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

7-019: Detection of *Xanthomonas campestris* pv. *campestris* on *Brassica* spp.

Published by: International Seed Testing Association (ISTA), Bassersdorf, Switzerland
2007

Crop: *Brassica* spp. (broccoli, cabbage, calabrese, canola, cauliflower, oilseed rape)

Pathogen: *Xanthomonas campestris* pv. *campestris* (black rot)

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Revision History: Version 1.0, 13 May 2003.
Version 2.0, 06 August 2004.
Version 3.0, 05 July 2006.

Background

This method is based on methods originally published by Franken *et al.* (1991) and in the 2nd edition of Working Sheet No. 50 in the ISTA Handbook of Seed Health Testing (Schaad and Franken, 1996). Compared to the 2nd edition of Working Sheet No. 50, this version incorporates a number of modifications resulting from comparative tests in 13 laboratories (Koenraadt *et al.,* 2004), a study done in a single laboratory (Roberts *et al.,* 2004), and experience of routine testing in a number of laboratories. Summary of modifications: no fungicides used in extraction buffer; NSCAA medium replaced by mCS20ABN; no centrifugation step after 5 min; continuous shaking instead of static incubation; only one plate of each medium per dilution; removal of check for antagonistic bacteria; minor changes to media preparation; simplified pathogenicity test method; removal of IF and direct plating assays; changes to format and layout. This method differs from that originally proposed in the validation report (Roberts and Koenraadt, 2003) by the omission of a centrifugation step, which may theoretically give a reduced analytical sensitivity (Roberts *et al.,* 2004). Users of this method should be aware that the values quoted for analytical sensitivity (detection limits) are theoretical; in practice the actual level of sensitivity achieved will vary with the background level of saprophytes.

Details of how to use cetrifugation with this method and other methods will be included in the ISTA Seed Health Handbook which is currently in preparation.

Validation Studies

Roberts, S.J. and Koenraadt, H. (2003)
Copies are available: by E-mail from ista.office@ista.ch; by mail from the ISTA Secretariat.

Reproducibility:	dispersion = 1
Repeatability:	dispersion = 1
Detection limits:	15 cfu/ml (theoretical, P=0.95)

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. 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

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

Note. *Brassica* seed subjected to a physical treatment, for example, hot water is regarded as treated 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 *Xanthomonas campestris* pv. *campestris* or standardised reference material.
Plates of FS medium	-	9.0 cm Petri dishes (3 plates of each medium per sub-sample + controls)
Plates of mCS20ABN medium	-	9.0 cm Petri dishes (3 plates of each medium per sub-sample + controls)
Plates of YDC	-	for sub-culture (at least 1 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 28-30°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	-	check accuracy and precision regularly
Brassica seedlings	-	susceptible to all races of the pathogen (e.g. *B. oleracea* cv. Wirosa) for pathogenicity test
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 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:

$$TSW = \frac{Wt\ of\ seed}{No\ of\ seed} \times 1000$$

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 pre-chilled (2-4°C) 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. Shake for 2.5 h at room temperature (20-25ºC) on an orbital shaker set at 100-125 rpm.
2. *Dilution and plating*
   2.1. Shake the flasks to mix just before dilution.
   2.2. Prepare two serial tenfold dilutions 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) (see General Methods).
   2.3. Pipette 100 µl of each dilution and the un-diluted seed extract onto plates of each of the selective media (FS, mCS20ABN) and spread over the surface with a sterile bent glass rod (see General Methods).
   2.4. Incubate plates at 28-30°C and examine after 3-4 d.
3. *Positive control (culture or reference material)*
   3.1. Prepare a suspension of a known strain of *X. campestris* pv. *campestris* in sterile saline or reconstitute standardised reference material according to the supplier's instructions.
   3.2. Dilute sufficiently to obtain dilutions containing approx. 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 each of the selective media (FS, mCS20ABN) and spread over the surface with a sterile bent glass rod.
   3.4. Incubate plates with the sample plates.
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 each of the 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. campestris* pv. *campestris* (*Xcc*) colonies by comparison with the positive control plates.
   5.3. On FS after 3-4 d, *Xcc* colonies are small, pale green, mucoid and surrounded by a zone of starch hydrolysis. This zone appears as a halo that may be easier to see with a black background (Fig. 1a). Colonies may show marked variation in size and may be visible on FS after 3 d; if not, incubate for an additional day.
   5.4. After 3-4 d on mCS20ABN, *Xcc* colonies are pale yellow, mucoid and surrounded by a zone of starch hydrolysis (Fig 1b). Colonies may show marked variation in size. Depending on the number of colonies present, it may be easier to evaluate plates after 3 d, before coalescence of starch hydrolysis zones which can make it more difficult to identify suspect colonies.
   5.5. Incubation of the plates at 4°C for several hours before recording may result in sharper zones of starch hydrolysis with some starch sources.
   5.6. Record the number of suspect and other colonies (see General Methods).
6. *Confirmation/identification of suspect colonies*
   6.1. Sub-culture suspect colonies to sectored plates of YDC. To avoid the potential for cross-contamination of isolates, use a new sectored 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 (**CCP**).
   6.2. Sub-culture the positive control isolate to a sectored plate for comparison.
   6.3. Incubate sectored plates for 24-48 h at 28-30°C.
   6.4. Compare appearance of growth with positive control. On YDC *X. campestris* pv. *campestris* colonies are pale yellow and mucoid/fluidal (Fig 2).
   6.5. Confirm the identity of isolates by pathogenicity on Brassica seedlings of known susceptibility (**CCP**).
   6.6. Record results for each colony sub-cultured.
7. *Pathogenicity*
   7.1. Grow seedlings of a Brassica cultivar known to be susceptible to all races of *X. campestris* pv. *campestris* (e.g. cabbage cv. Wirosa, see Vicente *et al.*, 2001) in small pots or modules until at least 3-4 true leaf stage.
   7.2. Scrape a small amount of bacterial growth directly from a 24-48 h YDC culture (e.g. sectored plate) with a sterile cocktail stick or insect pin.
   7.3. Inoculate six of the major veins at a point near the leaf edges on the two youngest leaves by stabbing with the cocktail stick or insect pin.
   7.4. The number of plants which should be inoculated will depend on the variability of the cultivar and experience of the operator, but 1-3 plants per isolate should usually be sufficient. It is better to inoculate more isolates with 1 plant per isolate than fewer isolates with 3 plants per isolate.
   7.5. Inoculate with the positive control isolate and stab with a sterile cocktail stick or insect pin as a negative control (**CCP**).
   7.6. Grow on plants at 20-25°C.

7.7 Examine plants for the appearance of typical progressive V-shaped, yellow/necrotic lesions with blackened veins after 10-14 d. (See Fig. 3). Symptoms may be visible earlier depending on temperature and the aggressiveness of the isolate. Compare with positive control (**CCP**). It is important to discriminate between the progressive lesions caused by the vascular pathogen *X. campestris* pv. *campestris* and the limited dark necrotic lesions at the inoculation site caused by leaf spot *Xanthomonas* (often classified as either *X. c.* pv. *armoraciae* or *X. c.* pv. *raphani* (see Kamoun *et al.* 1992; Alvarez *et al.,* 1994; Tamura *et al.*, 1994; Vicente *et al.,* 2001; Roberts *et al.,* 2004).

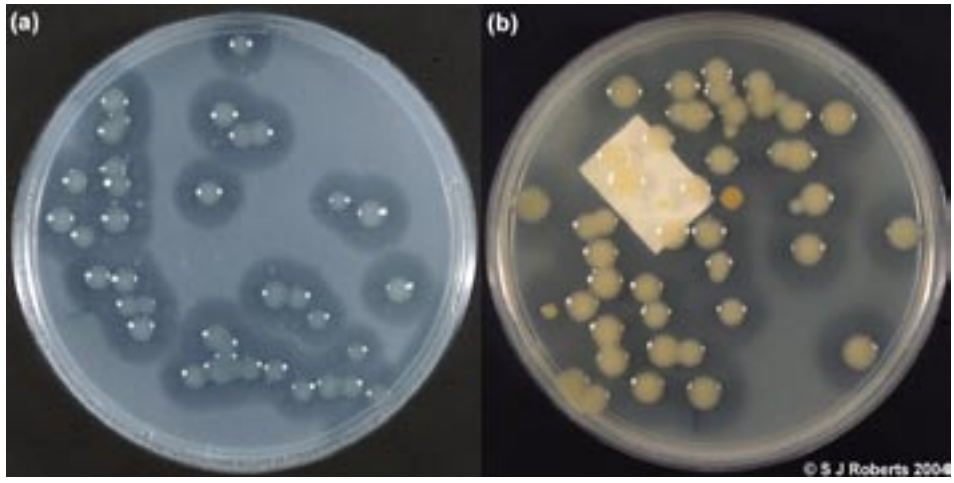

Fig. 1. Plates of FS (a) and mCS20ABN (b) media after 4 d incubation at 30°C showing typical colonies of *Xanthomonas campestris* pv. *campestris* surrounded by zones of starch hydrolysis.

Fig. 2. Typical pale yellow mucoid/fluidal growth of *Xanthomonas campestris* pv. *campestris* isolates on a sectored plate of YDC after 48 h at 30°C.

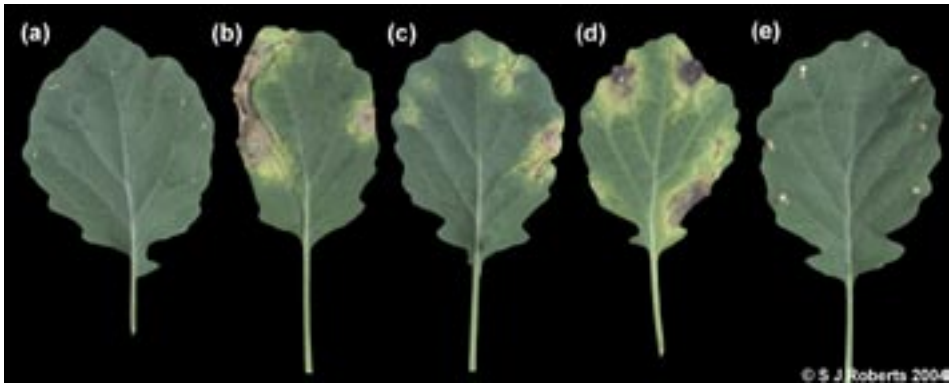

Fig. 3. Leaves of a cabbage seedlings cv. Wirosa, 10 d (at 22°C) after inoculation: (a) sterile control, (b) *Xanthomonas campestris* pv. *campestris* Race 1, (c) Race 3, (d) Race 5, (e) 'leaf spot' *Xanthomonas*.

General Methods (common to many test procedures)

1. *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
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).

Due to the potential for non-pathogenic isolates to be present in seedlots together with pathogenic isolates, it is essential to sub-culture (Step 6.1), if present, at least the minimum number of suspect colonies specified (six per sub-sample) and to test all *Xanthomonas*-like sub-cultured isolates for pathogenicity (Step 6.5).

Positive control isolates should be included in every pathogenicity test (Step 7.5).

The positive control isolate should give typical symptoms in pathogenicity test (Step 7.7).

The source of starch used in the selective media is critical for observation of starch hydrolysis. Verify that each new batch of starch gives clear zones of hydrolysis with reference cultures of *X. campestris* pv. *campestris* (FS and mCS20ABN media).

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 (FS and mCS20ABN media).

The activity of neomycin against some strains of *Xcc* is known to be affected by pH. It is essential that the pH of the medium is less than 6.6 (mCS20ABN medium, Step 3)

Preparation of sterile saliner

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. Dissolve and dispense into final containers.
4. Autoclave at 121°C, 115 psi for 15 min.
5. For extraction of seeds, add 20 µl of sterile Tween 20 per 100 ml after autoclaving

Storage

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

Preparation of mCS20ABN agar medium

Note. This medium is based on, but not identical to, the medium of Chang *et al.* (1991), from which it differs in starch source and concentration, cycloheximide concentration and solution media for antibiotics. The amounts of phosphate salts have also been adjusted to achieve the correct pH without further adjustment

Compound	g/l	g/ 500 ml
Soya Peptone (Oxoid L44)	2.0	1.0
Bacto Tryptone (Difco Bacto)	2.0	1.0
KH_2PO_4	1.59	0.79
$(NH_4)_2HPO_4$	0.33	0.17
$MgSO_4.7H_2O$	0.4	0.2
L-Glutamine (Sigma G-3126)	6.0	3.0
L-Histidine (Sigma H-8000)	1.0	0.5
D-Glucose (Dextrose)	1.0	0.5
Distilled/de-ionised water	1000 ml	500 ml
Soluble starch (Aldrich No 17,993-0) (**CCP**)	25.0	12.5
Bacto Agar (Difco)	15.0	7.5
Cycloheximide[a] (200 mg/ml 70% EtOH)	200 mg (1 ml)	100 mg (0.5 ml)
Neomycin[b] (40 mg/ml in 20% EtOH)	40 mg (1 ml)	20 mg (0.5 ml)
Bacitracin[c] (100 mg/ml in 50% EtOH)	100 mg (1 ml)	50 mg (0.5 ml)

[a, b, c] Added after autoclaving

Preparation

1. Weigh out all ingredients except agar, starch and 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 6.5, adjust if necessary (important, **CCP**).
4. Add starch and agar then steam to dissolve.
5. Autoclave at 121°C, 115 psi for 15 min.
6. Prepare antibiotic solutions if necessary.
7. Allow medium to cool to approx. 50°C and add antibiotic and methionine solutions.
8. Mix thoroughly but gently by inversion/swirling to avoid air bubbles and pour plates (22 ml per 9.0 cm plate).
9. Leave plates to dry in a laminar flow bench or similar before use.

*Antibiotics (amounts for guidance only, **CCP**)*

[a] Dissolve 2.0 g cycloheximide (Sigma C-7698) in 10 ml 70% ethanol. Add 1 ml/l (0.5 ml/500 ml).

[b] Dissolve 400 mg neomycin (Sigma N-1876) in 10 ml 20% ethanol. Add 1 ml/l (0.5 ml/500 ml).

[c] Dissolve 1.0 g bacitracin (Sigma B-0125, 66k units/g) in 10 ml 50% ethanol. Add 1 m/l (0.5 ml/500 ml).

Storage

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

Depending on the source of starch, pre-storage in the refrigerator for several days before use may result in more easily visible zones of starch hydrolysis.

Preparation of FS agar medium

Note. A number of different versions of this medium have been published; for the sake of consistency, the recipe given below is the one referred to (Schaad, 1989) in the previous working sheet.

Compound	g/l	g/ 500 ml
Bacto Agar	15.0	7.5
Soluble starch (Aldrich No 17,993-0) (**CCP**)	10.0	5.0
Bacto yeast extract (Difco)	0.1	0.05
K_2HPO_4	0.8	0.4
KH_2PO_4	0.8	0.4
$MgSO_4.7H_2O$	0.1	0.05
Methyl Green (1% aq.)	1.5 ml	0.75 ml
Distilled/de-ionised water	1000 ml	500 ml
Cycloheximide[a] (200 mg/ml 70% EtOH)	200 mg (1 ml)	100 mg (0.5 ml)
D-methionine[b] (3 mg/ml 50% EtOH)	3 mg (1 ml)	1.5 mg (0.5 ml)
Pyridoxine-HCl[c] (1 mg/ml 50% EtOH)	1 mg (1 ml)	0.5 mg (0.5 ml)
Cephalexin[d] (50 mg/ml 70% EtOH)	50 mg (1 ml)	25 mg (0.5 ml)
Gentamycin[e] (1 mg/ml H_2O)	0.4 mg (0.4 ml)	0.2 mg (0.2 ml)
Trimethoprim[f] (10 mg/ml 70% EtOH)	30 mg (3 ml)	15 mg (1.5 ml)

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

Preparation

1. Weigh out all ingredients except antibiotics and methionine into a suitable container.
2. Add 1000 ml (or 500 ml) of distilled/de-ionised water.
3. Steam to dissolve.
4. Autoclave at 121°C, 115 psi for 15 min.
5. Prepare antibiotic and methionine solutions and filter sterilise as appropriate
6. Allow medium to cool to approx. 50°C before adding antibiotic and methionine solutions.
7. Mix the molten medium gently 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 (amounts for guidance only, **CCP**)*

[a] Dissolve 2 g cycloheximide in 10 ml 70% ethanol. Add 1 ml/l (0.5 ml/500 ml).

[b] Dissolve 60 mg D-methionine in 10 ml distilled water, then add 10 ml ethanol. Add 1 ml/l (0.5 ml/500 ml).

[c] Dissolve 20 mg pyridoxine in 20 ml 50% ethanol. Add 1 ml/l (0.5 ml/500 ml).

[d] Dissolve 500 mg cephalexin in 10 ml 70% ethanol. Add 1 ml/l (0.5 ml/500 ml).

[e] Dissolve 10 mg gentamycin in 10 ml distilled water, filter sterilise. Add 0.4 ml/l (0.2 ml/500 ml).

[f] Dissolve/suspend 200 mg trimethoprim in 20 ml 70% ethanol. As the chemical may not dissolve fully it may be necessary to vortex the suspension immediately before adding it to the medium (3 ml/l or 1.5 ml/500 ml).

Storage

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

Depending on the source of starch, pre-storage in the refrigerator for several days before use may result in more easily visible zones of starch hydrolysis.

Preparation of Yeast Dextrose Chalk (YDC) agar medium

Compound	g/l	g/500 ml
Bacto Agar	15.0	7.5
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. Steam to dissolve.
4. Autoclave at 121°C, 115 psi for 15 min.
5. Allow medium to cool to approx. 50°C .
6. Swirl to ensure even distribution of $CaCO_3$ and avoid air bubbles, and pour plates (22 ml per 9.0 cm plate).
7. 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.

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