



International Rules for Seed Testing
Annexe to Chapter 7: Seed Health Testing Methods



7-020: Detection of *Xanthomonas hortorum* pv. *carotae* on *Daucus carota*

Published by: International Seed Testing Association (ISTA), Bassersdorf, Switzerland
2010

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 damage etc., resulting from the use of this method.

Crop: *Daucus carota* (carrot)

Pathogen: *Xanthomonas hortorum* pv. *carotae*
(bacterial leaf blight)

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Sponsored by: ISHI-Veg

Revision History: Version 1.0, 1 July 2005
Version 1.1, 2010-01-01
Editorial change: correction of autoclaving pressures

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 and Kuan, (1986); Williford and 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 and IF 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 PCR test is used to confirm suspect isolates.

Validation Studies

Asma, M. (2005)

Copies are available: by E-mail from ista.office@ista.ch; by mail from the ISTA Secretariat, Zürichstrasse 50, 8303 Bassersdorf, Switzerland.

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

Safety Precautions

Ensure you are familiar with hazard data and take appropriate safety precautions, especially during preparation of media, autoclaving, and weighing out of ingredients and handling of ethidium bromide. It is assumed that this procedure is being carried out in a microbiological laboratory by persons familiar with the principles of Good Laboratory Practice, Good Microbiological Practice, and aseptic technique. Dispose of all waste materials in an appropriate way (e.g. autoclave, disinfect) and in accordance with local health, environmental and safety regulations.

Treated Seed

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

Sample and sub-sample size

The sample (total number of seeds tested) and sub-sample size to be tested depends on the desired tolerance standard (maximum acceptable percentage of seeds infested) and detection limit (theoretical minimum number of pathogen propagules per seed which can be detected); in any case the maximum sub-sample size should be 10,000 seeds. A full discussion of these aspects can be found in Geng *et al.* (1987), Roberts *et al.* (1993) and Roberts (1999).

Materials

Reference material	-	known strain of <i>Xanthomonas hortorum</i> pv. <i>carotae</i> (<i>X. hortorum</i> pv. <i>carotae</i>) or standardised reference material
Plates of MKM medium	-	9.0 cm Petri dishes (8 plates of MKM medium per sub-sample + controls)
Plates of MD5A medium or mTBM medium	-	9.0 cm Petri dishes (8 plates of MD5A or 8 plates of mTBM medium per sub-sample + controls)
Plates of YDC	-	for sub-culture (at least 1 plate per sub-sample)
Conical flasks	-	of sterile saline (0.85% NaCl) plus Tween 20 (0.02% - 20µl per 100 ml) for soaking of seeds (10 ml per 1000 seeds)
Dilution bottles	-	containing 4.5 ml of sterile saline (2 per sub-sample); 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	-	susceptible to the pathogen for pathogenicity test e.g. cv. Napoli
PCR Primers (Meng <i>et al.</i> , 2004)	-	3Sforw 5' CAT.TCC.AAg.AAg.CAg.CCA 3' 3Srev 5' TCg.CTC.TTA.ACA.CCg.TCA 3'

Materials continued

Universal Primers - 1052F 5' gCA.Tgg.TTg.TCg.TCA.gCT.CgT 3'
(adapted from Eden *et al.*, 1991) 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

1. This can be done in advance of the assay.
2. 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 be achieved by swabbing/spraying equipment and gloved hands with 70% ethanol.
3. 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.
4. Count the number of seeds in a known weight. Estimate the Thousand Seed Weight (TSW) as:

$$TSW = \frac{\text{Weight of seed}}{\text{Number of seed}} \times 1000$$

5. Based on the TSW, weigh out sub-samples of the required size into new, clean polythene bags.

Method

[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 1,000 seeds).
 - 1.2. Soak sub samples 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 (10¹ dilution). Pipette 0.5 ml of the 10¹ dilution into another 4.5 ml of sterile saline and vortex to mix (10² dilution). Pipette 0.5 ml of the 10² dilution into another 4.5 ml of sterile saline and vortex to mix (10³ dilution) (see General Methods).
 - 2.3. Pipette 100 µl of each dilution and the un-diluted 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).

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- 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 10² to 10⁴ cfu/ml. 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, 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 number of suspect and other colonies (see General Methods).
 6. *Confirmation/identification of suspect colonies*
 - 6.1. Sub-culture suspect colonies to sector plates of YDC. To avoid the potential for cross-contamination of isolates, use a new sector plate for each sub-sample. The precise numbers of colonies sub-cultured will depend on the number and variability of suspect colonies on the plate: if present, at least six colonies should be sub-cultured per sub-sample.
 - 6.2. Sub-culture the positive control isolate to a sector plate for comparison.
 - 6.3. Incubate sector 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 sub-cultured.
 7. *Pathogenicity (CCP)*
 - 7.1. Grow seedlings of a carrot cultivar known to be susceptible to *X. hortorum* pv. *carotae* (e.g. cv. Napoli) in small pots or modules until at least 3-4 true leaf stage (approximately 3-4 weeks after sowing).
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- 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. *carotae* 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_{600nm} approximately 0.05) in 1.0 ml sterile distilled 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 by gel electrophoresis during 1.5 h at 150V on a 1.5% agarose gel in 0.5x 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 (common to many test procedures)

1. Preparation of ten-fold dilution series

Each dilution should be prepared by pipetting 0.5 ml ($\pm 5\%$) from a well-mixed seed extract or previous dilution into a universal bottle (screw-capped) or similar containing 4.5 ml ($\pm 2\%$) 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.

2. *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.

3. *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.

4. *Sectored Plates*

Using a laboratory marker pen draw lines on the base of a standard 9 cm plate (Petri dish) to divide it into six equal sectors. Sub-culture 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 sub-cultured to each sectored plate. Separate plates should be used for each sample/sub-sample. If the purity of sub-cultured isolates is doubtful, they should be further streaked out on whole plates.

5. *Reporting Results*

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

In the case of a negative result (pathogen not detected in any sub-samples), the results should be reported in terms of the tolerance standard and detection limit. The tolerance standard depends on the total number of seeds tested, n , and is approximately $3/n$ ($P=0.95$) (see Roberts *et al.*, 1993); the detection limit per sub-sample is equal to the detection limit per ml multiplied by the volume of extract.

In the case of a positive result, the report should indicate the mean number of pathogen propagules (cfu) per seed and either the number of positive sub-samples out of the total number tested and the sample size or the maximum likelihood estimate of the proportion of infested seeds.

Quality Assurance

General

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

[Identified by **CCP** in the methods]

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).

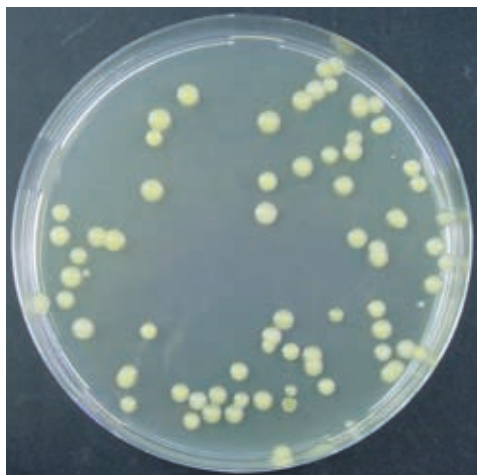


Fig. 1. *Xanthomonas hortorum* pv. *carotae* colonies on MKM plates after 6 d indicated by light yellow-cream, light brown to peach yellow, glistening, round colonies and 2-4 mm in diameter.

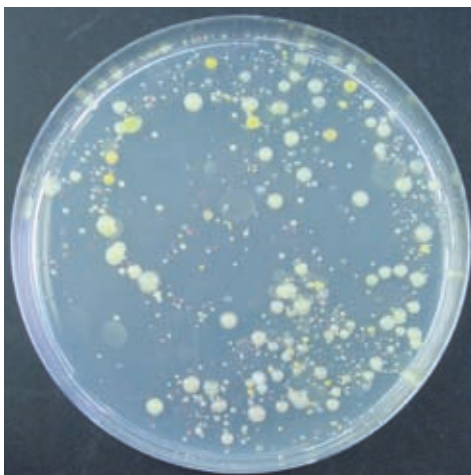


Fig. 2. *Xanthomonas hortorum* pv. *carotae* colonies on MD5A plates after 7 d indicated by straw yellow, glistening, round smooth, convex colonies with entire margins, and 2-3 mm in diameter.

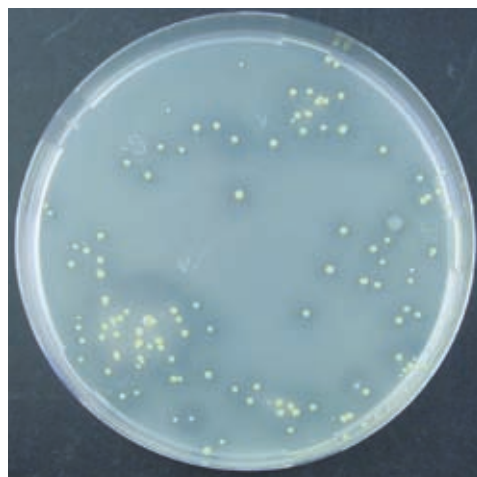


Fig. 3. *Xanthomonas hortorum* pv. *carotae* colonies on mTBM plates after 7 d indicated by white or yellow or white-yellow, glistening, round, smooth, convex colonies with entire margins, 1-2 mm in diameter and surrounded by a zone of casein hydrolysis.

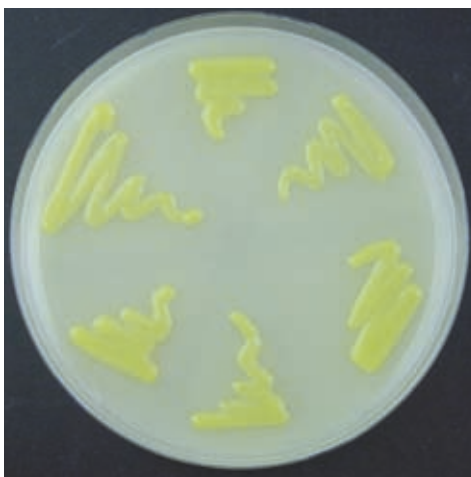


Fig. 4. Typical pale yellow and mucoid growth of *Xanthomonas hortorum* pv. *carotae* isolates on a sector plate of YDC after 72 h at 28°C.



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

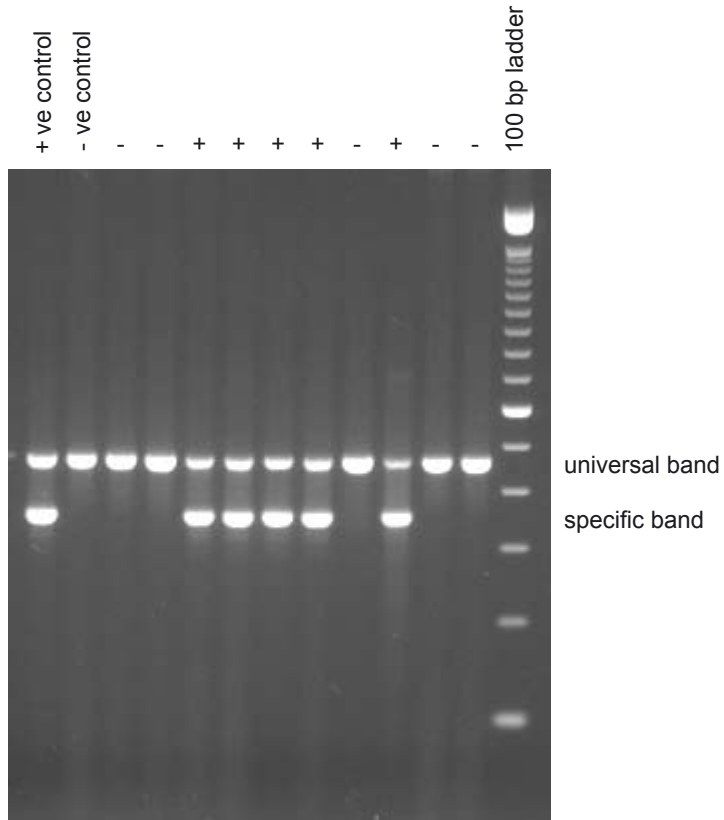


Fig. 6. Agarose gel showing *Xanthomonas hortorum* pv. *carotae* specific products of 355 bp and universal bacterial products of 441 bp. Two bands (specific and universal) = positive identification; one band (universal) = negative identification.

Preparation of sterile saline

Compound	g/l	g/500 ml
Sodium chloride (NaCl)	8.5	4.25
Distilled/de-ionised water	1000 ml	500 ml

Preparation

1. Weigh out all ingredients into a suitable container.
2. Add 1000 ml (or 500 ml) of distilled/de-ionised 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, 15 psi for 15 min.

Storage

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

Preparation of MKM agar 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	g/l	g/500 ml
NH ₄ Cl	1.0	0.5
K ₂ HPO ₄	1.2	0.6
KH ₂ PO ₄	1.2	0.6
Lactose monohydrate	10.0	5.0
D(+) trehalose dihydrate	4.0	2.0
Yeast extract	0.5	0.25
2-Thiobarbituric acid	0.2	0.1
Bacto agar	17.0	8.5
Tobramycin sulphate ^a	0.002	0.001
Cephalexin monohydrate ^b	0.010	0.005
Bacitracin ^c	0.050	0.025
Nystatin ^d	0.035	0.018

a, b, c, d Added after autoclaving

Preparation

1. Weigh out all ingredients except antibiotics into a suitable container.
2. Add 1000 ml (or 500 ml) of distilled/de-ionised water.
3. Dissolve and check pH which should be 6.6.
4. Autoclave at 121 °C, 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 9.0 cm plate).
8. Leave plates to dry in a laminar flow bench or similar before use.

Antibiotics and other additions (amounts 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.

Storage

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

Preparation of MD5A agar medium (Cubeta and Kuan, 1986)

Compound	g/l	g/500 ml
MgSO ₄ ·7H ₂ O	0.3	0.15
NaH ₂ PO ₄	1.0	0.5
NH ₄ Cl	1.0	0.5
K ₂ HPO ₄	3.0	1.5
Bacto agar	17.0	8.5
Cellobiose ^a	10.0	5.0
L-glutamic acid ^b	0.005	0.0025
L-methionine ^c	0.001	0.0005
Cephalexin monohydrate ^d	0.01	0.005
Bacitracin ^e	0.01	0.005
Nystatin ^f	0.035	0.018

a, b, c, d, e, f Added after autoclaving

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/de-ionised water.
3. Dissolve and check pH which should be 6.4, adjust if necessary.
4. Autoclave at 121 °C, 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 9.0 cm plate).
8. Leave plates to dry in a laminar flow bench or similar before use.

Antibiotics and other additions (amounts for guidance only, CCP)

- ^a Dissolve 10 g cellobiose in 100 ml of distilled/de-ionised water and filtersterilize.
- ^b Dissolve 50 mg L-glutamic acid in 10 ml distilled/de-ionised water and filtersterilize. Add 1.0 ml/l (0.5 ml/500 ml).
- ^c Dissolve 10 mg L-methionine in 10 ml distilled/de-ionised water and filtersterilize. 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.

Storage

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

Preparation of mTBM agar 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 CaCl₂.

Compound	g/l	g/500 ml
H ₃ BO ₃	0.3	0.15
KBr	10.0	5.0
Peptone	10.0	5.0
Skim milk powder ^a	10.0	5.0
Bacto agar	17.0	8.5
Tween 80 ^b	10.0 ml	5.0 ml
Cephalexin monohydrate ^c	0.065	0.033
5-Fluorouracil ^d	0.012	0.006
Nystatin ^e	0.035	0.018

^{a, b, c, d, e} Added after autoclaving

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/de-ionised water.
3. Dissolve and check pH which should be 7.4, adjust if necessary.
4. Autoclave at 121 °C, 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 9.0 cm plate).
8. Leave plates to dry in a laminar flow bench or similar before use.

Antibiotics and other additions (amounts 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.

Storage

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

Preparation of Yeast Extract Dextrose Chalk (YDC) medium (Schaad, 1988)

Compound	g/l	g/500 ml
Bacto Agar	17.0	8.5
Yeast Extract	10.0	5.0
CaCO ₃ (light powder)	20.0	10.0
D-Glucose (Dextrose)	20.0	10.0
Distilled/de-ionised 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/de-ionised water.
3. Autoclave at 121 °C, 15 psi for 15 min.
4. Allow medium to cool to approx. 50 °C .
5. Swirl to ensure even distribution of CaCO₃ and avoid air bubbles, and pour plates (22 ml per 9.0 cm 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.

Preparation of Reaction Mixture for PCR

Compound	Final concentration	Volume (μ l) in 10 μ l
Sterile MilliQ		3.42
10x Buffer	1x	1.00
MgCl ₂ (50 mM)	1.5 mM	0.30
dNTPs (10 mM total, 2.5 mM each)	200 μ M each	0.80
Primer 3Sforw (5 pmol/ μ l)	0.50 μ M	1.00
Primer 3Srev (5 pmol/ μ l)	0.50 μ M	1.00
Primer 1052F (5 pmol/ μ l)	0.10 μ M	0.20
Primer BacR (5 pmol/ μ l)	0.10 μ M	0.20
Taq Polymerase (5U/ μ l)	0.04 U/ μ l	0.08
Bacterial suspension		2.00

10x Buffer

Tris-HCL(pH 9.0)	750 mM
(NH ₄) ₂ SO ₄	200 mM
Tween 20	0.1% (v/v)

Preparation of Tris Borate EDTA (TBE) Buffer 0.5x

Compound	g/l	g/500 ml
Tris	5.39	2.70
Boric acid	2.75	1.38
Na ₂ EDTA.2H ₂ O	0.37	0.19

The pH is 8.3 and requires no adjustment.

Preparation of 1.5% agarose gel for electrophoresis

Compound	1 agarose gel (20x20 cm)	1 litre
Tris Borate EDTA (TBE) 0.5x	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/de-ionised 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 0.5x 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 minutes 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.

References

- Asma, M. (1999). Test Report Comparative Test *Xanthomonas campestris* pv. *carotae* in carrot seed. ISHI Report , Bejo Zaden BV, Research Report P9411.
- Asma, M. (2000a). Report of the ISHI-ISTA comparative test *Xanthomonas campestris* pv. *carotae* in carrot seed. ISHI Report, Bejo Zaden BV, Research Report P9417.
- Asma, M. (2000b) Additioneel onderzoek *Xanthomonas campestris* pv. *carotae*. ISHI Report, Bejo Zaden BV, Research Report P9317-15 / P9416.
- Asma, M. (2005). Proposal for a new method for detecting *Xanthomonas hortorum* pv. *carotae* on carrot seeds. ISTA Method Validation Reports **2**, 1-17.
- Asma, M., de Vogel, R., Woudt, B. and Krause, D. (2002). Evaluation of pathogenicity testing, rep-fingerprinting and PCR for the identification of *Xanthomonas campestris* pv. *carotae*, ISHI Report Bejo Zaden BV, Research Report P9317-16.
- Cubeta, M.S. and Kuan, T.L. (1986). Comparison of MD5 and XCS media and development of MD5A medium for detection of *Xanthomonas campestris* pv. *carotae* in carrot seed, *Phytopathology* **76**, 1109.
- Eden, P.A., Schmidt, T.M., Blakemore, R.P. and Pace, N.R. (1991). Phylogenetic Analysis of *Aquaspirillum magnetotacticum* using Polymerase Chain Reaction Amplified 16S rRNA specific DNA. *International Journal of Systematic Bacteriology* **41**, 324-325.
- Geng, S., Campbell, R.N., Carter, M. and Hills, M. (1987). Quality control programs for seedborne pathogens. *Plant Disease* **67**, 236-242.
- Kim, H.K., Sasser, M. and Sands, D.C. (1982). Selective medium for *Xanthomonas campestris* pv. *translucens*. *Phytopathology* **72**, 936.
- Kuan, T.L., Minsavage, G.V. and Gabrielson, R.L. (1985). Detection of *Xanthomonas campestris* pv. *carotae* in carrot seed. *Plant Disease* **69**, 758-760.
- Meng, X.Q., Umesh, K.C., Davis, R.M. and Gilbertson, R.L. (2004). Development of PCR based assays for detecting the carrot bacterial leaf blight pathogen *Xanthomonas campestris* pv. *carotae* from different substrates. *Plant Disease* **88**, 1226-1234.
- McGuire, R.G., Jones, J.B. and Sasser, M. (1986). Tween media for the semiselective isolation of *Xanthomonas campestris* pv. *vesicatoria* from soil and plant material. *Plant Disease* **70**, 887-891.
- Roberts, S.J., Phelps, K., Taylor, J.D. and Ridout, M.S. (1993). Design and interpretation of seed health assays. In: Sheppard, J.W., (Ed.) *Proceedings of the First ISTA Plant Disease Committee Symposium on Seed Health Testing*, Ottawa, Canada. pp. 115-125. Agriculture Canada, Ottawa, Canada.
- Roberts, S.J. (1999). Thresholds, standards, tests, transmission and risks. In: *Proceedings of 3rd ISTA Seed Health Symposium*, Ames, Iowa, USA, 16-19 August 1999. pp. 20-24. ISTA, Zurich, Switzerland.
- Schaad, N.W. (1988). Initial identification of common genera. In: Schaad, N.W. (ed.) *Laboratory Guide for Identification of Plant Pathogenic Bacteria*, pp. 1-15. APS Press, St. Paul, MN, USA.
- Williford, R.E. and Schaad, N.W. (1984). Agar medium for selective isolation of *Xanthomonas campestris* pv. *carotae* from carrot seeds. *Phytopathology* **74**, 1142.

