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Detection of *Pseudomonas syringae* pv. *pisi* on Pea Seed

DECEMBER 2020

Developed by ISHI-Veg

Detection of *Pseudomomonas syringae* pv. *pisi* on Pea Seed

Crop: Pea (*Pisum sativum*)

Pathogen(s): *Pseudomonas syringae* pv. *pisi*

Version: 2 (December 2020)

PRINCIPLE

Detection of viable *Pseudomonas syringae* pv. *pisi* (Psp) bacteria is done by dilution plating on two semi-selective media (KBBCA and SNAC). Extraction of bacteria from the surface and the funiculus of the seed is enhanced by soaking. Suspect bacterial colonies are then confirmed by a pathogenicity assay.

After dilution plating, a PCR assay may be used for identification of the suspect bacterial colonies. The test is complete if no Psp bacteria are detected. However, as PCR may detect both infectious and non-infectious bacterial particles, a positive PCR must be followed with a pathogenicity assay to determine if the suspect Psp isolate is pathogenic. The complete method process workflow is presented in Figure 1.

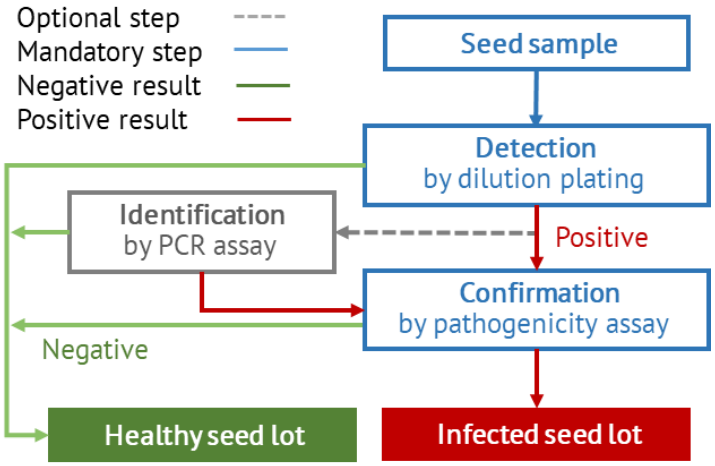

Figure 1. Method process workflow

METHOD VALIDATION

Version 1 of this method for detecting Psp on pea seed is an ISTA Rule (7-029) (https://www.seedtest.org/en/seed-health-methods-_content---1--1452.html), and seven laboratories participated in a comparative test to generate data on repeatability and reproducibility (ISTA, 2012). It is also an NSHS Standard A (https://seedhealth.org/seed-health-testing-methods/).

In this second version an optional PCR identification assay was validated (Lê Van 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. For PCR methods, in-house method optimization is often necessary by changing certain parameters as described in Best Practices for PCR Assays in Seed Health Tests.

This test method is suitable for untreated seed.

The ability to recover Psp on plates can be influenced by the presence of other microorganisms and/or inhibitory chemicals used for seed disinfestation/disinfection. It is the responsibility of the user to check for such antagonism and/or inhibition by analysis and sample spiking.

This test method has not been validated for seed treated with protective chemicals or biological substances or physical (such as hot water) or chemical (calcium or sodium hypochlorite) processes with the aim of disinfestation/disinfection. If a user chooses to test treated seed 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 the method results.

METHOD EXECUTION

To ensure process standardization and valid results, it is strongly recommended that the best practices developed by ISHI-Veg for PCR and Dilution Plating Assays in Seed Health Tests are followed.

SAMPLE AND SUBSAMPLE SIZE

The recommended minimum sample size is 5,000 seeds with a maximum subsample size of 1,000 seeds.

REVISION HISTORY

Version	Date	Changes (minor editorial changes not indicated)
1.0	January 2014	
1.1	January 2017	Step 6.1 and 6.2 of the dilution plating are modified.
2.0	December 2020	An optional PCR identification assay replaces the optional biochemical identification steps. The protocol is presented in accordance with ISHI-Veg's protocol guidelines

Protocol for detecting *Pseudomonas syringae* pv. *pisi* on Pea Seed

An older version of this protocol is available on ISTA's website: ISTA 7-029: Detection of *Pseudomonas syringae* pv. *pisi* in *Pisum sativum* (pea) seed (https://www.seedtest.org/en/seed-health-methods-_content---1--1452.html).

I. DETECTION BY DILUTION PLATING

Materials

- Polythene bags or containers
- Cold room or refrigerator operating at 4 °C
- Incubator: operating at 28–30 °C
- pH meter: capable of reading to the nearest 0.01 pH unit
- Dilution containers
- Sterile spreader
- Seed extraction buffer (Table I.1)
- Controls (Table I.2)
- Plates of SNAC (Table I.3) and KBBCA media (Table I.4)
- Lab disposables

Table I.1. Seed extraction buffer (0.85% NaCl (w/v) / 0.15M)

Compound	Amount/L
Sodium chloride (NaCl)	8.5 g
Distilled/de-ionized water	1000 mL

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

Table I.2. Types of controls used

Control type	Description
Positive Control (PC)	A known strain of Psp
Negative Process Control (NPC)	Seed extraction buffer

Table I.3. SNAC semi-selective medium

Compounds	Amount/L
Tryptone	5.0 g
Peptone	3.0 g
NaCl	5.0 g
Sucrose	50.0 g
H_3BO_3 (0.1 g/mL)	10.0 mL
Agar	15.0 g
Distilled/deionized water to a final volume of	1000 mL
Cephalexin monohydrate[a] (80 mg/mL in 70% (v/v) ethanol)	80.0 mg (1.0 mL)
Nystatin[a] (35 mg/mL in 70% (v/v) ethanol)	35.0 mg (1.0 mL)

[a] Added after autoclaving

Table I.4. KBBCA semi-selective medium

Compound	Amount/L
Proteose peptone (e.g.#3 Difco)	20.0 g
Glycerol	10.0 g
K_2HPO_4	1.5 g
$MgSO_4$ anhydrous	0.73 g
H_3BO_3	1.5 g
NaOH (1N)	2.0 mL
Agar	15.0 g
Distilled/deionized water to a final volume of	1000 mL
Cycloheximide[a,b] (50 mg/mL in 70% (v/v) ethanol)	100.0 mg (2.0 mL)
Cephalexin[a] (80 mg/mL in 50% (v/v) ethanol)	40 mg (0.5 mL)

[a] Added after autoclaving.

[b] Nystatin (1.0 mL of 35 mg/mL in 70% (v/v) ethanol) could be used as an alternative for cycloheximide to control fungi.

Media preparation

1. Weigh all ingredients, except the antibiotics.
2. Dissolve them in a suitable container in distilled/deionized water.
3. Autoclave at 15 psi for 15 min at 121 °C.
4. Prepare antibiotic solutions, and filter sterilize as appropriate.

   Note: 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 the autoclaved distilled/de-ionized water. Solutions prepared in 70% ethanol need no sterilization.
5. Allow medium to cool to approx. 50 °C prior to adding antibiotic solutions.
6. Mix the molten medium thoroughly but gently to avoid air bubbles and pour plates (18 mL per 90 mm plate).
7. Leave plates to cool down and dry under sterile conditions.

   Note: Storage conditions and duration may affect antibiotic activity, which can influence the performance of the test.

1. Seed extraction

1.1. Suspend each subsample of seeds in sterile seed extraction buffer in a polythene bag or container. The volume of seed extraction buffer in mL should be adjusted according to the number of seeds used (use 2.5 mL of buffer per 1 g of seeds).

1.2. Soak the subsamples overnight (18–24 hours) at 4 °C under agitation.

2. Dilution and plating

2.1. Shake the polythene bags or containers by hand to obtain a homogeneous extract before diluting.

2.2. Prepare two serial ten-fold dilutions from the seed extract by i.) Pipetting 0.5 mL of the extract into 4.5 mL of sterile seed extraction buffer to give a 10^1 dilution and ii.) Pipetting 0.5 mL of the 10^1 dilution into 4.5 mL of sterile seed extraction buffer to give a 10^2 dilution. Vortex well all dilutions.

2.3. Pipette 100 µL of each dilution and the undiluted seed extract onto two plates of each of the KBBCA and SNAC semi-selective media and spread over the surface.

2.4. Incubate inverted plates at 28±2 °C and examine after 4–5 days.

3. Positive Control (culture or reference material)

3.1. Prepare a suspension of a known Psp strain in sterile seed extraction buffer or reconstitute standardized reference material according to the supplier's instructions. Dilute sufficiently to obtain dilutions containing approx. 10^2 to 10^3 CFU/mL.

3.2. Pipette 100 µL of appropriate dilutions onto plates of both semi-selective media (KBBCA, SNAC) and spread over the surface.

3.3. Incubate plates with the sample plates (as in 2.4).

4. Negative Process Control (sterility check)

4.1. Prepare a dilution series from a sample of the seed extraction buffer, without seeds. Plate each dilution on the two semi-selective media, spread over the surface and incubate (as in 2.2 to 2.4).

5. Examination of the plates

5.1. Examine the sterility check (NPC) and positive control plates. Test results are only valid when all included controls give the expected result:

- No growth on dilution plates being used as a sterility check.
- Psp colonies on SNAC are circular, white to transparent, mucoid, dome shaped and levan positive (Figure I.1), and on KBBCA creamy and half-translucent (Figure I.2).
- The number of bacteria on the dilution plates are consistent with the dilution, i.e. it decreases approx. ten-fold with each dilution.

Note: Dilution plates prepared from the positive control isolate or reference material, should give single colonies with typical morphology. The numbers of colonies on dilution plates prepared from the positive control isolate or reference material should be similar on both media.

5.2. Examine the sample plates for the presence of typical Psp colonies by comparing them with the positive control plates.

Note: Verify that the plates are readable according to the dilution plating best practices and record the presence of suspect colonies.

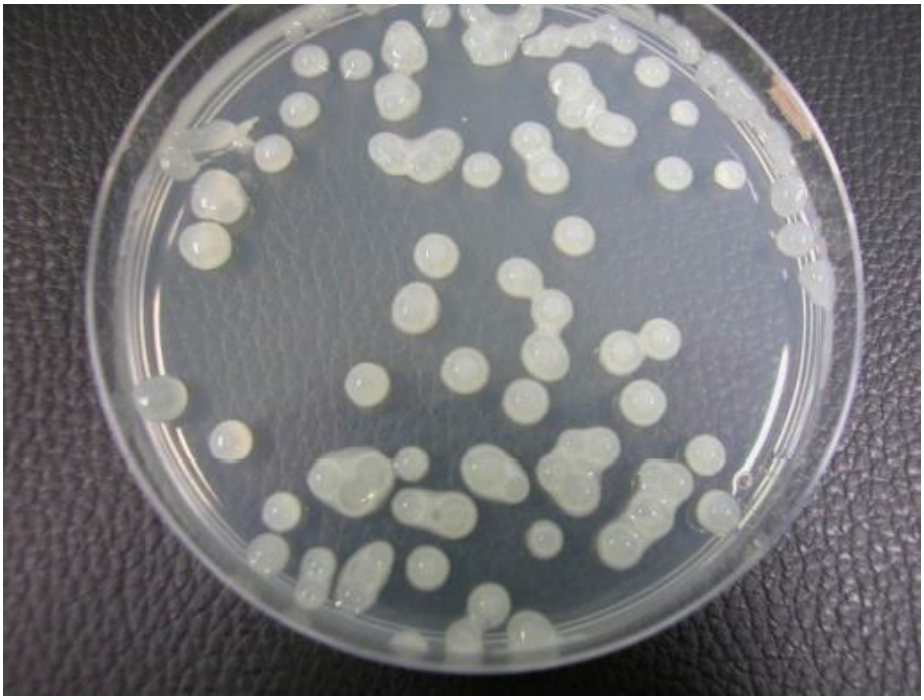

Figure I.1. Plate of SNAC medium after 4 days of incubation at 28±2 °C showing typical colonies of Psp that are levan positive.

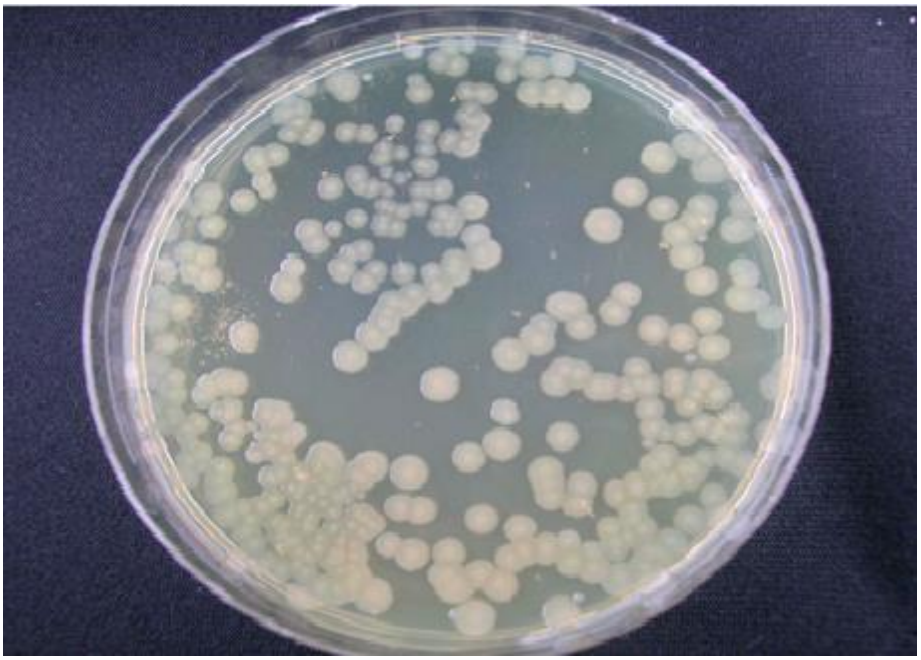

Figure I.2. Plate of KBBCA medium after 4 days of incubation at 28±2 °C showing typical colonies of Psp.

6. Confirmation/identification of suspect colonies

6.1. Pick up at least six suspect colonies per subsample grown on SNAC medium, if present, and subculture them on sectored plates of KBBCA medium.

6.2. Pick up at least six suspect colonies per subsample grown on KBBCA medium, if present, and subculture them on sectored plates of SNAC medium.

6.3. Repeat with the positive control colonies. Subculture one colony grown on SNAC medium on a sectored plate of KBBCA medium and subculture one colony grown on KBBCA medium on a sectored plate of SNAC medium.

6.4. Incubate sectored plates for 2–3 days at 28±2 °C.

6.5. Check colonies sub-cultured on SNAC medium for the typical Psp morphology and levan production. *P. syringae* pv. *pisi* colonies are levan positive (Figure I.1). Compare to the positive control.

6.6. Check colonies sub-cultured on KBBCA medium for the typical Psp morphology and optionally for blue fluorescence under UV light. Some Psp strains produce a blue fluorescent pigment under UV light whereas others are non-fluorescent (Figure I.3).

Note: The optional UV-light step can decrease the number of suspect colonies by only selecting blue fluorescent and non-fluorescent colonies.

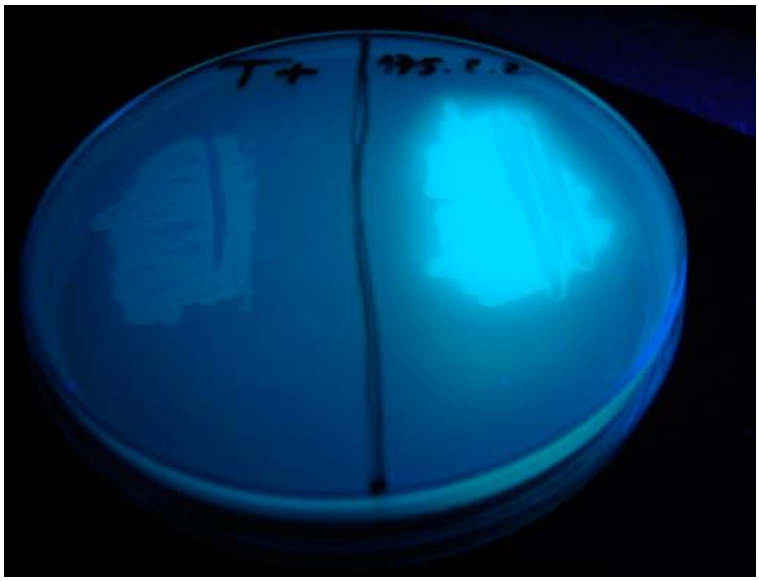

Figure I.3. Fluorescent and non-fluorescent Psp isolates under UV-light.

6.7. Record results for each colony sub-cultured.

6.8. The identity of the isolates can be confirmed by the PCR assay (section II). The pathogenicity of the isolates should be confirmed by a pathogenicity assay on pea seedlings of known susceptibility (section III).

Note: As non-pathogenic isolates may also be present in seed lots it is essential to subculture at least the minimum number of suspect colonies specified (six per subsample), and to test the pathogenicity of all *Pseudomonas*-like sub-cultured isolates by a pathogenicity assay.

II. IDENTIFICATION BY QPCR (OPTIONAL STEP)

For PCR methods, in-house method optimization is often necessary by changing certain parameters as described in Best Practices for PCR Assays in Seed Health Tests.

Materials

- sterile distilled/deionised water
- Controls (Table II.1)
- PCR mix, primers (Table II.2) and equipment
- Lab disposables

Table II.1. Types of controls used

Control type	Description
Positive Process Control (PPC)	Freshly prepared suspension of Psp
Negative Process Control (NPC)	Freshly prepared suspension of non-target colony
Internal Amplification Control (IAC)	Universal bacterial primers (Wu et al. 2008)
Non Template Control (NTC)	Nucleic acid-free water

Table II.2. Primer-sequences and references

Name	Sequence	Source
avrRps4-F	5' – GAG GCC AAC CCA GCC GAA A– 3'	Baldwin 2015
avrRps4-R	5' – TGA TTC TGC GGT CTT CGT TTC TG – 3'	
AN7/2	5' – AAC GGC GAG GGT TGT GGA AA – 3'	Arnold et al. 1996
Pspi3	5' – TCA CTC CGA GCT CCT CAC TA – 3'	
AN3/1	5' – CAC CCA GCG CAT TAC TAG GA – 3'	
AN3/2	5' – CCA GCA CCC AGA TTG AGA CT – 3'	
Wu-F	5' – CAA CGC GAA GAA CCT TAC C – 3'	Wu et al. 2008
Wu-R	5' – ACG TCA TCC CCA CCT TCC – 3'	

1. DNA isolation

The template DNA from single colonies for PCR can be obtained by several means. The method presented here was used for the validation of this protocol.

1.1. Make a bacterial suspension of 10^7-10^8 CFU/mL from the suspected cultures and the positive process control (Table II.1) in sterile distilled/deionized water. In addition, a non-suspect isolate should be used as a negative process control (NPC).

1.2. Incubate the suspensions for 10 min at 95 °C.

1.3. Suspensions can be stored at –20 °C until identification.

2. PCR

2.1. Use the avrRps4 primer set from Baldwin (2015) for Psp-specific DNA amplification (Table II.2). Amplification with these primers will give a product size of 114 bp.

Use the AN7 and AN3 primer sets from Arnold et al. (1996) for Psp-specific DNA amplification (Table II.2). Amplification with these primers will give a product size of 272 bp or 132 bp, respectively, depending on the genetic lineage of the Psp strain (either AN7 or AN3).

Use the universal primers from Wu et al. (2008) (Table II.2) to validate the PCR reaction. Amplification with these primers will give a product size of 228bp.

2.2. Prepare the reaction mixtures as indicated in Tables II.3, II.4 and II.5.

Table II.3. PCR mix avrRps4 SYBR-green (*P. syringae* pv. *pisi* specific reaction mix)

Component	For 1 reaction (in µL)	Final concentration
avrRps4-F (10 µM)	1.0	0.7 µM
avrRps4-R (10 µM)	1.0	0.7 µM
Qiagen Quantitect SYBR (2x)	7.5	1x
PCR grade H_2O	3.5	
Template DNA	2.0	
Total	**15.00**	

Table II.4. PCR mix AN3, AN7 SYBR-green (*P. syringae* pv. *pisi* specific reaction mix)

Component	For 1 reaction (in µL)	Final concentration
AN3/1 (10 µM)	1.0	0.7 µM
AN3/2 (10 µM)	1.0	0.7 µM
AN7/2 (10 µM)	1.0	0.7 µM
Pspi3 (10 µM)	1.0	0.7 µM
Qiagen Quantitect SYBR (2x)	7.5	1x
PCR grade H_2O	1.5	
Template DNA	2.0	
Total	**15.00**	

Table II.5. PCR mix Wu SYBR-green (Universal bacterial primer reaction mix)

Component	For 1 reaction (in µL)	Final concentration
Wu-F (10 µM)	1.0	0.7 µM
Wu-R (10 µM)	1.0	0.7 µM
Qiagen Quantitect SYBR (2x)	7.5	1x
PCR grade H_2O	3.5	
Template DNA	2.0	
Total	**15.0**	

2.3. Perform the PCR reaction in a real-time PCR instrument according to the PCR conditions (Table II.6).

Note: If different PCR mixtures and amplification programs are used, it is necessary to verify their performance. Validation studies have shown that PCR results are more dependent on laboratory conditions than on the PCR protocol when different PCR mixes and amplification products were used.

Table II.6. PCR conditions

Step	Temperature	Duration
hold	95 °C	15 min
35 cycles	94 °C	10 sec
	60 °C	15 sec
	72 °C	30 sec
Melt Curve	72 °C-95 °C	-

2.4. Determine the cut-off values. Cut-off values must be established by each laboratory for their positive and internal amplification controls (IAC) prior to the assay being used on routine samples. For recommendations on setting cut-off values, see Real-time PCR pre-screening in seed health methods.

Note: In the case of universal bacterial primers, positive reactions may occur in non-template controls (NTC) due to the presence of residual DNA in Taq enzyme reagents. The IAC Cq values from reactions on suspect isolates should at least be 3.3 Cq value lower than the IAC Cq values from the NTC reactions.

3. Interpretation and decisions

For interpretation and decision making, the results from all primer sets need to be taken into account, see Table II.7. Test results are only valid when all included controls presented in Table II.1 give the expected result.

Note: in the CT a PCR result was regarded positive with a Cq value of ⩽ 35, with a melt curve temperature identical (±1.5 °C) to the PPC and negative with a Cq value > 35 or a melt curve temperature different (±1.5 °C) from the PPC.

Table II.7. Interpretation and decision table for the PCR

avrRps4	AN3/AN7	Wu	qPCR Result	Follow-up
Positive	Positive	Positive or negative	Target DNA for Psp detected	Pathogenicity test for confirmation
Negative	Negative	Positive	No target DNA for Psp detected	Negative, no follow up
Negative	Negative	Negative	IAC failure	Repeat PCR
Positive	Negative	Positive or negative	Inconclusive	Pathogenicity test for confirmation
Negative	Positive	Positive or negative	Inconclusive	Pathogenicity test for confirmation

III. CONFIRMATION BY PATHOGENICITY ASSAY

Materials

- Pea seeds or seedlings susceptible to all races of the pathogen (e.g. cv. Kelvedon Wonder)
- Blotter paper
- Plastic bags
- Controls (Table III.1)
- Demineralised water
- Potting substrate or equivalent

Table III.1. Types of controls used

Control type	Description
Positive Process Control (PPC)	A known pathogenic strain of Psp
Negative Control (NC)	Demineralized water

1. Pathogenicity assay – option 1

1.1. Germinate seeds of a pea cultivar known to be susceptible to all races of Psp (e.g. cv. Kelvedon Wonder) in wet blotter paper. Roll the paper with the seeds and place it in a plastic bag. Incubate the closed bag at room temperature (18–20 °C) for 2-4 days to allow for seed germination. Make sure to germinate enough seeds for all the suspect colonies that will be tested.

1.2. Prepare a suspension of a 24–48 hours old suspect bacterial culture and the positive process control (from SNAC or KBBCA) in sterile demineralised water and dilute it to a concentration of 10^8 CFU/mL.

1.3. Cut and remove the root tips of the 2-4 day-old germinated pea seeds.

1.4. Incubate 3 seeds for 15 min in the bacterial suspension of the suspect colonies and the positive process control (PPC) and incubate 3 seeds for 15 min in sterile demineralised water as negative control (NC).

1.5. Remove the seeds from the bacterial suspension (for the suspect colonies and PPC) or sterile demineralised water (NC) and sow them in a labelled potting substrate or equivalent. Incubate at 20±5 °C with 12 hours light/12 hours dark or 16 hours light/8 hours dark and 100% saturating humidity.

1.6. Examine seedlings for typical greasy lesions on stems and leaflets after 5-9 days (Figure III.1). Compare to the positive and negative controls.

Test results are only valid when all included controls give the expected result. The PPC isolate should give typical symptoms and the NC should give no symptoms in the pathogenicity test.

1.7. Record the suspect colonies as positive if greasy lesions are observed.

Figure III.1. Typical greasy lesions on the stem of a pea seedling 'Kelvedon Wonder', 9 days after inoculation following pathogenicity assay - option 1.

2. Pathogenicity assay – option 2

2.1. Grow seedlings of a pea cultivar known to be susceptible to all races of Psp (e.g. cv. Kelvedon Wonder) in small pots or container with potting soil at 20–25 °C with sufficient light until 2 true leaf stage (approx. 8–10 days after sowing).

2.2. Prepare a suspension of a 24–48 hours old suspect bacterial culture and the positive process control culture (from SNAC or KBBCA) in sterile demineralised water and dilute it to a concentration of 10^6 CFU/mL.

2.3. Inject each bacterial suspension with a syringe and needle into the stem of at least 2 pea seedlings (2 seedlings per suspect colony). Inject sterile demineralised water into the stem of 2 pea seedlings to serve as the negative control. A negative control as well as a positive control isolate should be included in every pathogenicity test.

2.4. Incubate the inoculated seedlings at 20±5 °C with saturating humidity.

2.5. Examine seedlings for extended greasy lesion from the point of inoculation after 5–9 days. Compare to positive and negative controls.

Note:Test results are only valid when all included controls give the expected result. The PPC isolate should give typical symptoms and the NC should give no symptoms in the pathogenicity test.

2.6. Record the suspect colonies as positive if greasy lesions are observed.

REFERENCES

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