCR mixes (Sv and 18S rRNA gene) (Tables II.4 and II.5) using the PCR cycle described in Table II.6. Perform the PCR in duplicate for each isolate and for each PCR mix.

2.2. Perform a gel-based analysis of the obtained PCR products using a 2% (w/v) agarose gel in 0.8× Tris-borate-EDTA (TBE) buffer working concentration (Table II.1).

3. Interpretation and decisions

3.1. Compare the obtained results to the results of the PPC and NPC. The expected band size for the Sv PCR is 496 bp. The expected band size for the 18S rRNA gene is 150 bp.

Note: In case of DNA extraction using option 2, if less than six suspect isolates are tested and all are Sv PCR negative, repeat the DNA extraction using option 1.

Table II.4. Sv PCR mix.

Component	Per reaction (in µL)	Final concentration
PCR buffer 10×	2.0	1×
PCR grade water	12.7	
$MgCl_2$ (25 mM)	1.2	1.5 mM
dNTP (10 mM each)	0.4	0.2 mM
Pv-F (10 µM)	0.75	0.375 µM
Pv-R (10 µM)	0.75	0.375 µM
Taq Polymerase (5U/µL)	0.2	1 U
DNA	2.0	
Total	**20.0**	

Table II.5. 18S rRNA gene PCR mix.

Component	Per reaction (in µL)	Final concentration
PCR buffer 10×	2.0	1×
PCR grade water	12.7	
$MgCl_2$ (25 mM)	1.2	1.5 mM
dNTP (10 mM each)	0.4	0.2 mM
18S uni-F (10 µM)	0.75	0.375 µM
18S uni-R (10 µM)	0.75	0.375 µM
Taq Polymerase (5 U/µL)	0.2	1 U
DNA	2.0	
Total	**20.0**	

Table II.6. PCR cycling parameters for the Sv and 18S rRNA gene PCR.

Step	Temperature	Duration
Hold	95 °C	3 min
35 cycles	94 °C	45 sec
	59 °C	45 sec
	72 °C	1 min
Final elongation	72 °C	7 min
Post PCR	4 °C	Hold

3.2. Suspect mycelium that gives a positive Sv PCR are considered suspect isolates and should be confirmed by pathogenicity assay.

Note: Test results are only valid when all included controls presented in Table II.3 give the expected results: PPC/PAC positive Sv PCR, NPC/NTC negative Sv and 18S rRNA gene PCR, IAC (18S rRNA gene PCR) positive for all suspect mycelium when negative in Sv PCR.

III. CONFIRMATION BY PATHOGENICITY ASSAY

The pathogenicity assay is performed only for the suspects that amplify a target specific fragment in the Sv PCR assay.

Materials

- Healthy corn salad seeds from a *S. valerianellae* susceptible cultivar (e.g., Gala), with no invading saprophytes in order to avoid competition with the suspect isolate.
- Incubator at 20 ± 2 °C without light
- Malt agar medium (Table I.1)
- Controls (Table III.1)

Table III.1. Types of controls used.

Control type	Description
Positive control (PC)	*Stagonosporopsis valerianellae* isolate
Negative control (NC)	Malt agar portion free from mycelium

1. Pathogenicity assay

1.1. Cut a small portion of malt agar medium including the suspect isolate (around 0.5 cm^3 square), PC, and NC, and transfer each to the centre of a separate fresh malt agar medium plate.

1.2. In parallel, place six healthy corn salad seeds from a *S. valerianellae* susceptible cultivar in a fresh malt agar medium plate. Prepare enough plates to obtain two plates per isolate to test.

1.3. Incubate the plates from step 1.1 and 1.2 at 20 ± 2 °C for 7 days in the dark.

1.4. Inoculate a small part of 7 day-old developed mycelium from step 1.1 at the centre of two plates with germinated corn salad seeds from Step 1.2.

1.5. Incubate again at 20 ± 2 °C for 10 to 14 days in the dark.

1.6. Check the development of mycelium. The pathogenicity test can be analysed when the mycelium reaches at least half of the corn salad roots. If not, incubate for longer the plates.

1.7. Analyse the pathogenicity test. The suspected mycelium is pathogenic if at least one root in contact with the mycelium shows necroses (turns black) (Figure III.1).

Note: Results are only valid when the PC and NC give the expected results. The PC should give necrosed black root in the pathogenicity test. The NPC should not give these symptoms.

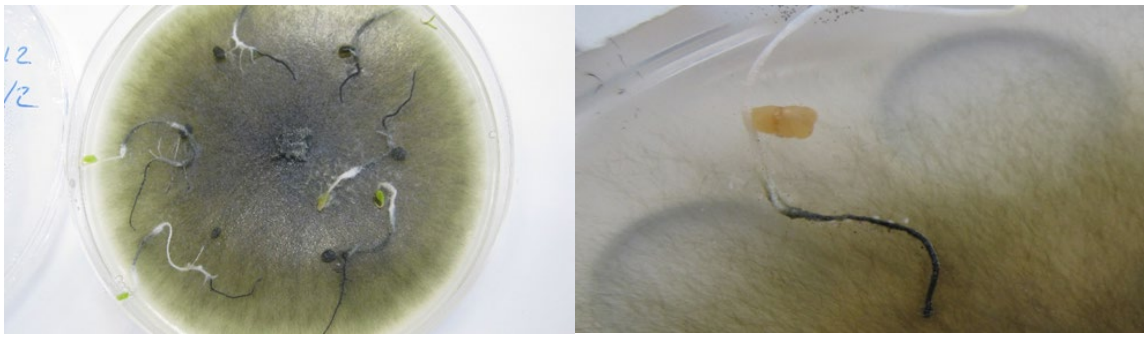

Figure III.1. Pathogenicity assay of *S. valerianellae*.

REFERENCES

Delisle, J. and Orgeur, G. (2024). Detection of *Stagonosporopsis valerianellae* in corn salad (*Valerianella locusta*) seed. Validation report, International Seed Federation (ISF), Nyon, Switzerland. https://www.worldseed.org/our-work/seed-health/ishi-method-development-and-validation/

Ioos, R., Fourrier, C., Iancu, G. and Gordon, T.R. (2009). Sensitive detection of *Fusarium circinatum* in pine seed by combining an enrichment procedure with a real-time polymerase chain reaction using dual-labeled probe chemistry. *Phytopathology*, **99**(5), 582–590.

Pellegrino, C., Gilardi, G., Gullino, M. L. and Garibaldi, A. (2010). Detection of *Phoma valerianellae* in lamb's lettuce seeds. *Phytoparasitica*, **38**, 159–165.