International Rules for Seed Testing 2027

Validated Seed Health Testing Methods

7-020: Detection of *Xanthomonas hortorum* pv. *carotae* in *Daucus carota* (carrot) seed

Including changes and editorial corrections adopted at the Ordinary General Meeting 2026 in Calgary, Canada

Effective from 1 January 2027

Validation reports

See References. Copies are available by e-mail from the ISTA Secretariat at ista.office@ista.ch.

Please send comments, suggestions or reports of problems relating to this method to the ISTA Seed Health Committee, c/o ISTA Secretariat.

Disclaimer

Whilst ISTA has taken care to ensure the accuracy of the methods and information described in this method description, ISTA shall not be liable for any loss or damage, etc. resulting from the use of this method.

Safety precautions

Ensure you are familiar with hazard data and take appropriate safety precautions, especially during weighing out of ingredients. It is assumed that persons carrying out this test are in a laboratory suitable for carrying out microbiological procedures and familiar with the principles of Good Laboratory Practice, Good Microbiological Practice, and aseptic techniques. Dispose of all waste materials in an appropriate way (e.g. autoclaving, disinfection) and in accordance with local health, environmental and safety regulations.

Note on the use of the translations

The electronic version of the International Rules for Seed Testing includes the English, French, German and Spanish versions. If there are any questions on interpretation of the ISTA Rules, the English version is the definitive version.

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7-020: Detection of *Xanthomonas hortorum* pv. *carotae* in *Daucus carota* (carrot) seed

Host: *Daucus carota* L.
Pathogen(s): *Xanthomonas hortorum* pv. *carotae* (Kendrick) Vauterin, Hoste, Kersters & Swings, syn. *X. campestris* pv. *carotae* (Kend) Dye

Prepared by: International Seed Health Initiative for Vegetable Crops, ISF (ISHI-Veg)

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Revision history

Version 1.0, 2005-07-01
Version 1.1, 2010-01-01: Editorial change: correction of autoclaving pressures
Version 1.2, 2013-01-01: Definition of sample size
Version 1.3, 2014-01-01: Common name of host added
Version 1.4, 2021-01-01: Safety precautions added; Sample size and Methods revised
Version 1.5, 2024-01-01: Sample size, Methods and General methods revised
Version 1.6, 2025-01-01: Sample size, Materials and Methods revised
Version 1.7, 2027-01-01: Flowchart added in Methods

Background

The most commonly used method in seed health testing laboratories for the detection of *Xanthomonas hortorum* pv. *carotae* is based on a seed wash dilution-plating assay. This method involves washing seeds in buffer and plating serial dilutions of the extract on a semi-selective medium. Various semi-selective media are currently used as described or adapted from the following papers: Cubeta & Kuan (1986); Williford & Schaad (1984); Kim *et al.* (1982); and McGuire *et al.* (1982). These media have been tested by ISHI-Veg and ISHI-Veg/ISTA in a number of comparative studies (Asma, 1999; Asma, 2000a and Asma, 2000b). In addition to comparing selective media the latter comparative study (Asma, 2000b) concluded that the confirmation method chosen had an effect on test results, with ELISA (enzyme-linked immunosorbent assay) and IF (immunofluorescence) giving false positive confirmations due to poor specificity of antisera. The 2000 study (Asma, 2000b) also looked at the effect of antibiotics and agar source on the performance of the test. Further work by Asma *et al.* (2002) has shown PCR to be a reliable and quick confirmation method when compared to pathogenicity tests.

This method is derived from the previous comparative tests and the validation studies carried out by ISHI-Veg in 2003 (Asma, 2005). For routine testing of carrot seed a combination of two semi-selective media, MKM/MD5A or MKM/mTBM is recommended. If nystatin at a concentration of 35 mg/l is not enough to completely inhibit fungal growth, cycloheximide should be used. Either a pathogenicity test or a polymerase chain reaction (PCR) test is used to confirm suspect isolates.

Safety precautions

Dispose of all waste materials in an appropriate way (e.g. autoclaving, disinfection) and in accordance with local health, environmental and safety regulations.

Ethidium bromide

Ethidium bromide is carcinogenic. If possible, use an alternative chemical e.g. Gel Red™ (Biotium). Use ethidium bromide according to safety instructions. It is recommended to work with solution instead of powder. Some considerations are mentioned below.

- Consult the Material Safety Data Sheet on ethidium bromide before using the chemical.
- Always wear personal protective equipment when handling ethidium bromide. This includes wearing a lab coat, nitrile gloves and closed toe shoes.
- Leave lab coats, gloves, and other personal protective equipment in the lab once work is complete to prevent the spread of ethidium bromide or other chemicals outside the lab.
- All work with ethidium bromide is to be done in an 'ethidium bromide' designated area in order to keep ethidium bromide contamination to a minimum.

Ultraviolet light

Ultraviolet (UV) light must not be used without appropriate precautions. Ensure that UV protective eyewear is utilised when working with ethidium bromide.

Treated seed

Chemical seed treatments may affect the performance of this test. It should only be performed on untreated seed.

Sample size

The sample size (total number of seeds to be tested) and subsample size depend on intended use, the maximum acceptable infection level and the analytical sensitivity of the method. The minimum recommended working sample size is 30 000 seeds and the maximum subsample size must be 10 000 seeds.

Materials

Reference material: known strain of *Xanthomonas hortorum* pv. *carotae* (*X. hortorum* pv. *carotae*) or standardised reference material

Plates of MKM medium: 90 mm Petri dishes (8 plates of MKM medium per subsample + controls)

Plates of MD5A medium or mTBM medium: 90 mm Petri dishes (8 plates of MD5A or 8 plates of mTBM medium per subsample + controls)

Plates of YDC (yeast dextrose chalk) medium: for subculture (at least 1 plate per subsample)

Conical flasks: of sterile saline (0.85 % NaCl) plus Tween™ 20 (0.02 % – 20 µl per 100 ml) for soaking of seeds (10 ml per 1000 seeds)

Dilution bottles: containing 4.5 ml of sterile saline (2 per subsample); other volumes may be acceptable, see General methods

70 % ethanol: for disinfection of surfaces, equipment

Incubator: operating at 28 °C

Balance: capable of weighing to the nearest 0.001 g

pH meter: capable of being read to the nearest 0.01 pH unit

Automatic pipettes: capable of pipetting to the nearest 0.001 ml; check accuracy and precision regularly

Carrot seedlings: use a cultivar proven to be susceptible to all races of the pathogen (e.g. 'Napoli') for pathogenicity test

PCR Primers (Meng *et al.*, 2004):
3Sforw 5' CAT.TCC.AAG.AAG.CAG.CCA 3'
3Srev 5' TCG.CTC.TTA.ACA.CCG.TCA 3'

Universal Primers (adapted from Eden *et al.*, 1991):
1052F 5' GCA.TGG.TTG.TCG.TCA.GCT.CGT 3'
Bac R 5' TAC.GGC.TAC.CTT.GTT.ACG.ACT.T 3'

Thermal cycler: ramping time 3 °C/s

Agarose electrophoresis equipment

Orbital shaker

Sterile pipette tips

Sterile bent glass rods

Sample preparation

This can be done in advance of the assay.

It is vital to exclude any possibility of cross-contamination between seed samples. It is therefore essential to disinfect all surfaces, containers, hands, etc. both before and after handling each sample. This can achieved by swabbing/spraying equipment and gloved hands with 70 % ethanol.

If the submitted sample is received in several packets, these should be combined by emptying into a new, clean polythene bag and mixing by hand to give a composite sample.

1. Count the number of seeds in a known weight. Estimate the thousand-seed weight (TSW) as:
   TSW = (weight of seeds / number of seeds) × 1000
2. Based on the estimated TSW, weigh out subsamples of the required size into new, clean polythene bags or containers.

Methods

Critical control points are indicated by CCP.

1. Extraction

1.1 Suspend seeds in sterile saline plus Tween™ 20 (0.02 % v/v) in a conical flask. The volume of saline should be adjusted according to the number of seeds used (10 ml per 1000 seeds).

1.2 Soak subsamples overnight (16–18 h) at 4–7 °C.

1.3 Shake for 5 min at room temperature (20–25 °C) on an orbital shaker set at 200 rpm.

2. Dilution and plating

2.1 Shake the flasks to mix just before dilution.

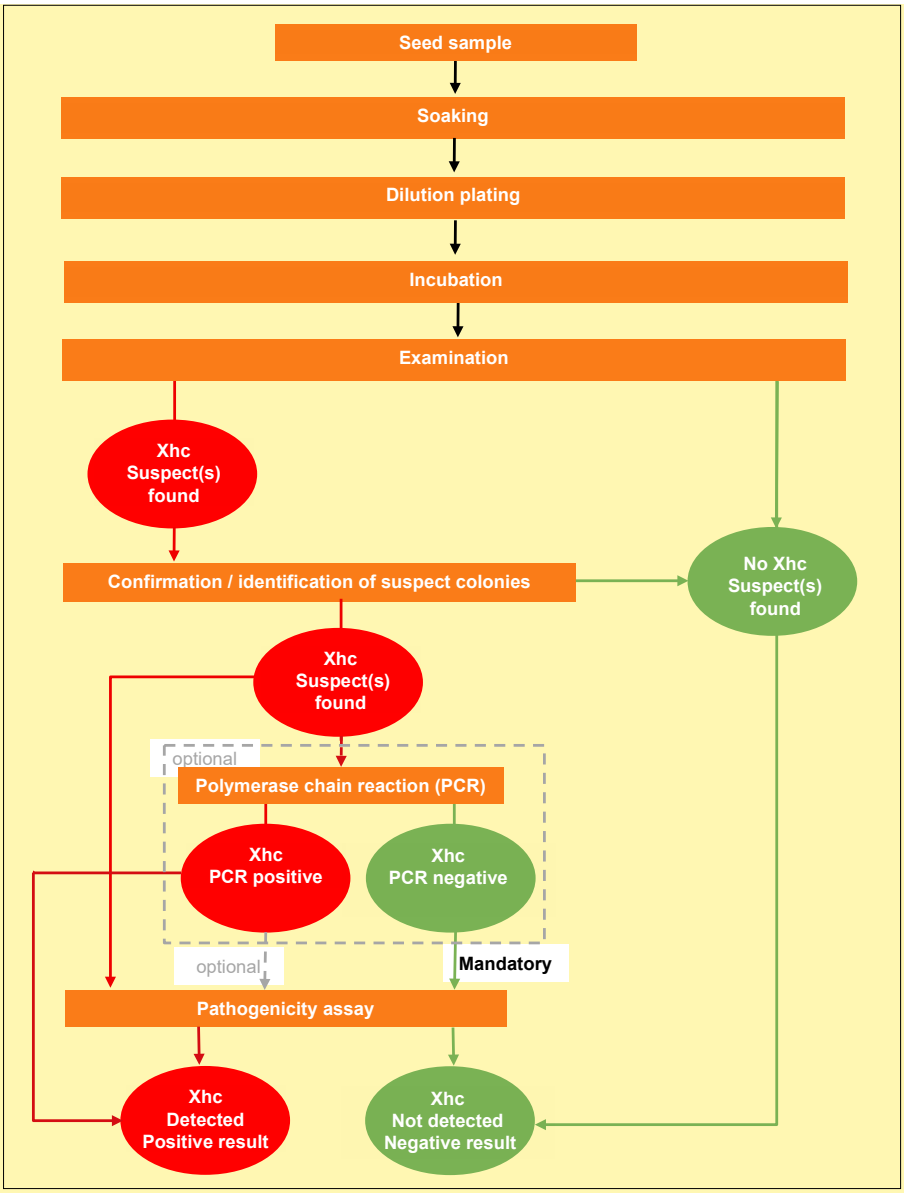
2.2 Prepare a tenfold dilution series from the seed extract. Pipette 0.5 ml of the extract into 4.5 ml of sterile saline and vortex to mix (10^{-1} dilution). Pipette 0.5 ml of the 10^{-1} dilution into another 4.5 ml of sterile saline and vortex to mix (10^{-2} dilution). Pipette 0.5 ml of the 10^{-2} dilution into another 4.5 ml of sterile saline and vortex to mix (10^{-3} dilution) (see General methods).

2.3 Pipette 100 µl of each dilution and the undiluted seed extract onto two plates of each of the two chosen semi-selective media either MKM medium with MD5A medium or MKM medium with mTBM medium and spread over the surface with a sterile bent glass rod (see General methods).

2.4 Incubate plates with positive control plates (Section 3) at 28 °C and examine after 4–8 d.

3. Positive control (culture or reference material)

3.1 Prepare a suspension of a known strain of *X. hortorum* pv. *carotae*, e.g. NCPPB 425, in sterile saline or reconstitute standardised reference material according to the supplier's instructions. If a lyophilised culture is



used, culture at least once on a non-selective medium prior to use to check the viability and morphology.

3.2 Dilute sufficiently to obtain dilutions containing approximately 10^{-2} to 10^{-4} colony-forming units (cfu) per millilitre. This may require up to seven ten-fold dilutions from a turbid suspension.

3.3 Pipette 100 µl of appropriate dilutions onto plates of both semi-selective media (MKM/MD5A or MKM/mTBM) and spread over the surface with a sterile bent glass rod.

3.4 Incubate plates with the sample plates (Section 2).

4. Sterility check

4.1 Prepare a dilution series from a sample of the extraction medium (i.e., saline plus Tween™ 20), containing no seeds, and plate on both semi-selective media as for samples.

5. Examination of the plates

5.1 Examine sterility check and positive control plates (CCP).

5.2 Examine the sample plates for the presence of typical *X. hortorum* pv. *carotae* colonies by comparison with the positive control plates.

5.3 On MKM after 4–6 d, *X. hortorum* pv. *carotae* colonies appear light yellow-cream, light brown to peach yellow, glistening, round and 2–4 mm in diameter (Fig. 1).

5.4 On MD5A after 7–8 d, *X. hortorum* pv. *carotae* colonies appear straw yellow, glistening, round smooth, convex with entire margins, and 2–3 mm in diameter (Fig. 2).

5.5 On mTBM after 7–8 d, *X. hortorum* pv. *carotae* colonies appear white or yellow or white-yellow, glistening, round, smooth, convex with entire margins,

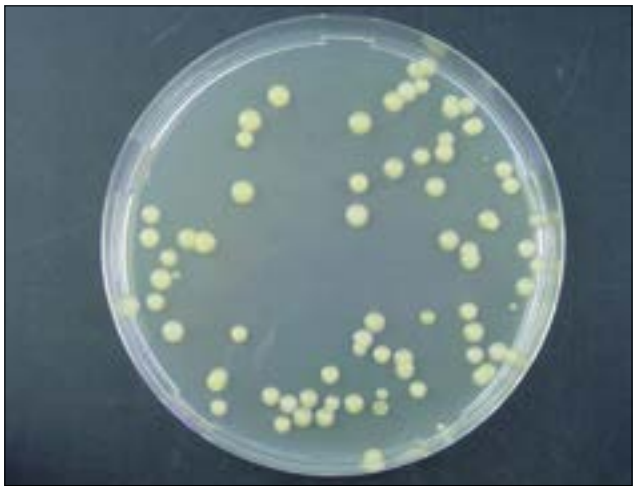


Figure 1. *Xanthomonas hortorum* pv. *carotae* colonies on MKM plates after 6 d indicated by light yellow-cream, light brown to peach yellow, glistening, round colonies and 2–4 mm in diameter.

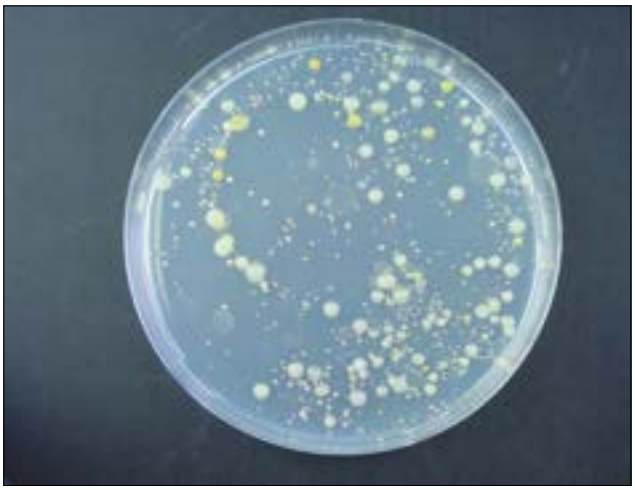


Figure 2. *Xanthomonas hortorum* pv. *carotae* colonies on MD5A plates after 7 d indicated by straw yellow, glistening, round smooth, convex colonies with entire margins, and 2–3 mm in diameter.

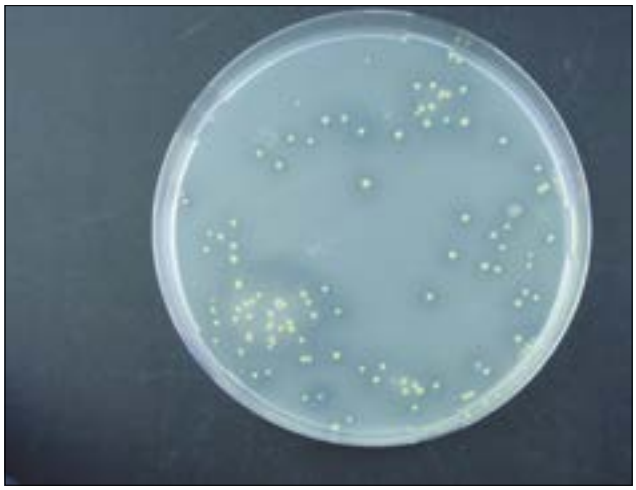


Figure 3. *Xanthomonas hortorum* pv. *carotae* colonies on mTBM plates after 7 d indicated by white or yellow or white-yellow, glistening, round, smooth, convex colonies with entire margins, 1–2 mm in diameter and surrounded by a zone of casein hydrolysis.



Figure 4. Typical pale yellow and mucoid growth of *Xanthomonas hortorum* pv. *carotae* isolates on a sectored plate of YDC after 72 h at 28 °C.

1–2 mm in diameter and surrounded by a large clear zone of casein hydrolysis (Fig. 3). Casein hydrolysis on mTBM is not always present.

5.6 The colony size and colour can differ within a sample.

5.7 Record the presence of suspect colonies (see General methods). If necessary, estimate the number of cfu of suspect and other colonies.

6. Confirmation/identification of suspect colonies

6.1 Subculture suspect colonies to sectored plates of YDC. To avoid the potential for cross-contamination of isolates, use a new sectored plate for each subsample. The precise numbers of colonies subcultured will depend on the number and variability of suspect colonies on the plate: if present, at least six colonies should be subcultured per subsample.

6.2 Subculture the positive control isolate to a sectored plate for comparison.

6.3 Incubate sectored plates for 48–72 h at 28 °C.

6.4 Compare appearance of growth with positive control. On YDC *X. hortorum* pv. *carotae* colonies are pale yellow and mucoid (Fig. 4).

6.5 Confirm the identity of isolates by pathogenicity on carrot seedlings of known susceptibility or by polymerase chain reaction (PCR).

6.6 Record results for each colony subcultured.



Figure 5. Typical *Xanthomonas hortorum* pv. *carotae* symptoms in a pathogenicity test indicated by small brown irregular areas surrounded by a yellow halo.

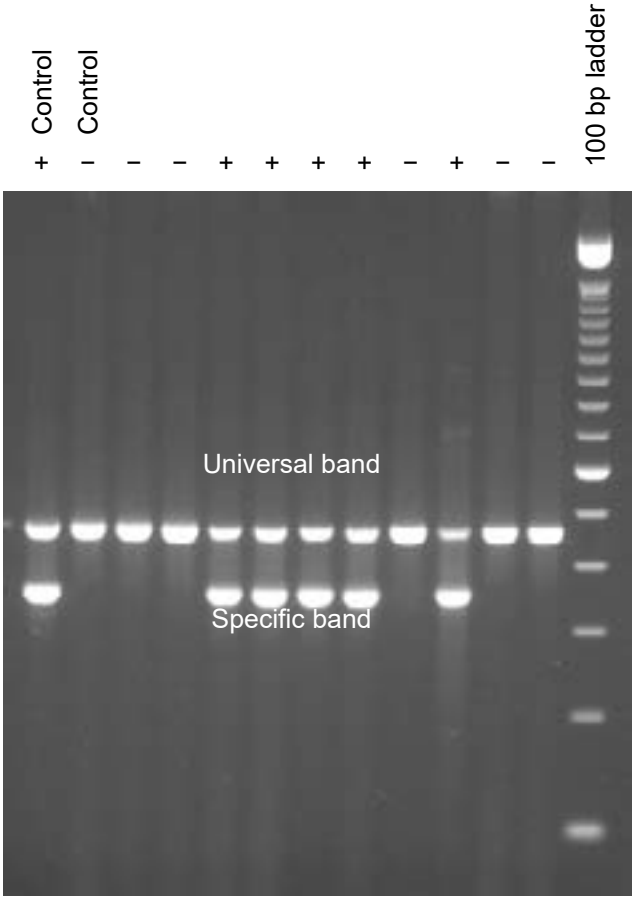


Figure 6. Agarose gel showing *Xanthomonas hortorum* pv. *carotae* specific products of 355 bp and universal bacterial products of 441 bp. Two bands (specific and universal) = positive identification; one band (universal) = negative identification.

6.7 All negative PCR results must be confirmed with a pathogenicity test in order for the PCR results to be valid. All positive PCR results are valid and a pathogenicity test is optional.

7. Pathogenicity (CCP)

7.1 Grow seedlings of a carrot cultivar known to be susceptible to *X. hortorum* pv. *carotae* (e.g. 'Napoli') in small pots or modules until at least 3–4 true leaf stage (approximately 3–4 weeks after sowing).

7.2 Prepare a suspension in sterile tap water from each suspect bacterial culture on YDC medium and dilute to a concentration containing approximately 2×10^6 cfu/ml. The same procedure should be used for the positive control isolate.

7.3 Inoculate plants by spraying until runoff. Use one small pot with 3–4 plants per isolate. Include the positive control and a negative control. It is important not to rub the leaves after spraying, since this will cause false positive results (CCP).

7.4 Incubate inoculated plants at 27–28 °C enclosed in plastic bags (to provide conditions near 100 % RH). After 48 h, remove the bags during daytime and replace at night.

7.5 Record symptoms after 7–10 d incubation. Typical *X. hortorum* pv. *carota*e symptoms first appear as small irregular yellowish water-soaked areas with a tiny light brown spot in the centre on inoculated leaves. Later, affected areas enlarge, become brown, and are often surrounded by a yellow halo (Fig. 5). Compare with positive control (CCP).

8. Polymerase chain reaction (PCR)

8.1 Make a slightly turbid cell suspension ($OD_{600\ nm}$ approximately 0.05) in 1.0 ml sterile distilled/deionised water from the suspect cultures on YDC medium and the positive control. In addition a non-suspect isolate should be used as a negative control. The suspensions can be stored at –20 °C until identification.

8.2 Use the following *X. hortorum* pv. *carotae* specific primers (Meng *et al.*, 2004):
3Sforw 5' CAT.TCC.AAG.AAG.CAG.CCA 3'
3Srev 5' TCG.CTC.TTA.ACA.CCG.TCA 3'

8.3 Universal bacterial Primers should be used to validate the PCR reaction. These primers will give a product size of 441 bp (adapted from Eden *et al.*, 1991) compared to the 355 bp product from the *X. hortorum* pv. *carotae* specific primers:
1052F 5' GCA.TGG.TTG.TCG.TCA.GCT.CGT 3'
Bac R 5' TAC.GGC.TAC.CTT.GTT.ACG.ACT.T 3'

8.4 Prepare the reaction mixture (page 7-020–17). Carry out PCR reactions in 0.2 ml thin walled PCR tubes in a final volume of 10 µl (8 µl reaction mixture + 2 µl bacterial suspension).

8.5 PCR profile: An initial 5 min incubation at 95 °C followed by 35 cycles of 15 s at 94 °C, 15 s at 58 °C and 30 s at 72 °C. A final 5 min incubation at 72 °C and 20 min at 20 °C

8.6 Fractionate 10 µl of the PCR products, the negative process control and sterile water (negative PCR control) by gel electrophoresis during 1.5 h at 150 V on a 1.5 % agarose gel in 0.5× Tris borate EDTA (TBE buffer) stained with ethidium bromide. Include a 100 bp ladder.

8.7 Analyse the amplification products for a *X. hortorum* pv. *carotae* specific product of 355 bp and a universal bacterial product of 441 bp with an ultraviolet transilluminator. Two bands (specific and universal) = positive identification; one band (universal) = negative identification; no bands = bacterial template absent, repeat reaction.

General methods

Preparation of ten-fold dilution series: Each dilution should be prepared by pipetting 0.5 ml (±5 %) from a well-mixed seed extract or previous dilution into a universal bottle (screw-capped) or similar containing 4.5 ml (±2 %) of sterile diluent and then vortexing to mix prior to the next dilution step. A new sterile pipette tip should be used for each dilution step. Pipettes should be checked regularly for accuracy and precision and re-calibrated as necessary. It is acceptable to prepare ten-fold dilutions using other volumes provided that the laboratory can demonstrate that the required accuracy and precision can be achieved.

Plating of dilutions: This should be done as soon as possible after dilutions have been prepared and certainly within 30 min. Working from the highest (most dilute) dilution to the undiluted extract, 0.1 ml is pipetted onto the centre of a surface-dry, labelled agar plate. The liquid should then be spread evenly over the entire surface of the medium with a bent glass rod. If care is taken to work from the highest to the lowest dilution (or undiluted extract) a single pipette tip and a single bent glass rod can be used for each sample. Ensure that all liquid has been absorbed by the agar before inverting and incubating plates. If necessary allow plates to dry under a sterile air-flow in a microbiological safety cabinet or laminar flow hood.

Recording of dilution plates: Record the results for all dilution plates. The most accurate estimate of bacterial numbers should be obtained from spread plates with total number between 30 and 300 colonies. However this may be further complicated depending on the relative numbers of suspect pathogen and other colonies. In order to minimise effort, start recording with the highest dilution (most dilute) and count the number of suspect and the number of other colonies. If the total number of colonies on a plate greatly exceeds 300 there is little value in trying to make a precise count if a more reliable count has already been obtained from a more dilute plate, in which case it is sufficient to record the number of colonies as 'm' (many) if they are still separate or 'c' (confluent) if they have run together.

Sectored plates: Using a laboratory marker pen, draw lines on the base of a standard 90 mm plate (Petri dish) to divide it into six equal sectors. Subculture single colonies from dilution plates and make a single zigzagged streak within a single sector on the plate. Take care to leave sufficient space between each isolate to ensure the growth does not coalesce. Thus six suspect colonies can be subcultured to each sectored plate. Separate plates should be used for each sample/subsample. If the purity of subcultured isolates is doubtful, they should be further streaked out on whole plates.

Reporting results: The result of a seed health test should indicate the scientific name of the pathogen detected and the test method used. When reported on an ISTA Certificate, results are entered under 'Other Determinations'.

The report must indicate the number of seeds tested.

In the case of a negative result (pathogen not detected in any subsample), the results must be reported as 'not detected'.

In the case of a positive result, the report must indicate the number of positive subsamples out of the total number tested. The number of cfu can be indicated.

Quality assurance

A record should be kept of the date and results of pipette calibration checks.

It is essential that operators have received appropriate training and use automatic pipettes correctly.

Critical control points (CCP)

- Dilution plates prepared from positive control isolate(s) or reference material, should give single colonies with typical morphology (Step 5.1).
- The numbers of colonies on dilution plates prepared from the positive control isolate(s) or reference material should be similar on both media (Step 5.1).
- Numbers of bacteria on dilution plates should be consistent with the dilution (i.e. should decrease approx. ten-fold with each dilution)(Step 5.1).
- There should be no growth on dilution plates prepared as a sterility check (Step 5.1).
- Most cultivars of bunching carrots are susceptible to *X. hortorum*. pv. *carotae* (Step 7.1).
- Positive control isolates should be included in every pathogenicity test (Step 7.3).
- The positive control isolate should give typical symptoms in a pathogenicity test (Step 7.5).
- The activity (units/mg) of some antibiotics may vary between batches. It may be necessary to adjust the weight or volume added to ensure that the final number of units per litre of medium is consistent (MKM, MD5A).

Media and solutions

Sterile saline

Compound	Amount/l	Amount/500 ml
Sodium chloride (NaCl)	8.5 g	4.25 g
Distilled/deionised water	1000 ml	500 ml

Preparation

1. Weigh out all ingredients into a suitable container.
2. Add 1000 ml (or 500 ml) of distilled/deionised water.
3. (For extraction of seeds, add 200 µl of sterile Tween™ 20 per 1000 ml).
4. Dissolve and dispense into final containers.
5. Autoclave at 121 °C and 15 psi for 15 min.

Storage

Provided containers are tightly closed, may be stored for several months before use.

MKM medium

Note: This medium is a modification of the KM-1 medium (Kim *et al.*, 1982) from which it differs in antibiotics composition and concentration. The amounts of phosphate salts have also been adjusted to achieve the correct pH without further adjustment.

Compound	Amount/l	Amount/500 ml
NH_4Cl	1.0 g	0.5 g
K_2HPO_4	1.2 g	0.6 g
KH_2PO_4	1.2 g	0.6 g
Lactose monohydrate	10.0 g	5.0 g
D(+) Trehalose dihydrate	4.0 g	2.0 g
Yeast extract	0.5 g	0.25 g
2-Thiobarbituric acid	0.2 g	0.1 g
Agar (BD Bacto™ Agar)	17.0 g	8.5 g
Tobramycin sulphate[a]	0.002 g	0.001 g
Cephalexin monohydrate[b]	0.010 g	0.005 g
Bacitracin[c]	0.050 g	0.025 g
Nystatin[d]	0.035 g	0.018 g

[a–d] Added after autoclaving. Amounts for antibiotics and other additions are for guidance only (CCP).

[a] Dissolve 20 mg tobramycin sulphate (Sigma T-1783 or Duchefa T-0153) in 10 ml 70 % ethanol. Add 1 ml/l (0.5 ml/500 ml).

[b] Dissolve 200 mg cephalexin monohydrate (Sigma C-4895) in 10 ml 70 % ethanol. Add 0.5 ml/l (0.25 ml/500 ml).

[c] Dissolve 500 mg bacitracin (Sigma B-0125 66K units/g or Duchefa B-0106, 70 K units/g) in 10 ml 70 % ethanol. Add 1.0 ml/l (0.5 ml/500 ml).

[d] Dissolve 100 mg nystatin (Sigma N-3503, Duchefa N-0138) in 10 ml 70 % ethanol. Add 3.5 ml/l (1.75 ml/500 ml). Use 100 mg/l cycloheximide, instead of nystatin, when fungal growth on the selective media is not completely inhibited by 35 mg/l nystatin.

Preparation

1. Weigh out all ingredients except antibiotics into a suitable container.
2. Add 1000 ml (or 500 ml) of distilled/deionised water.
3. Dissolve and check pH which should be 6.6.
4. Autoclave at 121 °C and 15 psi for 15 min.
5. Prepare antibiotic solutions.
6. Allow medium to cool to approximately 50 °C and add antibiotic solutions.
7. Mix thoroughly but gently by inversion/swirling to avoid air bubbles and pour plates (22 ml per 90 mm plate).
8. Leave plates to dry in a laminar flow bench or similar before use.

Storage

Store prepared plates inverted in polythene bags at 4 °C and use within two weeks of preparation to ensure activity of antibiotics.

MD5A medium

(Cubeta & Kuan, 1986)

Compound	Amount/l	Amount/500 ml
$MgSO_4 \cdot 7H_2O$	0.3 g	0.15 g
NaH_2PO_4	1.0 g	0.5 g
NH_4Cl	1.0 g	0.5 g
K_2HPO_4	3.0 g	1.5 g
Agar (BD Bacto™ Agar)	17.0 g	8.5 g
Cellobiose[a]	10.0 g	5.0 g
L-glutamic acid[b]	0.005 g	0.0025 g
L-methionine[c]	0.001 g	0.0005 g
Cephalexin monohydrate[d]	0.01 g	0.005 g
Bacitracin[e]	0.01 g	0.005 g
Nystatin[f]	0.035 g	0.018 g

[a–f] Added after autoclaving. Amounts for antibiotics and other additions are for guidance only (CCP)

[a] Dissolve 10 g cellobiose in 100 ml of distilled/deionised water and filter sterilise.

[b] Dissolve 50 mg L-glutamic acid in 10 ml distilled/deionised water and filter sterilise. Add 1.0 ml/l (0.5 ml/500 ml).

[c] Dissolve 10 mg L-methionine in 10 ml distilled/deionised water and filter sterilise. Add 1.0 ml/l (0.5 ml/500 ml).

[d] Dissolve 200 mg cephalexin monohydrate (Sigma C-4895) in 10 ml 70 % ethanol. Add 0.5 ml/l (0.25 ml/500 ml).

[e] Dissolve 500 mg bacitracin (Sigma B-0125, 66K units/g or Duchefa B-0106, 70 K units/g) in 10 ml 70 % ethanol. Add 0.2 ml/l (0.1 ml/500 ml).

[f] Dissolve 100 mg nystatin (Sigma N-3503, Duchefa N-0138) in 10 ml 70 % ethanol. Add 3.5 ml/l (1.75 ml/500 ml). Use 100 mg/l cycloheximide, instead of nystatin, when fungal growth on the selective media is not completely inhibited by 35 mg/l nystatin.

Preparation

1. Weigh out all ingredients except antibiotics, L-glutamic acid, L-methionine and cellobiose into a suitable container.
2. Add 900 ml (or 450 ml) of distilled/deionised water.
3. Dissolve and check pH which should be 6.4, adjust if necessary.
4. Autoclave at 121 °C and 15 psi for 15 min.
5. Prepare antibiotic, L-glutamic acid, L-methionine and cellobiose solutions.
6. Allow medium to cool to approximately 50 °C before adding solutions.
7. Mix thoroughly but gently by inversion/swirling to avoid air bubbles and pour plates (22 ml per 90 mm plate).
8. Leave plates to dry in a laminar flow bench or similar before use.

Storage

Store prepared plates inverted in polythene bags at 4 °C and use within two weeks of preparation to ensure activity of antibiotics.

mTBM medium

Note: This medium is a modification of Tween™ Medium B (McGuire *et al.*, 1986) from which it differs in adding 10.0 g/l skim milk powder and leaving out 0.4 mg/l tobramycine and 0.25 g/l $CaCl_2$.

Compound	Amount/l	Amount/500 ml
H_3BO_3	0.3 g	0.15 g
KBr	10.0 g	5.0 g
Peptone	10.0 g	5.0 g
Skim milk powder[a]	10.0 g	5.0 g
Agar (BD Bacto™ Agar)	17.0 g	8.5 g
Tween™ 80[b]	10.0 ml	5.0 ml
Cephalexin monohydrate[c]	0.065 g	0.033 g
5-Fluorouracil[d]	0.012 g	0.006 g
Nystatin[e]	0.035 g	0.018 g

[a–c, d, e] Added after autoclaving. Amounts for antibiotics and other additions are for guidance only (CCP)

[a] Autoclave solution (10.0 g/100 ml) separate. The quality of skim milk powder greatly affects the capabilities of mTBM. Milk sources that work well are BBL, Oxoid or Sigma.

[c] Dissolve 200 mg cephalexin monohydrate (Sigma C-4895) in 10 ml 70 % ethanol. Add 3.25 ml/l (1.625 ml/500 ml).

[d] Dissolve 100 mg 5-fluorouracil (Sigma F-6627, Duchefa F-0123) in 10 ml 70 % ethanol. Add 1.2 ml/l (0.6 ml/500 ml).

[e] Dissolve 100 mg nystatin (Sigma N-3503, Duchefa N-0138) in 10 ml 70 % ethanol. Add 3.5 ml/l (1.75 ml/500 ml). Use 100 mg/l cycloheximide, instead of nystatin, when fungal growth on the selective media is not completely inhibited by 35 mg/l nystatin.

Preparation

1. Weigh out all ingredients except skim milk powder, antibiotics and Tween™ 80 into a suitable container.
2. Add 900 ml (or 450 ml) of distilled/deionised water.
3. Dissolve and check pH which should be 7.4, adjust if necessary.
4. Autoclave at 121 °C and 15 psi for 15 min.
5. Prepare antibiotic and skim milk powder solutions.
6. Allow medium to cool to approximately 50 °C before adding skim milk powder, antibiotic solutions and Tween™ 80.
7. Mix thoroughly but gently by inversion/swirling to avoid air bubbles and pour plates (22 ml per 90 mm plate).
8. Leave plates to dry in a laminar flow bench or similar before use.

Storage

Store prepared plates inverted in polythene bags at 4 °C and use within two weeks of preparation to ensure activity of antibiotics.

Yeast extract dextrose chalk (YDC) medium

(Schaad, 1988)

Compound	Amount/l	Amount/500 ml
Agar (BD Bacto™ Agar)	17.0 g	8.5 g
Yeast Extract	10.0 g	5.0 g
$CaCO_3$ (light powder)	20.0 g	10.0 g
D-Glucose (dextrose)	20.0 g	10.0 g
Distilled/deionised water	1000 ml	500 ml

Preparation

1. Weigh out all ingredients into a suitable oversize container (i.e. 250 ml of medium in a 500 ml bottle/flask) to allow swirling of medium just before pouring.
2. Add 1000 ml (or 500 ml) of distilled/deionised water.
3. Autoclave at 121 °C and 15 psi for 15 min.
4. Allow medium to cool to approx. 50 °C .
5. Swirl to ensure even distribution of $CaCO_3$ and avoid air bubbles, and pour plates (22 ml per 90 mm plate).
6. Leave plates to dry in a laminar flow bench or similar before use.

Storage

Store prepared plates inverted in polythene bags at room temperature. Prepared plates can be stored for several months provided they do not dry out.

Reaction mixture for PCR

Compound	Final concentration	Volume (µl) in 10 µl
Sterile MilliQ		3.42
10× buffer	1×	1.00
$MgCl_2$ (50 mM)	1.5 mM	0.30
dNTPs (10 mM total, 2.5 mM each)	200 µM each	0.80
Primer 3Sforw (5 pmol/µl)	0.50 µM	1.00
Primer 3Srev (5 pmol/µl)	0.50 µM	1.00
Primer 1052F (5 pmol/µl)	0.10 µM	0.20
Primer BacR (5 pmol/µl)	0.10 µM	0.20
Taq Polymerase (5U/µl)	0.04 U/µl	0.08
Bacterial suspension		2.00

10× Buffer

Tris HCL(pH 9.0): 750 mM
$(NH_4)_2SO_4$: 200 mM
Tween™ 20: 0.1 % (v/v)

Tris borate EDTA (TBE) buffer 0.5×

Compound	Amount/l	Amount/500 ml
Tris	5.39 g	2.70 g
Boric acid	2.75 g	1.38 g
$Na_2EDTA \cdot 2H_2O$	0.37 g	0.19 g

The pH is 8.3 and requires no adjustment.

1.5 % agarose gel for electrophoresis

Compound	1 agarose gel (20×20 cm)	Amount/l
Tris borate EDTA (TBE) 0.5×	160 ml	1000 ml
Agarose	2.4 g	15.0 g
Ethidium bromide[a]	8.0 µl	50.0 µl

[a] Dissolve 100 mg ethidium bromide in 10 ml distilled/deionised water. Add 50 µl/l of this solution. Ethidium bromide is carcinogenic!

Preparation

1. To calculate the volume of the gel (in ml), multiply the area of the gel by the required thickness (0.4 cm).
2. Make sure that the gel tray is clean and dry before use. Seal the ends of the tray with tape.
3. Weigh out the desired amount of agarose and place in an Erlenmeyer flask with a measured amount of electrophoresis buffer, e.g. for a 100 ml gel add 1.5 g of agarose and 100 ml of 0.5× TBE buffer to a 200 ml

flask. The larger flask insures against the agarose boiling over.
4. Dissolve the agarose in a boiling water bath or in a revolving-plate microwave oven. All the grains of agarose should be dissolved and the solution clear.
5. Allow the medium to cool to approx. 60 °C before adding ethidium bromide[a] stock solution, mix well and pour a gel with 0.4 cm thickness immediately. Wear suitable gloves when adding ethidium bromide.
6. Place the gel comb(s) in position in the gel tray.
7. After the gel is completely set (approximately 30 min at room temperature) carefully remove the gel comb(s).
8. Remove the tape from the ends of the gel tray and put the tray in position in a agarose electrophoresis unit. For electrophoresis in a 'submarine' mode, pour enough electrophoresis buffer into the apparatus to cover the gel to a depth of at least 1 mm.
9. The same electrophoresis buffer used in the gel must also be used for the running buffer.

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