VERSION: 2.1	**DATE:** 07/2024
PATHOGEN: Xanthomonas hortorum pv carotae (syn Xanthomonas campestris pv. carotae)	
HOST: carrot (Daucus carotae)	
COMMON NAME: bacterial leaf blight	
METHOD: Rb 1.1 ISTA seed wash and plating method 7-020: ver 1.4, 2022	
METHOD CLASS: STANDARD (A)	
SAMPLE: The minimum sample size should be 10,000 seeds and the maximum subsample size should be 10,000 seeds.	
SUBMITTED BY: plating assay adapted from ISTA Method 7-020, ver 1.4, 2022; qPCR assay by the U.S. ISHI Veg committee	

REVISION HISTORY: NSHS Version 1.1: ISTA Method 7-020, 2021-01-01: Safety precautions added; Sample size and Methods revised.
NSHS Version 2.0: Dilution plating and pathogenicity test from ISTA Method 7-020, ver 1.4, 2022. The qPCR confirmation assay is from ISHI Method Detection of *Xanthomonas hortorum* pv. *carotae* on Carrot Seed, December 2021. The ISTA conventional PCR confirmation assay was removed from the NSHS method due to occasional false negative results. NSHS Version 2.1: Treated seed section added.

TREATED SEED:

This method is suitable for untreated seed. Chemical seed treatments may affect the performance of this test.

MATERIALS:

Dilution Plating

- Reference material: known strain of *Xanthomonas hortorum* pv. *carotae* or standardized reference material
- Plates of MKM medium
- Plates of MD5A or mTBM medium
- Plates of YDC medium
- Conical flasks
- Dilution bottles
- 70% ethanol
- Incubator - 28°C

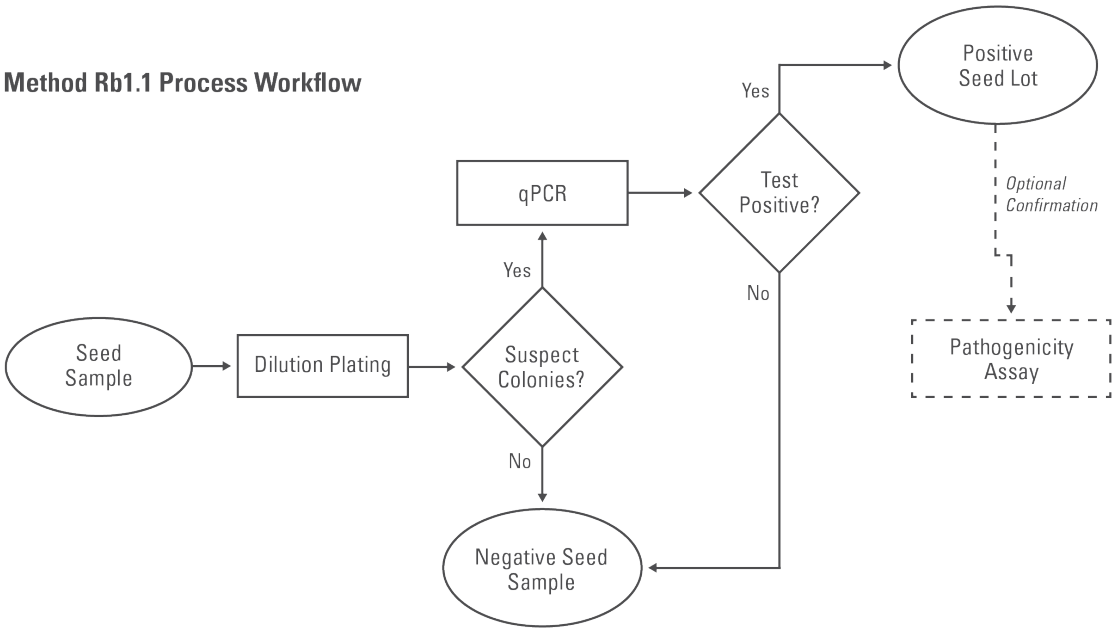
- Balance
- pH meter
- Pipettes
- Orbital shaker
- Sterile pipette tips
- Sterile bent glass rods

qPCR

- Centrifuge
- qPCR thermocycler
- DNA Extraction kit - Qiagen
- Primers – q2, MVSXhc, and Wu
- Probes
- qPCR Mix
- PCR-grade water
- Xhc positive control, negative process control, internal amplification control, and non-template control
- Optical Density (OD) meter
- Lab disposables

Pathogenicity (optional)

- Carrot seedlings – susceptible to pathogen, e.g. 'Napoli'
- Spraying device
- Sterile tap water
- Controls



PROCEDURE:

PROTOCOL- Dilution Plating

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 hours) 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 10^1 dilution into another 4.5 ml of sterile saline and vortex to mix (10^3 dilution).

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.

2.4. Incubate plates with positive control plates at 28°C and examine after 4 – 8 days.

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 standardized reference material according to the supplier's instructions. If a lyophilized 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 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.

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.

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

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

5.2.2. On MD5A after 7-8 days, *X. hortorum* pv. *carotae* colonies appear straw yellow, glistening, round smooth, convex with entire margins, and 2-3 mm in diameter (Figure 2).

5.2.3. On mTBM after 7-8 days, *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 (Figure 3). Casein hydrolysis on mTBM is not always present.

5.3. The colony size and color can differ within a sample.

5.4. Record the number of suspect and other colonies.

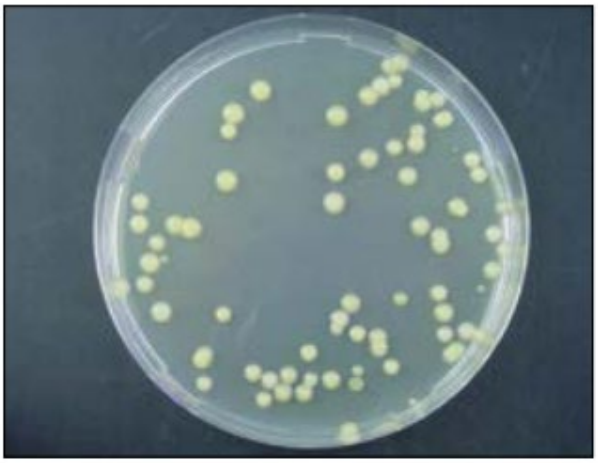


Figure 1. *Xanthomonas hortorum* pv. *carotae* colonies on MKM plates after 6 d indicated by light yellow-cream, light brown to peach yellow, glistening, round colonies and 2–4 mm in diameter.

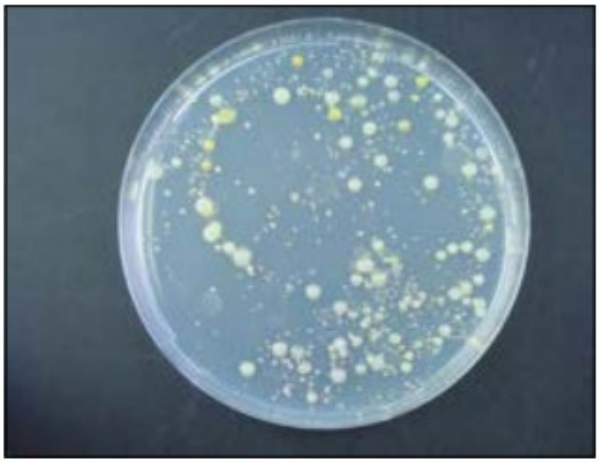


Figure 2. *Xanthomonas hortorum* pv. *carotae* colonies on MD5A plates after 7 d indicated by straw yellow, glistening, round smooth, convex colonies with entire margins, and 2–3 mm in diameter.

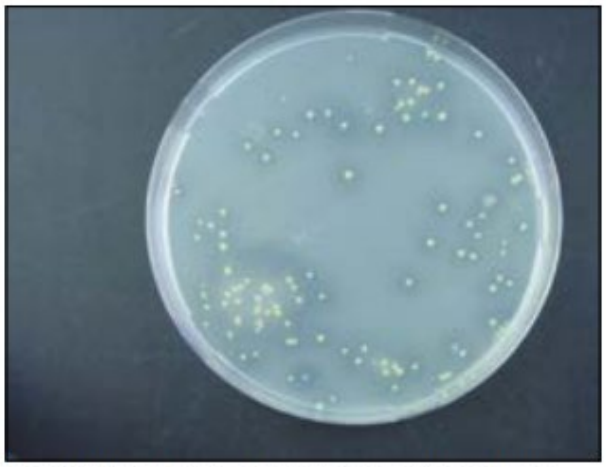


Figure 3. *Xanthomonas hortorum* pv. *carotae* colonies on mTBM plates after 7 d indicated by white or yellow or white-yellow, glistening, round, smooth, convex colonies with entire margins, 1–2 mm in diameter and surrounded by a zone of casein hydrolysis.

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.

Pictures from ISTA Method 7-020: Detection of Xanthomonas hortorum pv. carotae in Daucus carota (carrot) seed

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 hours at 28°C.

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

6.5. Confirm the identity of isolates by qPCR followed by an optional pathogenicity test.

6.6. Record results for each colony subcultured.

Identification by qPCR of suspect Xhc colonies

7. DNA Isolation

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

7.1. Make a slightly turbid cell suspension (e.g. OD 600 nm approximately 0.05) in 0.3 – 0.5 mL PCR-grade water from the suspected cultures on YDC medium and the positive controls

(Table 1). In addition, a non-suspect isolate should be used as a negative process control (NPC).

7.2. Incubate for 10 min at 95°C.

7.3. Suspensions can be stored at -20°C until identification.

8. qPCR

8.1. Use the Xhc specific primers and probes from Barnhoorn 204 (MVSXhc3 set) and from Temple et al. 2013 (Xhc-q2 set) (Table 2).

8.2. Use the universal primers and probes from Wu et at. (2008) (Table 2) to validate the PCR reaction.

8.3. Prepare the reaction mixture (Table 3).

8.4. Perform the PCR reaction in a real-time PCR instrument according to the PCR conditions (Table 4). Note: if different PCR mixtures and amplification programs are used, it is necessary to verify their performances.

8.5. Samples will be considered positive with a Cq value ≤ 32. Cq values of positive controls should consistently be lower than 32. The cut-off Cq value of the internal amplification control (IAC) should be below 35, and the expected range is to be determined by the user based on experimental data. Notes: 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.

8.6. 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 indicate at least be 3.3 Cq value lower than the IAC Cq values from the NTC reactions.

8.7. For interpretation and decision making, the results from both primer sets need to be considered, see Table 5. Test results are only valid when all included controls presented in Table 2 give the expected result.

9. Pathogenicity

9.1. Grow seedlings of a carrot cultivar known to be susceptible to *Xanthomonas 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).

9.2. Prepare a suspension in sterile tap water from each suspect bacterial culture on YDC medium and dilute to a concentration containing approximately $2x10^6$ cfu/ml. The same procedure should be used for the positive control isolate. See General Methods for preparation of ten-fold dilution series.

9.3. 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.

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

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

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

Picture from ISTA Method 7-020: Detection of Xanthomonas hortorum pv. carotae in Daucus carota (carrot) seed

Table 1. Types of controls used

Control Type	Description
Positive Process Control (PPC)	Freshly prepared suspension of Xhc
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 2. TaqMan PCR Primer and probe sequences

Name	Dye	Sequence 5' – 3'	Quencher	Reference
MVSXhc3F (10 µM)		5' – CCA AAG CAG TCG CAA ACT TGA – 3'		Barnhoorn 2014
MVSXhc3R (10 µM)		5' – AAT TGC GGA TTC CCA ACA AA – 3'		
MVSXhc3P (10 µM)	Vic*	5' – TGG CCC TAA GCT TCA A – 3'	MGB(NFQ)*	
Xhc -q2F (10 µM)		5' – GCA TGA AGG CAA TAC AGC G – 3'		Temple et al., 2013
Xhc -q2R (10 µM)		5' – CGA TCC AGC TGA TGT TCT CCG AA – 3'		
Xhc -q2P (10 µM)	FAM*	5' – TCA AGC TCA GAC GAA ACC GGC GTC – 3'	BHQ1*	
Wu-F (10 µM)		5' – CAA CGC GAA GAA CCT TAC C – 3'		Wu et al., 2008
Wu-R (10 µM)		5' – ACG TCA TCC CCA CCT TCC – 3'		
Wu-P1 (10 µM)	TxRd*	5' – ACG ACA ACC ATG CAC CAC CTG – 3'	BHQ2*	
Wu-P2 (20 µM)	TxRd*	5' – ACG ACA GCC ATG CAG CAC CT – 3'	BHQ2*	

* Recommended dyes and quenchers. Differing ones can be used as long as proven effective by the laboratory.

Table 3. Example qPCR mix: MVSHhc3 / Xhc-q2 / Wu triplex

Component	Final Concentration	For 1 reaction (volume in µL)
MVSXhc3F (10 µM)	**0.9 µM**	**2.25**
MVSXhc3R (10 µM)	**0.9 µM**	**2.25**
MVSXhc3P (10 µM)	**0.25 µM**	**0.625**
Xhc -q2F (10 µM)	**0.4 µM**	**1.0**
Xhc -q2R (10 µM)	**0.4 µM**	**1.0**
Xhc -q2P (10 µM)	**0.2 µM**	**0.50**
Wu-F (10 µM)	**0.2 µM**	**0.50**
Wu-R (10 µM)	**0.2 µM**	**0.50**
Wu-P1 (10 µM)	**0.2 µM**	**0.50**
Wu-P2 (10 µM)	**0.2 µM**	**0.50**
ABI Gene Expression Master mix (2x)*	**1x**	**12.50**
PCR grade H_20		**0.875**
Template DNA		**2.00**
Total		**25.00**

Note: Make sure the template DNA is at room temperature when added to the mix to prevent temperature linked chemical reactions prior to PCR.

Table 4. qPCR conditions

Step	Temperature	Duration
Hold	95°C	10 minutes
40 cycles	95°C	15 seconds
	60°C	30 seconds

Table 5. Interpretation and decision table for the qPCR

MVSXhc3	Xhc-q2	Wu	qPCR Result	Follow-up
Positive	Positive	Positive*	Target DNA for Xhc detected	Optional pathogenicity test for confirmation
Negative	Positive	Positive*	Target DNA for Xhc detected	Optional pathogenicity test for confirmation
Positive	Negative	Positive*	Target DNA for Xhc detected	Optional pathogenicity test for confirmation
Negative	Negative	Positive	No target DNA for Xhc detected	Negative, no follow up
Negative	Negative	Negative	Invalid	Repeat qPCR

*** If the Wu primers are negative, the qPCR assay should be rerun or a pathogenicity test conducted.**

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.

Media and Solutions

Sterile Saline

Compound	Amount/L	Amount/500 mL
Sodium chloride (NaCl)	8.5 g	4.25 g
Distilled/deionized water	1000 mL	500 mL

Preparation:

1. Weigh out all ingredients into a suitable container.
2. Add 1000 mL (or 500 mL) of distilled/deionized 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 minutes.

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

MKM Medium

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]	0.002g	0.001 g
Cephalexin monohydrate[b]	0.010g	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.

[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 66 K units/g or Duchefa B-0106, 70 K units/g) in 10 mL 70% ethanol. Add 1 mL/L (0.5 mL/500 mL).

[d] Dissolve 100 mg nystatin (Sigma N-3503 or 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. Weight out all ingredients except antibiotics into a suitable container.
2. Add 1000 mL (or 500 mL) of distilled/deionized water.
3. Dissolve and check pH which should be 6.6.
4. Autoclave at 121°C, 15 psi for 15 minutes.
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 (22mL per 90 mm plate).
8. Leave plates to dry in a laminar flow bench or similar before use.

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

MD5A Medium

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.
[a] Dissolve 10 g cellobiose in 100 mL of distilled/deionized water and filter sterilize.
[b] Dissolve 50 mg L-glutamic acid in 10 mL of distilled/deionized water and filter sterilize. Add 1.0 mL/L (0.5 mL/500 mL)
[c] Dissolve 10 mg L-methionine in 10 mL of distilled/deionized water and filter sterilize. 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 66 K 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 or 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. Weight 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/deionized water.
3. Dissolve and check pH which should be 6.4, adjust if necessary.
4. Autoclave at 121°C, 15 psi for 15 minutes.
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 (22mL per 90 mm plate).
8. Leave plates to dry in a laminar flow bench or similar before use.

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-e] Added after autoclaving. Amounts for antibiotics and other additions are for guidance only.
[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 or 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. Weight out all ingredients except skim milk powder, antibiotics and Tween 80 into a suitable container.
2. Add 900 mL (or 450 mL) of distilled/deionized water.
3. Dissolve and check pH which should be 7.4, adjust as necessary.
4. Autoclave at 121°C, 15 psi for 15 minutes.
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 (22mL per 90 mm plate).
8. Leave plates to dry in a laminar flow bench or similar before use.

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

Compound	Amount/L	Amount/500 mL
Agar (BD Bacto™ Agar)	17.0 g	8.25 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/deionized water	1000 mL	500 mL

Preparation:

1. Weigh out all ingredients into a suitable oversized 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/deionized water.
3. Autoclave at 121°C, 15 psi for 15 minutes.
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 (22mL per 90 mm plate).
6. Leave plates to dry in a laminar flow bench or similar before use.

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

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(ISTA) International rules for seed testing 7-020: Xanthomonas hortorum pv. carotae in Daucus carota (carrot) seed. Effective from 1 January 2022, ver 1.4.

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