

**Methods for the detection and quantification of *Erwinia carotovora*
subsp. *atroseptica* (*Pectobacterium carotovorum* subsp. *atrosepticum*)
on potatoes:
a laboratory manual**



edited by

M .C. M. PEROMBELON & J. M. VAN DER WOLF

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M. C. M. P.

Foreword

Potato blackleg, a seed tuber-borne disease, is caused by *Erwinia carotovora* subsp. *atroseptica* (Eca) in temperate regions. Since chemotherapy and thermotherapy are impractical, and in the absence of resistant cultivars, disease control relies primarily on the production and utilisation of healthy seed. As Eca contamination of potatoes is widespread and blackleg incidence is related to seed tuber contamination level, it is generally accepted that seed health is better assessed by determining the numbers of Eca on seed tubers than by simply checking for the presence or absence of the pathogen on these tubers or by inspecting parental crops for blackleg plants and selecting crops with no or a low disease incidence for seed (Pérombelon, 1992). Methods to determine Eca contamination of tubers commonly in use commercially, namely colony count on selective growth media and ELISA, suffer from poor specificity and/or inadequate sensitivity well above the threshold level for blackleg development, 10^2 - 10^3 cells ml^{-1} tuber peel extract (Bain *et al.*, 1990).

Four methods with the required degree of specificity, sensitivity and user friendliness have been recently developed and evaluated by the authors of this manual, mainly for commercial testing of potato seed stocks for Eca contamination. Three of the methods rely on Eca-specific antibodies for detection, namely immunomagnetic separation of Eca cells followed by growth at differential temperatures on a selective-diagnostic crystal violet pectate medium (IMS-CVP), immunofluorescence Eca colony staining (IFC) and Eca enrichment prior to detection by ELISA (E-ELISA). The fourth method is based on Eca DNA amplification by the polymerase chain reaction (PCR) using Eca-specific DNA primers. The choice of a method depends primarily on both the scale of the operation and its cost, which is in part related to available expertise and facilities as well as the degree of accuracy required.

This manual describes the four methods and is aimed primarily at potato seed certification agencies, nuclear potato seed-producing organisations and plant clinics involved in seed health assessment. In addition, procedures enabling the isolation and identification of Eca, using traditional methods, are also given. Research workers interested in *Erwinia* ecology and epidemiology might also find the manual useful. The format adopted is designed to enable the beginner to undertake the work with little need of further training. The procedures are described in full in a “cook book” fashion with further details of growth media, reagents and sources of supply and brands of materials, including detection probes and detection kits, shown to be satisfactory by the authors, given in an Appendix after each Section. However, this does not imply that alternative sources of materials cannot be used if, after checking, they have been found to be satisfactory substitutes.

Comprehensive evaluations of the four methods have been made by Toth *et al.* (1996) and Pérombelon *et al.* (1998) and further general background practical information on the techniques used can be found in Hampton *et al.* (1990), Sambrook *et al.* (1989) and Schaad (1988).

Notes on Revised Version 2002

Recently the soft rot erwinias have been classified in the resuscitated genus *Pectobacterium* as *P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *atrosepticum* and *P. chrysanthemi* based on 16S rDNA sequence studies (Hauben *et al.*, 1998). As the nomenclature has not yet been widely accepted by plant pathologists, the original names have been retained in this revised version.

Since publication, recent findings concerning erwinia diagnosis justify revision of the laboratory manual. The incidence of Eca serogroup I in several countries (Finland, Norway, Poland and Scotland) has been found not to be as high as previously believed and can be as low as 60 %. This implies that the current commercially available antibodies may not be specific enough to determine Eca seed health status in many countries because of the high risk of false negative results. Until specificity is improved, for example, by the production of antibodies against other Eca serogroups, PCR-based methods, using Eca-specific DNA primers, appear to be the most satisfactory method of detection. Meanwhile, the immunological methods described in Sections 2, 3 and 4 can be used only in regions where it has been shown that Eca serogroup I predominates or in epidemiological studies involving inoculation with strains of that serogroup. Because of the considerable progress made in PCR detection of Eca, Section 5, which deals with DNA detection methods, has had a major update. This includes a quantification PCR (Q-PCR) method, as well as a simpler commercial kit for DNA extraction. The ProbeliaTM Eca detection kit has not been marketed and, therefore, it has been omitted. Since the polypectate used in the preparation of the selective-diagnostic crystal violet pectate (CVP) medium is no longer available, the revised formulation for the preparation of the medium to allow the use of a new source of polypectate is given. Finally, minor changes, mostly to correct typing errors, have been made.

Eca diagnostics has been critically reviewed recently (Pérombelon, 2000).

June 2002

M. C. M. Pérombelon
L. J. Hyman

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Section 1

Potato tuber peel extract preparation and detection probes

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Introduction

The source and preparation of the test material are of crucial importance when determining potato seed tuber health for blackleg. Results obtained are as dependent on the quality of the test material as on the method used, how representative the samples are and the viability of the cells during the time between tissue extract preparation and testing. As the recommended methods rely on *E. c.* subsp. *atroseptica* (Eca)-specific detection probes with different characteristics, it is relevant to briefly describe them, outlining the reasons which make them more suitable for use with one method rather than another.

A. Tuber peel extract preparation

Background

Tuber contamination by Eca and two other related soft rot erwinias, *E. carotovora* subsp. *carotovora* (Ecc) and *E. chrysanthemi* (Ech), is almost unavoidable and occurs mostly superficially in lenticels and suberised wounds, where they can survive over the winter storage period until planting time (Pérombelon, 1992). A peel extract obtained by dry abrasion of samples of 10-12 pooled tubers in a mechanical potato peeler (Pérombelon *et al.*, 1987) or by grinding peel strips from individual tubers in a press, will contain most of the bacteria. Although other methods, such as grinding in a mortar and pestle, can be used, they are labour intensive and breakdown of the peel, hence bacterial extraction, tends to be variable. As more information can be obtained when the tubers are examined individually than when pooled (Jones *et al.*, 1994), procedures for the former are recommended.

Variation in tuber Eca contamination level is not so great as to make sampling impractical (Jones *et al.*, 1994). The probability of detecting infection is a function of both sample size and infection incidence. It is common practice to take between 25 and 75 tubers as randomly as possible from seed stocks of up to 25 tonnes. For example, there is a 90 % chance of detecting at least one contaminated tuber with sample sizes of 10, 24 and 76 tubers when infection levels are 20, 9 and 3 % respectively. Alternatively, when pooled tubers are peeled, it is usual to prepare peel extract from three to five lots of tubers per seed stock. Stocks can be sampled any time during storage after the curing period, usually ca. 3 weeks after harvest, when drying of the tubers results in a significant reduction of superficially sited erwinias and saprophytic bacterial populations.

Protocol

Materials

1. Tuber peeling equipment:
 - Mechanical potato peeler (Imperial Machine Company (Peelers) Ltd)* (Fig. 1.1 a).
 - Common kitchen hand-held potato peeler able to remove a 2 mm thick tuber peel strip.
 - Pollähne press (Meku), consisting of two interlocking and revolving rollers which can be rinsed automatically between each preparation* (Fig 1.1 b).
2. Phosphate buffered saline (PBS)*.
3. Antioxidants:
 - Dithiothreitol (DTT) stock solution (1.5 %)*.
 - Sodium diethyldithiocarbamate (DIECA) stock solution (0.02 %)*.
4. Ethanol (70 %).
5. Muslin cloth (ca. 8 x 8 cm squares).
6. Automatic adjustable pipettes and tips: (20-200 µl, 200-1000 µl)*.
7. Sterile universal vials (ca. 10 ml)*.

* Details in Appendix.

Procedures

1. Store tuber samples on receipt in paper bags in the dark at 4 °C until tested, usually within two weeks.
2. Briefly rinse test tubers individually under running tap water to remove excess soil.
- 3a. Prepare peel extract by peeling pooled tubers:
 - (i) Peel lots of 10-12 tubers, depending on size and turgidity, in the mechanical potato peeler for 2-5 min without addition of water, until all the peel has been removed.
 - (ii) Remove the upper casing of the peeler, scrape off some of the ground peel pulp with a sterile wooden spatula, squeeze through muslin cloth to collect ca. 1.5 ml of peel extract in a universal vial, and add antioxidant (see below).
 - (iii) Wash peeler thoroughly with running tap water for ca. 2 min and allow to drain before peeling the next sample of tubers.
- 3b. Peel extract preparation by peeling individual tubers:
 - (i) One or more peel strips are removed with a hand-held potato peeler from one tuber at a time to include both the heel and rose ends of the tuber.
 - (ii) Immediately hand feed the strips into the Pollähne press, crush to express ca. 1.5 ml into a vial and add antioxidant (see below). More than one strip per tuber may be needed to obtain enough peel extract when tubers are old and non-turgid.

(a)



(b)



Fig. 1.1 (a) Mechanical potato peeler (Imperial Machine (Peelers) Company Ltd).
(b) Pollähne press (Meku).

- (iii) Wash hand-held peeler in water and sterilise in 70 % ethanol before re-using. Rinse the press rollers thoroughly with running tap water while turning for ca. 1 min before re-using.

Note: Thorough rinsing of the peelers with tap water is usually sufficient to dilute any Eca present to below the sensitivity limit of the different methods, thereby avoiding cross-contamination from one tuber sample to another. However, when peel extract is prepared for PCR testing, it is advisable to use preferentially the Pollähne press as it is more easily cleaned than the mechanical potato peeler. It is advisable to wash the press surface after each extraction step, first, with tap water followed by 0.2 M NaOH, a second rinse with tap water, then 96 % ethanol and a third and final rinse with water.

4. Place the vials on crushed ice for 10 min to allow starch and other peel debris to settle before pipetting out 1 ml of the supernatant to a new tube.

Note: Depending on which detection method is used, it is advisable to add an antioxidant to retard Eca cell death by toxic substances (mainly phenolics) present in peel extract. However, it is still advisable to process the test material within 30 min. With IMS-CVP, IFC and PCR following Eca enrichment methods, 50 μ l of DTT stock solution is added to 1 ml peel extract to achieve a final concentration of 0.075 %; with E-ELISA, PBS-DIECA is added to an equal volume of peel extract (see Sections 2, 3, 4 and 5). The addition of an antioxidant is not needed when the Q-PCR assay is done on extracted DNA.

Expression of Eca contamination results

When quantifying contamination, results can be expressed in terms of number of Eca cells ml^{-1} peel extract or g^{-1} peel, when Eca numbers ml^{-1} peel extract is multiplied by a factor of 0.7 to convert to g^{-1} , as ca. 70 % of the liquid present in peel is recovered in the peel extract (Pérombelon *et al.*, 1987). Alternatively, contamination may be expressed per tuber when it is necessary to estimate the mean average weight of peel pulp per tuber obtained by weighing the pooled tubers before and after peeling or by weighing the peel strip/s from each tuber before crushing in the Pollähne press.

Usually, only the order of magnitude of contamination is sufficient. Overall stock contamination may be given by the average contamination level, or it is sometimes convenient to express contamination level as being high, intermediate or low, based on the limits arbitrarily adopted to define the three classes; for example, low: $<10^3$ Eca cells ml^{-1} , intermediate: $>10^3$ - $<10^4$ cells ml^{-1} and high: $>10^5$ cells ml^{-1} (10^2 - 10^3 Eca cells ml^{-1} is the blackleg development threshold level [Bain *et al.*, 1990]). Contamination may also be expressed in terms of the proportion of test tubers in the different classes of contamination levels weighted to reflect arbitrarily the relative blackleg risk of each class (Jones *et al.*, 1994).

B. Detection probes

Immunological probes

As the three soft rot erwinias commonly associated with potatoes, especially Eca and Ecc, are immunologically related, it is not surprising that most antisera produced against Eca using standard procedures (Hyman & Pérombelon, 1990) also contain Ecc cross-reacting antibodies against soluble antigens. This greatly reduces their usefulness when detecting Eca in peel extract by IFC and E-ELISA but not by the IMS-CVP method, which relies on both Eca-specific antibodies against cell wall-bound surface antigens and on differential growth temperatures to differentiate Eca from the other erwinias (see below).

The use of rabbits with a diverse genetic background has allowed the production of antisera at Plant Research International in the Netherlands specific to the homologous Eca serogroup I strain. These antisera have been cross-absorbed with a Eccserogroup I strain to remove common antibodies. They can be used in IFC and to a lesser extent in E-ELISA. Two monoclonal antibodies, 4F6 produced in Canada (De Boer & McNaughton, 1987) and marketed by LINARIS GMBH (Agdia) and IVIA-4G4 produced in Spain (Gorris *et al.*, 1994) and marketed by IVIA/REAL-DURVIZ, bind to Eca cell wall epitopes and are specific to the homologous serogroup I only. They can be used in both E-ELISA and IFC methods but not in the IMS-CVP method, probably because of the lower number of epitopes involved in binding compared to polyclonal antibodies. When >90% of Eca strains in a given region belong to one of nine known Eca serogroups (serogroup I), these antibodies can be used commercially for Eca testing. For example, in the Netherlands where recent studies have shown that this is the case. However, it has been found that the frequency of occurrence of Eca serogroup I in several other countries (Finland, Norway, Poland and Scotland) can be as low as 60% (Pérombelon, 2000). In this situation, the use of these antibodies would be inadvisable as the risk of false negative results would be too high to be commercially acceptable. However, they can be used under special conditions, for example in etiological and epidemiological studies involving inoculation with strains of that serogroup. When Eca serogroup distribution in a region is not known, it is recommended to ascertain whether serogroup I also predominates before initiating a testing programme involving the use of serological methods. If not, it would be necessary to produce antibodies against the most common serogroups which could then be pooled before use. This entails the determination of distribution frequency of the different serogroups. Finally, these Eca antibodies can also bind to a few soil and potato tuber-associated saprophytic bacterial species which, although not widespread, may give rise occasionally to false positive results.

Storage of antibodies

It is recommended to store crude antisera mixed 1:1 with 87 % glycerol and antibodies (ca. 1 mg ml⁻¹ in phosphate buffered saline containing 43 % glycerol), both in small aliquots (0.5 ml) in cryotubes at -20 °C or -80 °C. Thaw at room temperature and mix before use but avoid repeated freezing and thawing.

Alternatively, add 0.05 % sodium azide as a preservative, when storage can be at 4 °C.

Note: (i) Addition of glycerol or sodium azide to antisera for use in IFC is not recommended as colony staining quality can be affected as well as cell viability when re-isolation of Eca is required for confirmation purposes.

(ii) Sodium azide is a toxic substance and can cause an explosion when dry; take care to dispose of all spent liquid safely. Use recommended safety precautions when weighing and handling.

Molecular probes

Two sets of DNA primers specific for Eca, regardless of serogroup, have been produced and extensively tested: ECA1f and ECA2r of De Boer & Ward (1995) with unrestricted use, and Y45 and Y46 which are patented (Fréchon *et al.*, 1995). Use of Fréchon *et al.*'s primers is best restricted to research purposes unless clearance has been obtained from the patent holders.

Sequences of PCR Eca primers:

ECA1f:	5'-CGG CAT CAT AAA AAC ACG-3'
ECA2r:	5'-GCA CAC TTC ATC CAG CGA-3'
Y45:	5'-TCA CCG GAC GCC GAA CTG TGG CGT-3'
Y46:	5'-TCG CCA ACG TTC AGC AGA ACA AGT-3'

Primers may be synthesised commercially by a number of specialised laboratories (e.g. Pharmacia, Genset, MWG-Biotech UK Ltd).

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Appendix

Antibodies

- (i) Polyclonal antibodies: Plant Research International
- (ii) Monoclonal antibodies: IVIA-4G4 (REAL-DURVIZ)
4F6 (LINARIS GMBH)

Phosphate buffered saline (PBS)

10x concentrated stock solution (0.1 mol l⁻¹, pH 7.2-7.4)

NaCl	80.0 g
KH ₂ PO ₄	2.0 g
Na ₂ HPO ₄ .12H ₂ O	28.0 g
KCl	2.0 g
Distilled water	to 1000 ml

Dissolve salts, aliquot if necessary into smaller volumes, and sterilise by autoclaving at 120 °C for 15 min. Store at room temperature. When required, dilute 1 in 10 in distilled water.

DTT antioxidant stock solution

Prepare a 1.5 % solution of dithiothreitol (Sigma, D 9163) in sterile distilled water and store at 4 °C for about one week.

DIECA antioxidant stock solution

Prepare a 0.02 % solution of DIECA (sodium diethyldithiocarbamate; BDH, 10244) in PBS. Store at 4 °C for up to one week.

Equipment

Mechanical potato peeler (Imperial Machine Company [Peelers] Ltd).

Pollähne press (Meku).

Automatic adjustable pipettes and tips:

Gilson (Anachem) or Finpipette (Life Sciences).

Sterile Universal vials (BDH, 275/0460/06).

Immunomagnetic separation-colony count on CVP medium (IMS-CVP)

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Introduction

This is an improved version of the method based on colony counts on a selective-diagnostic medium, crystal violet pectate (CVP; Hyman *et al.*, 2002) and incubated at differential temperatures to identify the different soft rot erwinias (Pérombelon *et al.*, 1987). The two main disadvantages of the original method, namely *E. c.* subsp. *atroseptica* (Eca) inhibition by overcrowding by saprophytic bacteria and the inability to identify Eca colonies when outnumbered by those of *E. c.* subsp. *carotovora* (Ecc) and/or *E. chrysanthemi* (Ech), have been overcome by introducing an immunomagnetic separation (IMS) step prior to plating on CVP (Van der Wolf *et al.*, 1996). In IMS, complexes are formed consisting of Eca cells bound to Eca-specific surface-directed antibodies which themselves bind to protein A coupled to paramagnetic particles. These complexes can be recovered, when subjected to a strong magnetic force, thereby allowing the rapid and efficient recovery and concentration of Eca cells (Fig. 2.1). Protein A binds strongly to IgG molecules, which is the main component of polyclonal rabbit antisera.

Greater recovery is obtained in IMS with polyclonal antibodies (antisera) than with monoclonal antibodies. Recovery efficiency is also affected by the quality and titre of Eca antibodies, the nature and concentration of the magnetic particles, the IMS system and the number of wash cycles applied to remove non-target components. As only antibodies against the cell wall surface components are involved in IMS and because more of these antibodies present in Eca antisera are against Eca serogroup I than against other serogroups, the source of antisera against Eca serogroup I is not as critical as in other immunological methods. Removal of Ecc/ Ech may be incomplete when large numbers are present. Therefore, it is advisable that detection and identification of Eca also relies on the growth pattern on CVP at different temperatures. On CVP, only soft rot erwinias can form characteristic cavities after 48 h but, whereas Eca generally forms cavities at 27 °C only, Ecc and Ech also do so up to temperatures of 33.5 and 37 °C respectively (Pérombelon & Hyman, 1986; Fig. 2.2). Colonies/cavities formed at 33.5 °C by the few Ecc and Ech bacteria which have escaped the IMS procedure can readily be subtracted from total counts at 27 °C to determine more precisely Eca contamination levels.

The main advantages of the IMS-CVP method are that it is simple to use, little extra equipment to that already found in most laboratories is required, live Eca cells are detected and it has adequate sensitivity at $<10^2$ Eca cells ml⁻¹ peel extract with little risk of interference from cross-reacting saprophytic bacteria. Eca-specificity of the antisera is not as critical as in the other immunological methods. Disadvantages include inconsistency in recovery efficiency because of some loss of parti-

cles during the washing stage, which will affect the reliability of the quantification, and Eca identification by cavity formation pattern at differential temperatures is not always reliable as some strains of Eca can form cavities at 33.5 °C, which would result in an under-estimation of the population.

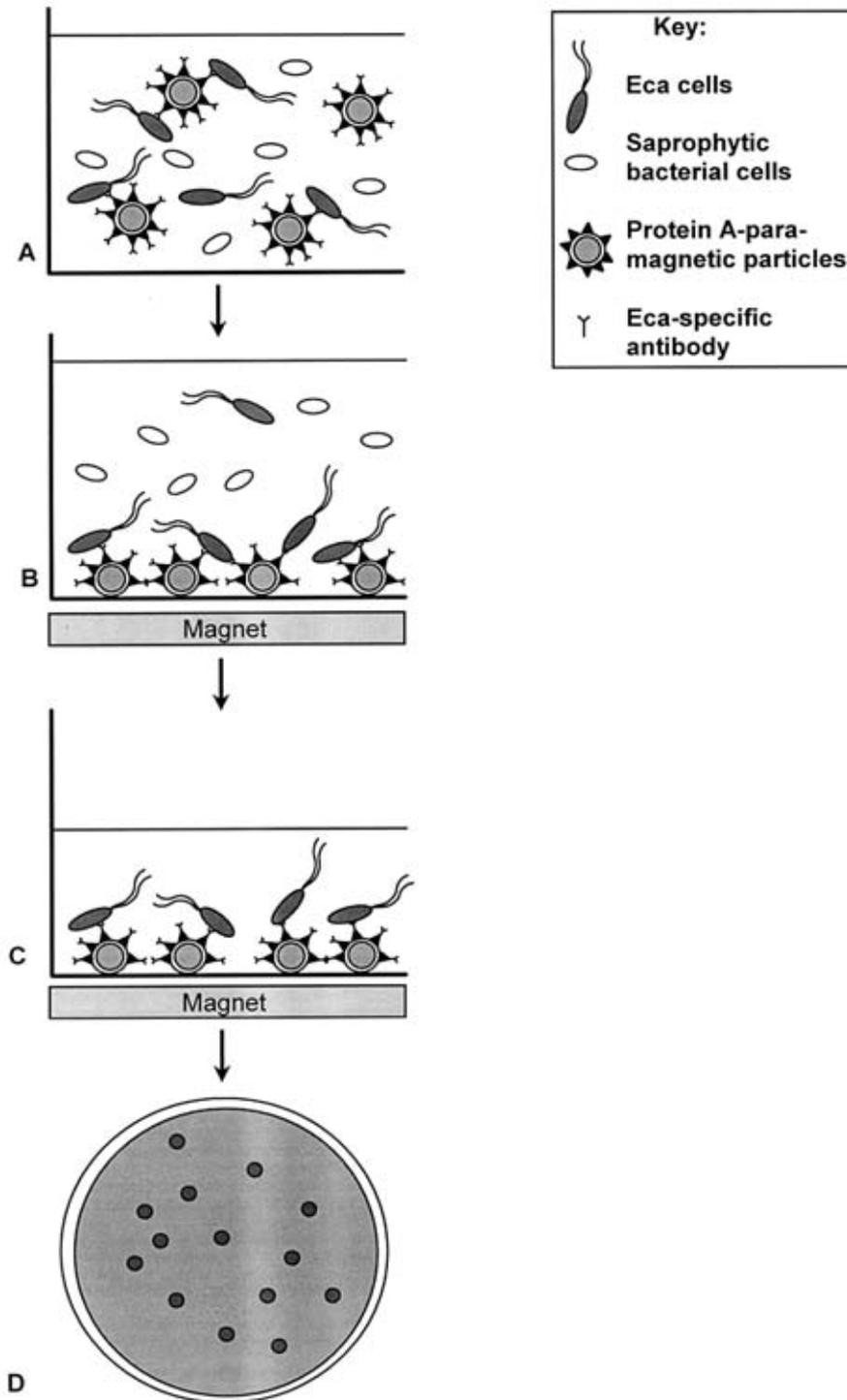


Fig. 2.1 Diagram of IMS-CVP: Bacterial cells, Eca-specific antibodies and protein A-magnetic particles in peel extract in microcentrifuge tube:
 A Before magnet applied.
 B After magnet applied.
 C As B but unbound bacteria washed away.
 D Magnet removed and bound Eca cells resuspended and inoculated on to CVP medium.

(a)



(b)



Fig. 2.2(a) Cavities formed by soft rot *Erwinia* colonies on CVP medium after 48 h incubation at 27 °C viewed from underside of Petri dish.

(b) Pattern of cavity formation by Ecc, Eca and Ech on CVP medium at different temperatures: each plate was inoculated with three strains of Eca (top row), Ecc (middle row) and Ech (bottom row) and plates 1, 2 and 3 incubated for 48 h at 27, 33.5 and 37 °C respectively.



Fig. 2.3 Dynal particle concentrator: Microcentrifuge tube holder containing tubes, with magnet bar half way in place.

A similar situation would arise if more than one Eca cell attach to each magnetic particle leading to the production of a single colony. Lastly, in common with the other immunologically based methods, only Eca serogroup I is detected.

Protocol

Materials

1. Diluent: Ringer solution/Tween/bovine serum albumin (RTB)*.
2. Antiserum against Eca serogroup I*.
3. Paramagnetic particles conjugated with protein A*.
4. Dynal MPC-M magnetic particle concentrator suitable for 10 microcentrifuge tubes (Fig. 2.3)*.
5. Automatic adjustable pipettes and tips (20-200 μ l, 200-1000 μ l)*.
6. Microcentrifuge tubes: 1.5 ml*.
7. Microcentrifuge tube rotary shaker*.
8. Vortex mixer*.
9. Microcentrifuge.
10. Pasteur pipettes.
11. Phosphate buffered saline (PBS)*.
12. CVP plates (dried)*.
13. Hot air ventilated incubator to dry CVP plates*.
14. Platform shaker*.
15. Incubators (27 °C and 33.5 °C).

* Details given in Appendix.

Procedures

1. Add 500 μ l of peel extract containing antioxidant DTT (see Section 1), 500 μ l of Eca anti-serum at the appropriate dilution made in RTB and 50 μ g ml⁻¹ protein A magnetic particles to a 1.5 ml microcentrifuge tube. Close, mix well on a vortex mixer and incubate for 1 h at room temperature while shaking on a platform or microcentrifuge tube shaker. Up to 10 tubes may be processed at a time when using the Dynal IMS system (Fig 2.2).
2. Remove any liquid in the tube cap by a pulse centrifugation or by a sharp hand shaking action.
3. Place tube(s) vertically in the Dynal particle concentrator, slide in the magnet bar and allow to stand for 2.5 min at room temperature inverting the rack twice during that time. The particles, bound to antibodies and Eca cells, together with some non-target bacteria and peel debris held by surface tension, are located in a spot on the side of the tube next to the magnet.
4. Remove supernatant while the magnet is still in place with a Pasteur pipette, taking care to leave behind a drop to avoid losing any particles while pipetting.
5. Remove magnet, wash the particles by adding 1 ml of RTB, mix as in step 1 and incubate for ca. 2 min at room temperature.
6. Wash a second and if necessary a third time by repeating steps 2 to 5, but in the last wash cycle

add 500 µl of RTB to obtain the original peel extract volume or a smaller RTB volume to concentrate the extract for an increase in sensitivity.

7. Prepare decimal dilutions (10^{-1} and 10^{-2}) of IMS-treated peel extract in phosphate buffered saline, for example by adding 100 µl to 900 µl buffer in a microcentrifuge tube, and immediately plate the undiluted IMS-treated peel extract and each dilution on to four CVP plates, two incubated at 27 °C and two at 33.5 °C for 48 h.
8. Determine cavity counts on all plates, using preferably those in the range of 25-200 colonies/cavities per plate, to calculate Eca numbers ml^{-1} peel extract.

Comments

- (i) The optimal antiserum dilution is dependent on antiserum source/batch and usually lies between 1/100 and 1/2000. If uncertain, the concentration should be estimated beforehand by determining the recovery level of Eca by IMS-CVP from aqueous suspensions of the bacterium (see Section 6), using serial dilutions of the antiserum within the mentioned range. Antiserum dilutions should be prepared on the day of testing.
- (ii) Cheaper alternatives to protein A magnetic particles are goat anti-rabbit antibody magnetic particles, but recovery by IMS is poorer.
- (iii) Mixing and shaking may be done by hand if facilities are not available provided they are done thoroughly and consistently.
- (iv) Confirmation of results may be done by streak-planting presumptive Eca colonies on CVP followed by identification using traditional biochemical tests as in Section 6 or directly by PCR assay as in Section 5.

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Appendix

Crystal Violet Pectate (CVP) medium (Hyman *et al.*, 2002)

Two CVP formulations based on Slendid type 440 polypectate from M. Burger Enterprises are available: CVP-S2 medium is more transparent and firmer, hence with reduced risk of early liquefaction in the presence of large numbers of erwinia colonies than CVP-S1 but it involves the mixing of two preparations sterilised separately.

(a) *CVP-S1 (One step CVP medium):*

Tryptone (Oxoid L42)	1.0 g
Tri-sodium citrate	5.0 g
NaNO ₃	2.0 g
CaCl ₂ .2H ₂ O (10 % aqueous solution)	10.2 ml
Crystal violet (0.075 % aqueous solution)	2.0 ml
NaOH (5 mol l ⁻¹)	2.6 ml
Agar (BDH Agar Powder)	4.0 g
Sodium polypectate (Slendid type 440)	18.0 g
Distilled water`	1000 ml

Preparation:

1. It is advisable to prepare not more than 500 ml at a time for ease of pouring.
2. Dissolve the ingredients, except the agar and pectate, in water after adjusting concentrations as appropriate, using a magnetic stirrer.
3. Add the polypectate slowly while the magnetic stirrer is on at near full speed to avoid lump formation.
4. Add the agar with the magnetic stirrer at half speed.
5. Autoclave for 15 min at 120 °C. Restore pressure slowly to avoid bubble formation within the medium. Mix gently by rotating the flask by hand.
6. Pour immediately ca. 18 ml per 9 cm petri dish as the medium cannot be readily re-melted. It is advisable to check the pH which should be ca. 7.0.
7. Chill plates for at least 2 h at 4 °C after pouring if to be used straight away. Otherwise, store plates at 4 °C for up 2 months in a sealed polythene bag.
8. Dry plates thoroughly before use to remove all surface water: place plates with lids ajar for 1 h at 45-50 °C in a ventilated oven, or in a laminar flow cabinet at room temperature for 4 h.

(b) *CVP-S2 (Two step CVP medium):*

Mix A:

Tryptone (Oxoid L42)	1.0 g
Tri-sodium citrate	5.0 g
NaNO ₃	2.0 g
CaCl ₂ .2H ₂ O (10 % aqueous solution)	10.2 ml
Crystal violet (0.075 % aqueous solution)	2.0 ml
Agar (BDH Agar Powder)	4.0 g
Distilled water`	500 ml

Mix B:

NaOH (5 mol l ⁻¹)	2.5 ml
Sodium polypectate (Slendid type 440)	18.0 g
Distilled water	500 ml

Preparation:

1. It is advisable to prepare not more than 500 ml at a time for ease of mixing and pouring.
2. Mix A: Dissolve the ingredients in water after adjusting concentrations as appropriate, using a magnetic stirrer.
3. Mix B: To the same volume of water as for Mix A, add NaOH first, then the polypectate slowly while the magnetic stirrer is on at near full speed to avoid lump formation.
4. Autoclave both Mix A and Mix B separately for 15 min at 120 °C. Restore pressure slowly to avoid bubble formation within the medium.
5. While still hot, mix by pouring Mix A in Mix B, shake gently by rotating the flask.
6. Pour immediately ca. 18 ml per 9 cm petri dish as the medium cannot be readily re-melted. It is advisable to check the pH which should be ca. 7.0.
7. Chill plates for at least 2 h at 4 °C after pouring if to be used straight away. Otherwise, store plates at 4 °C for up 2 months in a sealed polythene bag.
8. Dry plates thoroughly before use to remove all surface water: place plates with lids ajar for 1 h at 45-50 °C in a ventilated oven, or in a laminar flow cabinet at room temperature for 4 h.

Notes:

- (i) Store CaCl₂ (prepared fresh each week) and crystal violet stock solutions at 4 °C.
- (ii) The above recipe applies only if Slendid type 440 polypectate is used. If a different source is to be used, check first for toxicity and adjust the ratio of polypectate and agar and the pH to obtain the diagnostic cavities formed by soft rot erwinias.
- (iii) The final pH of CVP should be 6.9-7.2. If necessary, adjust by adding 1 mol l⁻¹ NaOH before pouring. It is easier to raise than to lower the pH in this medium.
- (iv) Tri-sodium citrate reduces growth and pit formation by pectolytic *Pseudomonas* species.
- (v) Although selectivity is affected by the addition of tryptone, this is necessary if Slendid pectate is used, to overcome toxicity attributed to the pectate preparation.
- (vi) In addition to BDH Agar Powder, Difco Agar Granulated and Oxoid L28 preparations can be used satisfactorily, but not Gibco-BRL Select Agar or Difco Agar Noble.

Buffers

(a) *Ringer-Tween-Bovine serum albumin (RTB)*:

1. Prepare quarter strength Ringer solution using Oxoid tablets (BR 52).
2. Add Tween 20: Sigma (P 1379) to a final concentration of 0.1 %.
3. Add Bovine serum albumin: Sigma (A 4378) to a final concentration of 0.1 %.

(b) *Phosphate buffered saline (pH 7.2-7.4)*:

As in Section 1: Appendix.

Polyclonal antibodies

Plant Research International, Adgen and most other sources, but it is best to check reaction to the different Eca serogroups.

Paramagnetic particles

Biomag protein A particles: Advanced Magnetics (AM4600B): store at 4 °C and shake well before use.

Equipment

Dynal particle concentrator (MPC-M).

Hot air circulating incubator (Vindon Scientific Ltd).

Magnetic particle concentrator (Dynal, 120.09).

Microcentrifuge tube rotary shaker (Dynal, MX3-159.09).

Automatic adjustable pipettes and tips (Gilson [Anachem] or Finpipette [Life Sciences]).

Vortex mixer: Stuart Scientific Co. Ltd (SA6).

Platform shaker: BDH (330/0114/00).

Microcentrifuge tubes: 1.5 ml and 2.0 ml: Fisher Scientific (TUL-150-150N and TUL-150-290U).

Immunofluorescence colony staining (IFC)

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Introduction

This method is a combination of immunofluorescence staining and colony counts in pour-plate selective medium (Van Vuurde, 1987; Fig. 3.1). Pour-plating reduces interference by non-target bacteria of growth of target bacteria because of the greatly reduced biomass per colony and physical separation of the colonies. As the aim is to obtain micro-colonies which are readily formed in pour-plates, it is possible to miniaturise the system by using multi-well tissue-culture plates, with considerable savings in handling time and antibodies.

The main advantage of the IFC method to quantify *E. c.* subsp. *atroseptica* (Eca) in peel extract is its sensitivity and consistency. As few as 10-50 Eca viable cells ml⁻¹ of peel extract can be detected, mostly independent of the background microflora (Van Vuurde & Roozen, 1990; Jones *et al.*, 1994). The method is also flexible, especially if the medium has been dried after colony growth, allowing staining and colony counting to be done at any time. Suspect results may be checked at a later date using different identification methods. Lastly, simultaneous detection of two pathogens, e.g. Eca and *E. chrysanthemi* (Ech), in the same preparation may be done with a double fluorescence staining procedure using two different antibodies and two fluorescent dyes. However, IFC suffers from several drawbacks, the most important being the requirement for antibodies against Eca cell surface components, which implies that only certain antibodies can be used, for example, IVIA-4G4 and 4F6 monoclonal antibodies or Plant Research International polyclonal antiserum, which are specific to only Eca serogroup I. This method at present does not allow the detection of the other Eca serogroups. In addition, high amounts of antibodies are needed if the standard procedure is used, a UV compound or stereo-microscope to detect fluorescein isothiocyanate (FITC)-stained colonies and, in the absence of automated counting facilities, labour intensive visual colony counting is required for precise Eca quantification.

Protocol

Materials

1. Semi-selective growth medium: PT*.
2. Dilution buffer: PBS-Ta*.
3. Washing buffer: PBS-Tb*.

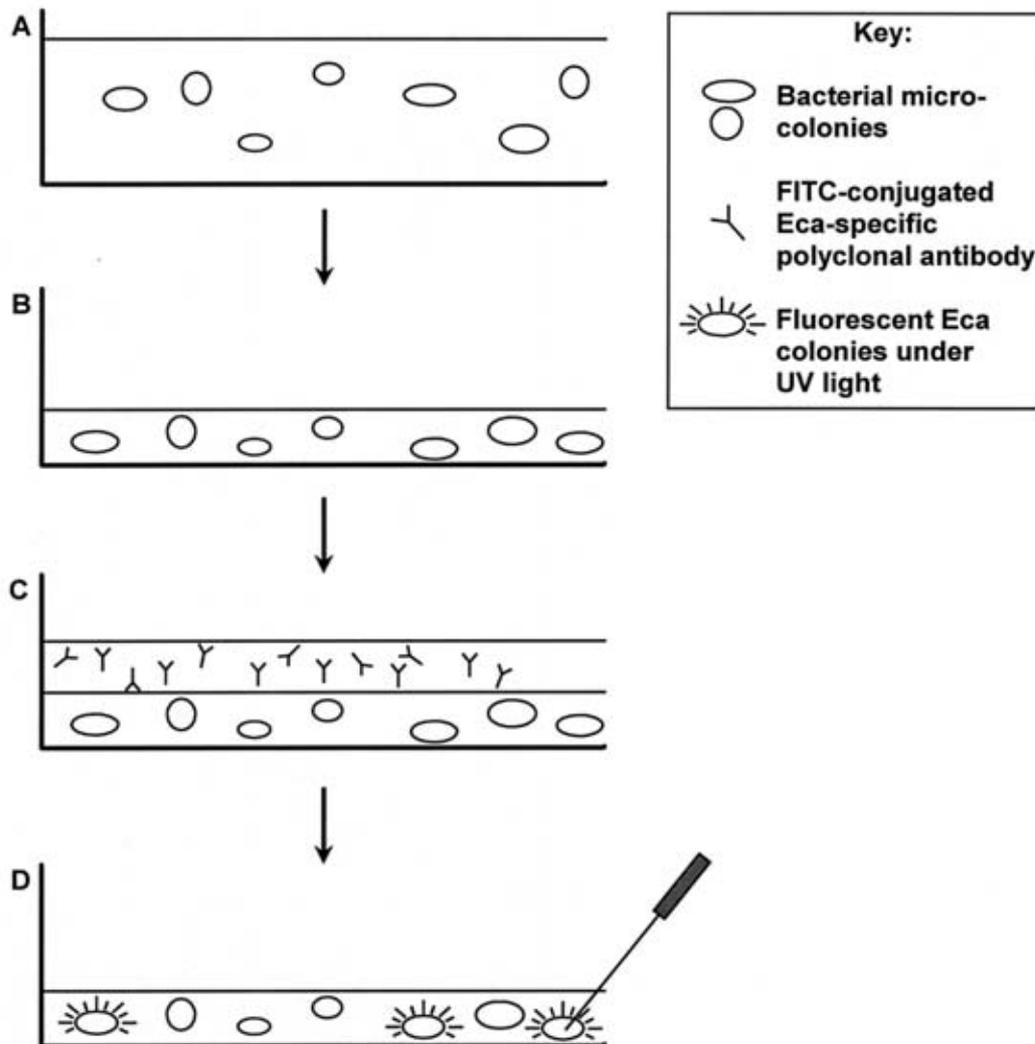


Fig. 3.1. Diagram of IFC (Direct):

A Bacterial colonies growing in PT medium in tissue culture well.

B PT medium after drying in hot air.

C Incubation with FITC-conjugated Eca-specific antibodies.

D Observation under UV or incident blue light at low magnification.

If necessary, the identity of a fluorescent colony may be verified by puncturing the colony with a needle or a fine capillary, followed by identification testing.

4. Eca-specific antibodies:
 Polyclonal antiserum: FITC-conjugated or non-conjugated
 (Plant Research International).
 Monoclonal antibody: IVIA-4G4 (REAL-DURVIZ)*.
 Polyclonal antiserum:
 goat anti-rabbit FITC-conjugated*.
 goat anti-mouse FITC-conjugated*.
5. Agarase stock solution*.
6. Phosphate buffered saline*.
7. Automatic adjustable pipettes and tips (2-20 μ l, 20-200 μ l, 200-1000 μ l)*.
8. Microcentrifuge tubes (1.5 ml)*.
9. Multi-well tissue-culture plates: 24- or 48- well plates*.
10. ELISA-plate shaker*.
11. Water bath.
12. Circulating hot air ventilated incubator set at ca. 50 °C to dry tissue culture plates*.
13. UV compound microscope or a stereo-microscope with blue light*.

* Details given in Appendix.

Procedures

1. Melt the PT medium and hold in a water bath at ca. 48 °C (see Note 1 below).
2. Prepare serial dilutions of peel extract containing antioxidant DTT (see Section 1): 10^{-1} and 10^{-2} dilutions obtained by adding 100 μ l of peel extract in 900 μ l phosphate buffered saline in microcentrifuge tubes.
3. Add 100 μ l or 50 μ l of undiluted and diluted peel extract to one well of 24-well or 48-well tissue culture plate respectively (see Note 2 below). Usually one well per dilution is adequate in routine testing.
4. Place tissue culture plate on plate shaker and, while shaking gently at low speed add, as soon as possible, 250 μ l or 125 μ l of molten PT medium to 24-well or 48-well plate respectively. Stop shaking ca. 5 sec after filling the last well, remove from shaker and replace lid. If a shaker is unavailable, ensure that PT medium and test material are mixed well by gentle hand rotation before the agar sets.
5. Incubate at 27 °C for ca. 40 h.
6. Dry tissue-culture plates with the lid removed in the drying oven for long enough to obtain a thin (ca. 1 mm thick) agar film. If in doubt, it is safer initially to under-dry than over-dry. If preferred, the drying step can be omitted (see Note 3 below).
7. Staining and washing: several protocols may be followed:

Prepare a working dilution of Plant Research International polyclonal antiserum and 4G4 monoclonal antibody in dilution buffer (PBS-Ta) containing agarase, if required, to a final concentration of 1 unit ml⁻¹ (see Note 4 below).

A. *Direct staining: using Plant Research International FITC-conjugated polyclonal antiserum*

- (i) Add 250 μ l or 125 μ l of antibody dilution to each well of 24- or 48-well tissue culture plates respectively.
- (ii) Incubate at 20 °C for 18 h or at 37 °C for 4 h with gentle shaking.
- (iii) Aspirate or gently invert the plate to remove the antibody dilution. Wash the wells by filling with washing buffer (PBS-Tb) and allow to stand for ca. 15 sec before removing the buffer. Repeat the procedure twice, but allow the plate to stand in the dark at room temperature for 15 min each time with dried plates or for 2 h each time in the case of non-dried plates, preferably with occasional gentle shaking (see Note 5 below). Take care that any torn fragment of the medium is not lost during washing especially when agarase is present.
- (iv) After the last wash, fill wells with 0.5 ml wash buffer (PBS-Tb), seal the plate with tape and store at 4 °C until required.

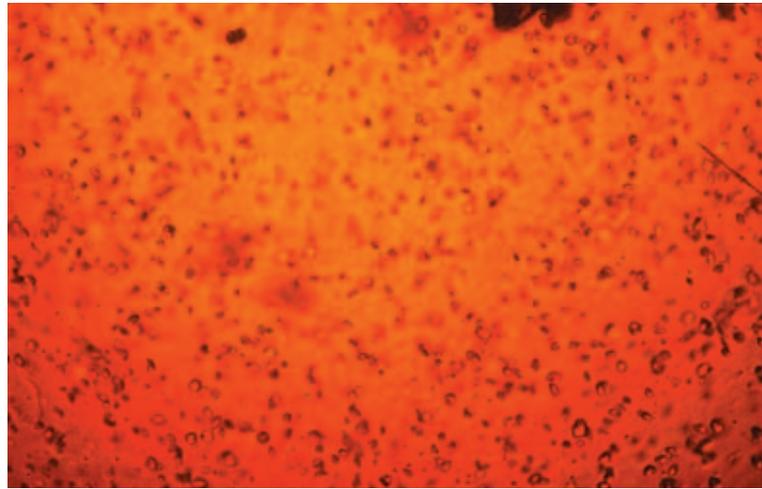
B. *Indirect staining: using monoclonal antibody IVIA-4G4 and goat anti-mouse FITC-conjugated antibodies*

- (i) Add monoclonal antibody dilution as in A (i).
 - (ii) Incubate as in A (ii).
 - (iii) Wash as in A (iii).
 - (iv) Add the FITC-conjugated antibody as in A (i).
 - (v) Incubate as in A (ii).
 - (vi) Wash as in A (iii).
 - (vii) Store plates as in A (iv).
8. Observe the stained preparation directly in the wells using a UV microscope at ca. 40 times magnification. FITC-stained colonies appear as bright green discs or cigar-shaped in a dark background, depending on their orientation in the medium (Fig. 3.2). The mean count from five random fields per well is multiplied by a factor (see Note 6 below) to obtain a count ml^{-1} peel extract. More reliable results are obtained with counts within the range of 5-25 colonies per field.

Notes:

- 1. The temperature of the PT medium should be 48-49 °C to avoid any deleterious effect on Eca cell viability whilst preventing rapid solidification of the medium which would inhibit mixing with the test material. It is also important that the final

(a)



(b)

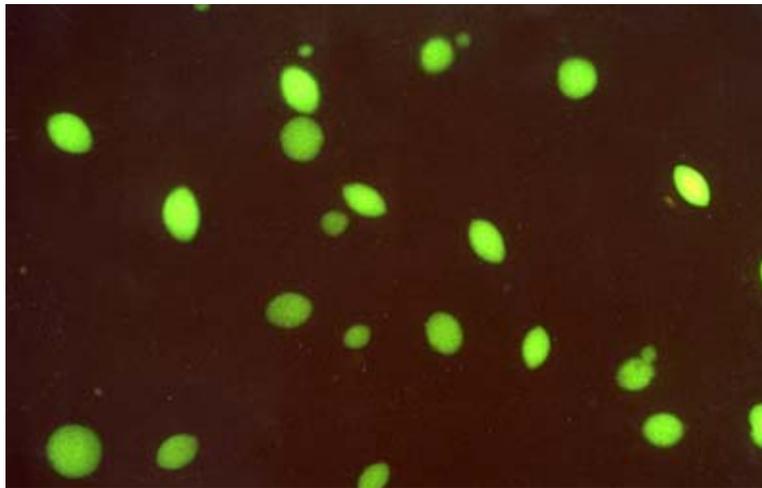


Fig. 3.2 Bacterial colonies in dried PT medium stained with FITC-conjugated Eca-specific antibodies, magnified 10 x 4 times:
(a) Total bacterial colonies under normal incident light.
(b) FITC-stained Eca colonies of same field under UV incident light.

concentration of agar in the wells should be in the range of 0.8-0.9 %, depending on the source, to facilitate drying, staining and washing.

2. The choice between 24- or 48-well tissue culture plates depends on the degree of accuracy required and the cost of the test: the larger the well, the greater the sample volume, but more antibodies are used.
3. The drying temperature and time depend primarily on the type of drying oven used and should be optimised for each oven, taking into account the degree of drying required. Drying favours faster staining and washing. However, if the agar film is over dried, distortion of the colonies occurs and reduced penetration of the antibodies into the medium may affect staining. Non drying of the medium overcomes these problems but constant refocussing to visualise the fluorescent colonies is necessary.

4. The working dilution of the different antibodies needs to be determined beforehand as it is dependent on their source and batch. As a guide, FITC-conjugated antiserum from Plant Research International and the commercial FITC-conjugated goat anti-rabbit or anti-mouse antibodies may be used at around 1 in 50 dilution whereas monoclonal antibody IVIA-4G4 is best used at 1-2 $\mu\text{g IgG ml}^{-1}$, e.g. IVIA/REAL-DURVIZ product diluted 1 in 100. If the amount of antibody is considered too high for large scale routine use, it is advisable to add agarase, which facilitates staining by softening the agar, hence allowing the use of more dilute antibodies. Working dilutions may be reduced by a factor of 2 to 4 depending on the quality of the antibody preparation.
5. Staining and washing proceed faster at 37 °C than at 20 °C. Washing procedures must be adapted to respond to the degree of interference caused by background fluorescence attributed to unbound antibodies especially in indirect IFC. The pH of the washing buffer should be 7.2 or higher for an optimal fluorescence. At a lower pH fluorescence is reduced but can be restored by washing again with a high pH buffer.
6. The multiplication factor to convert number of colonies per microscope field to number of colony forming units ml^{-1} is determined as follows: count the total number of colonies per well (x), then determine the mean number of colonies (y), from at least 5 random microscopic fields. The multiplication factor is obtained by dividing x by y. Repeat on several preparations to arrive at a reliable figure.

Comments

- (i) In routine testing, it may be sufficient to determine only the order of magnitude of contamination by making rough estimates with samples containing more than 10-20 fluorescent colonies per well.
- (ii) The tedium of colony counting may be overcome through automation using a computer-linked camera mounted on the microscope. As the cost of such facilities is steadily falling, they should become more affordable in the near future, even for relatively small scale operations.
- (iii) It is advisable to include a positive control in each plate consisting of peel extract to which ca. 10^3 Eca serogroup I cells ml^{-1} have been added (Van Vuurde & Van der Wolf, 1995).
- (iv) The presence of certain cross-reacting saprophytic bacteria, more prevalent in some countries than in others, may cause false positive results. As the numbers present are generally low, below the threshold density (10^2 - 10^3 cells ml^{-1}) for blackleg development (Bain *et al.*, 1990), these bacteria are unlikely to affect test results. However, it may be desirable to occasionally check the results, which can readily be done as follows: the identity of IFC-positive colonies can be confirmed by puncturing a colony from IFC preparations, briefly dried beforehand with tissue paper, under the microscope with a fine capillary tube or a platinum needle followed by isolation and characterisation using traditional biochemical tests as in Section 6 or by PCR assay as in Section 5 (Van Vuurde & Van der Wolf, 1995; Van der Wolf *et al.*, 1996). Isolation is more likely to be successful when the medium has not been dried.

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Appendix

PT Medium (PT), pH 7.0

Polygalacturonic acid (Sigma, P 1879)	5.0 g
Tryptone (Oxoid, L42)	0.5 g
NaNO ₃	1.0 g
K ₂ HPO ₄	4.0 g
MgSO ₄ .6H ₂ O	0.2 g
Agar (BDH Agar Powder)	8.0 g
Tween 20 (Sigma, P 9416)	0.1 ml
1 mmol ml ⁻¹ NaOH	17.0 ml
Distilled water	to 700 ml

1. Melt medium by steaming, check and adjust the pH if necessary, prepare aliquots of 10-20 ml and autoclave at 120 °C for 20 min.
2. When required, melt by steaming and hold in water bath at ca. 48 °C during use.

Note: Agar concentration may have to be altered if a different source, hence gelling capacity, is used.

Phosphate buffered saline (PBS), 10x concentrated stock solution (0.1 M, pH 7.2-7.4)

Stock solution 1; as in Section 1: Appendix.

Dilution buffer (PBS-Ta)

Dilute PBS stock solution 1 in 10 with distilled water and add Tween 20 to a final concentration of 0.01 %.

Washing buffer (PBS-Tb)

Dilute PBS stock solution 1 in 100 with distilled water and add Tween 20 to a final concentration of 0.1 %.

Agarase stock solution

1. Dissolve lyophilised agarase (Sigma, A 6306) in distilled water to a final concentration of 600 units ml⁻¹.
2. Filter sterilise using a 0.2 µ filter (Nalgene, ISN Biomedicals Ltd, 402 0800 11) and store in a screw capped vial at 4 °C.
3. When required, aseptically prepare enough of a decimal dilution for immediate use and add the necessary volume to the antibody solution for a final concentration of 1 unit ml⁻¹.

Antibody solutions

Eca-specific polyclonal antiserum and FITC-conjugated polyclonal antiserum

(Plant Research International)

- Storage: as in Section 1: Eca-specific antibodies.
- When required, prepare a working dilution, usually 1 in 50 in PBS-Ta without agarase, or 1 in 100 to 1 in 200 in PBS-Ta with agarase. Prepare only enough for immediate use.

Monoclonal antibodies (REAL-DURVIZ IVIA-4G4)

- Storage: as in Section 1: Eca-specific antibodies.
- When required, prepare a working dilution, usually 1 in 50 containing 1-2 µg ml⁻¹ in PBS-Ta without agarase. Prepare only enough for immediate use.

FITC-conjugated goat anti-mouse antibodies (Sigma, F 0257)

- Storage: as in Section 1: Eca-specific antibodies.
- When required, prepare a working dilution, usually 1 in 50 in PBS-Ta without agarase or 1 in 100 or 1 in 200 in buffer with agarase. Prepare only enough for immediate use.

Equipment

Multi-well micro plates:

24-well (BDH, 402/0450/10).

48-well (BDH, 402/0450/09).

Automatic adjustable pipettes and tips:

Gilson (Anachem) or Finpipette (Life Sciences).

Multi-well plate shaker:

Stuart Scientific (SO5) or Labsystems (Wellmix shaker, WM 506).

Circulating hot air incubator (Vindon Scientific Ltd)

Magnification system:

UV compound microscope: (Leica, Nikon, Olympus or Zeiss) equipped with 4x and 10x objective lenses, filterpack for blue light/FITC and epifluorescence illumination with pressure mercury lamp (50, 100 or 200 watts).

Epi-fluorescent stereo microscope: Leica or recent Wild stereo microscope models with zoom system up to 50x objective lens 1x or 1.5 equipped with a Leica fluorescence set containing a GFP plus filterpack and epifluorescent illumination with high pressure mercury lamp (50 watts).

Notes

The objectives of the compound microscope should have a Numeric Aperture of 0.12 or higher.

The working distance of the 10x objective is too short to look into the well, but may be used by inverting the plate and looking through the bottom.

Special objectives with magnifications of 10x and higher and sufficiently long working distances are advisable, e.g. Nikon 10x and 20x with working distance of 20 mm.

Alternatively, the advocated Leica stereo fluorescence microscope allows one 16 mm well to be observed in one microscope field with the possibility to zoom in for the smaller colonies with increasing light intensity. The system is superior to the compound microscope set up for isolation because of the large working distance and the non-mirror inverted view.

Section 4

Enrichment ELISA (E-ELISA)

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Introduction

The enzyme-linked immunosorbent assay (ELISA) is a commonly used method to detect and identify, but not to quantify, bacterial plant pathogens. Its low sensitivity, $>10^6$ cells ml⁻¹ peel extract (Hyman & Pérombelon, 1990), makes it unsuitable for assessing *E. c. subsp. atroseptica* (Eca) tuber contamination occurring at lower levels. Sensitivity may be increased by incorporating an enrichment step for Eca *in situ* in the microtitre plate wells before the ELISA (Gorris *et al.*, 1994); provided the initial Eca cell density is $>10^2$ ml⁻¹, the pathogen can multiply to $>10^6$ cells ml⁻¹ when it becomes detectable. The selective enrichment, a patented procedure by IVIA, improves immunological and molecular detection and isolation of plant pathogenic bacteria (López *et al.*, 1997). Although the standard ELISA does not differentiate between viable and non-viable cells, living cells are detected following enrichment unless the equivalent of $>10^6$ dead cells or cell debris ml⁻¹ are initially present. However, as *E. c. subsp. carotovora* (Ecc) is often present and will also be enriched and because most Eca polyclonal antisera contain some Ecc cross-reacting soluble antibodies, it is advisable to use Eca specific monoclonal antibodies when using E-ELISA for Eca detection.

In the recommended method, the Double Antibody Sandwich Indirect (DASI) format of ELISA is applied using first, polyclonal Eca antibodies to coat the wells and capture the bacteria and second, Eca serogroup I specific monoclonal antibodies to bind to the target bacteria/antigens (Fig 4.1). Only one known cross-reacting saprophytic bacterium, *Comamonas* sp., out of hundreds tested, has been found to react with the monoclonal antibodies, but it fails to multiply under the enrichment conditions. Enrichment is generally not affected by such factors as cultivar and potato stocks harbouring different microflora, but anaerobic incubation conditions are essential to favour growth of the soft rot erwinias (Gorris *et al.*, 1994). However, as the available monoclonal antibodies, IVIA-4G4 and 4F6, are specific to only Eca serogroup I, it is inadvisable to use this method to detect Eca contamination of seed potatoes in regions where serogroup I does not predominate. The method can be used in epidemiological studies involving inoculation with strains of this serogroup.

The degree of Eca quantification which can be achieved with E-ELISA depends on the protocol followed; in that described in the IVIA/REAL-DURVIZ kit, DASI-ELISA is applied to parallel peel extract samples, one before and the other after enrichment. Contamination level is divided into three categories: non-enriched and enriched samples yielding -/+ results respectively indicate a contamination level $>10^2$ and $<10^6$ Eca live cells ml⁻¹, +/+ results indicate the presence of $>10^6$ live and/or dead cells ml⁻¹ and -/- results indicate $<10^2$ Eca cells ml⁻¹. Alternatively, the method can be made more

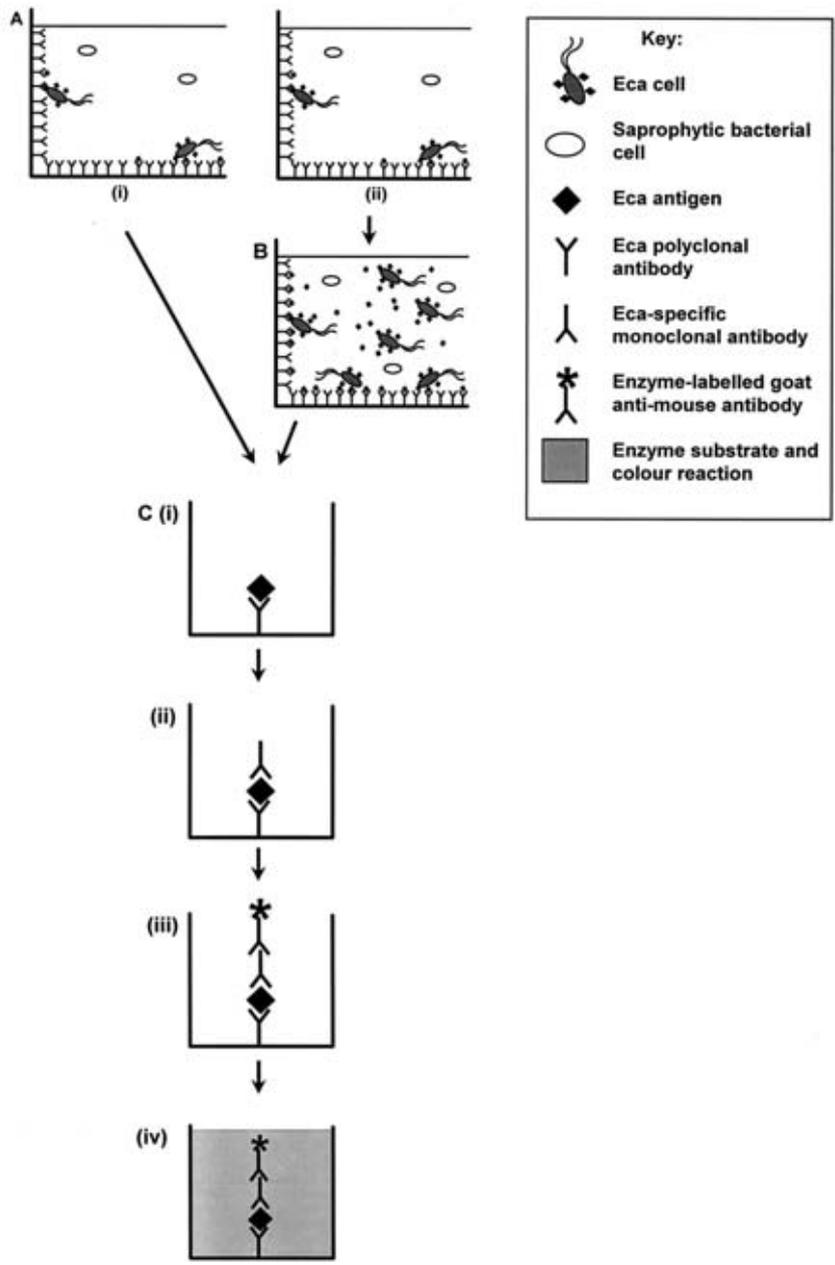


Fig. 4.1 E-ELISA diagram:

A Bacterial cells in tuber peel extract in microtitre plate well precoated with Eca-specific polyclonal antibodies:

- (i) Without D-PEM enrichment medium.
- (ii) With D-PEM enrichment medium.

B Preferential enrichment of Eca after 40 h anaerobic incubation at 27 °C.

C DASI ELISA:

- (i) Well washed.
- (ii) Eca-specific monoclonal antibodies added, incubated and well washed.
- (iii) Alkaline phosphatase-conjugated goat anti-mouse antibodies added, incubated and well washed.
- (iv) Alkaline phosphatase substrate added, incubated and colour intensity read on an ELISA plate reader.

quantitative by additionally enriching serial dilutions of the peel extract, thereby allowing determination of contamination levels between $>10^2$ and $<10^6$ Eca cells ml^{-1} (Table 4.1), assuming that the next positive dilution above a negative one can be expected to contain at least 10^2 Eca cells ml^{-1} . Experience suggests that instances when large numbers of dead cells are present rarely occur especially when washed potato tubers are tested. Finally, although contamination levels can only be semi-quantified, which in most cases would be adequate, an important advantage of E-ELISA is that it utilizes a known technique already widely used and which can be automated.

Table 4.1. Grades of peel extract contamination by *E. c. subsp. atroseptica* (Eca) determined from E-ELISA results, assuming that the lowest detection levels before and after enrichment are $>10^6$ and $>10^2$ Eca cells ml^{-1} respectively.

Non-enriched peel extract dilution	Enriched peel extract dilution				Eca peel extract contamination (2x cells ml^{-1})
	1/2 ^a	1/20	1/200	1/2000	
-	+	-	-	-	$>10^2 <10^3$ live cells
-	+	+	-	-	$>10^3 <10^4$ live cells
-	+	+	+	-	$>10^4 <10^5$ live cells
-	+	+	+	+	$>10^5 <10^6$ live cells
+	+	+	+	+	$>10^6$ live cells
+	+	-	-	-	$>10^6$ dead cells & $>10^2 <10^3$ live cells

^a Peel extract diluted by half initially by addition of an equal volume of PBS-DIECA.

Protocol

Materials

1. Eca polyclonal antibodies**.
2. Eca monoclonal antibodies (IVIA-4G4)**.
3. Alkaline phosphatase-conjugated goat anti-mouse antibodies**.
4. Carbonate buffer*.
5. Enrichment medium (D-PEM) (double strength)**.
6. Positive and negative controls**.
7. Extraction buffer (PBS-0.02% DIECA)*.
8. PBS buffer: see Section 2: Appendix.
9. Washing buffer (PBS-T)*.

10. Monoclonal antibody dilution buffer (PBS-BSA)*.
11. Alkaline phosphatase substrate (*p*-nitrophenyl phosphate)*.
12. Alkaline phosphatase substrate buffer*.
13. ELISA microtitre plates*.
14. Microtitre plate sealing film*.
15. Microtitre plate washer*.
16. Microtitre plate reader*.
17. Automatic adjustable pipettes (2-20 μ l, 20-200 μ l, 200-1000 μ l), multichannel pipette (50-200 μ l) and tips*.

Reagent troughs*.

19. Microcentrifuge tubes (1.5 ml)*.

Magnetic stirrer.

Vortex mixer.

22. Incubators (25 °C and 37 °C).
- 23.. Airtight bag or gas jar (BDH) with anaerobic gas system and indicator (Oxoid)*.
24. IVIA/REAL-DURVIZ kit*.

* Details given in Appendix.

** Also included in IVIA/REAL-DURVIZ kit.

Procedures

(IVIA/REAL-DURVIZ kit protocol modified by enriching additionally peel extract dilutions).

Five sequential steps are involved:

- | | |
|---|-------|
| (a) Coating wells with capture antibodies | Day 1 |
| (b) Preparation of peel extract dilution/s | Day 2 |
| (c) Setting up peel extract enrichment | Day 2 |
| (d) DASI-ELISA on non-enriched peel extract | Day 2 |
| (e) DASI-ELISA on enriched peel extract | Day 3 |

(a) Coating wells with capture antibodies

1. Prepare polyclonal antibodies for coating wells by diluting in carbonate buffer. Use preferably purified immunoglobulins, usually 1-2 μ g ml⁻¹, or as recommended in the kit (1 in 100 dilution).
2. Add 200 μ l of coating antibody solution to each well of two microtitre plates for testing enriched and non-enriched peel extract using a multi-channel pipette, seal tightly with a microplate sealing film and incubate overnight at 4 °C or 4 h at 37 °C.
3. Wash wells three times by hand or preferably in a plate washer with washing buffer (PBS-T). Ensure that the plate is dry after last wash by washer aspiration or by thorough pounding of the plate by hand on the absorbent tissue paper. Avoid touching the underside of the wells.

(b) Preparation of peel extract dilutions

Add 1 ml of PBS-DIECA to an equal volume of peel extract immediately after extraction (see Section 1). If a more precise quantification is required, prepare two or three decimal dilutions in PBS of above 1:1 dilution of peel extract to give a final concentration of 1/20, 1/200 and 1/2000 (e.g. 100 μ l + 900 μ l PBS in a microcentrifuge tube).

(c) *Setting up peel extract enrichment*

1. Add 100 µl of peel extract dilutions to two replicate wells per test material in one of the two antibody-coated microtitre plates. Add also 100 µl of at least two positive and negative controls per microtitre plate (see Comments below).
2. Add 100 µl of D-PEM to all wells, using a multichannel pipette.
3. Incubate microtitre plates covered with a loosely fitted lid anaerobically in a bag or gas jar at 25-27 °C for 40 h according to manufacturers instructions. Ensure that the plates remain horizontal at all times.

(d) *DASI-ELISA on non-enriched peel extract*

1. Add 100 µl of peel extract diluted 1:1 with PBS-DIECA to two replicate wells per test material in the second antibody-coated microtitre plate. Add also 100 µl of at least two positive and negative controls per microtitre plate (see Comments below).
2. Add 100 µl PBS to all wells using a multichannel pipette.
3. Seal plates with sealing film and incubate for 4 h at 37 °C or overnight at 4 °C.
4. Remove sealing film and wash wells as in (a) 3.
5. Add to each well 200 µl of monoclonal IVIA-4G4 at the recommended concentration, 0.1 µg ml⁻¹ or 1 in 1000 PBS-BSA when using kit monoclonal antibody preparation. Seal plates and incubate at 37 °C for 2 h.
6. Wash wells as in (a) 3.
7. Add to each well 200 µl of the recommended dilution of commercial anti-mouse alkaline phosphatase-conjugated IgG (0.1 µg ml⁻¹ or 1 in 1000 in PBS-BSA when using kit antibody preparation. Seal plates and incubate at 37 °C for 2 h.
8. Wash wells as in (a) 3.
9. Prepare a 1 mg ml⁻¹ alkaline phosphatase substrate solution of *p*-nitrophenyl phosphate diluted in alkaline phosphatase substrate buffer, and add 200 µl to each well.
10. Incubate sealed plates at room temperature in the dark and monitor hydrolysis of the substrate (*p*-nitrophenyl phosphate) to *p*-nitrophenol, which under alkaline conditions confers a yellow coloration to the wells, by carrying out sequential readings of absorbance values of the wells in a plate reader at A405 nm after 15, 30, 60 and 90 min. Note that use of some plate reader models allow automatic computation and printing of the results or their down-loading to a computer.

A useful cut-off point for positive results is when absorbance values are at least twice as great as those of the negative control with an absorbance value < 0.2-0.3.

(e) *DASI-ELISA on enriched peel extract*

After step (c) 3, proceed as for DASI-ELISA on non-enriched peel extract, steps (d) 4 to (d) 10.

Estimation of Eca numbers in peel extract

DASI-ELISA results obtained with non-enriched and enriched serially diluted or non-diluted peel extract are considered jointly when determining Eca numbers in peel extract as outlined in Introduction and summarised in Table 4.1.

Comments

- (i) When using the IVIA/REAL-DURVIZ kit, use the recommended dilutions of both the polyclonal and monoclonal (IVIA-4G4) antibodies provided. When other antibodies are to be used, such as the Eca-specific monoclonal antibody, 4F6, (see Section 1) and a different polyclonal antiserum, first optimise their concentrations by testing a range of dilutions on Eca-free peel extract and peel extract inoculated with 10^6 cells ml⁻¹ of a known Eca serogroup I strain. The optimal dilutions are those which give the least background with negative controls and absorbance readings of ca. 1.0 in 30-60 min for positive controls.
- (ii) *Controls*
Positive controls:
(a) Suspension containing 10^7 cells ml⁻¹ of an Eca serogroup I strain or positive control in kit.
(b) Eca-free peel extract inoculated with Eca strain in comment (a) to a final concentration of 10^7 cells ml⁻¹.
Negative controls:
(a) PBS buffer or negative control in kit.
(b) Eca-free peel extract (stock extract can be stored in small volumes at -20 °C for up to one year).
- (iii) Avoidance of cross-contamination between wells is important. Care should be taken when pipetting reagents into wells not to touch the wells with the pipette tips, especially when adding the enrichment medium. Check that adjustable pipettes are in good working order and calibrated properly. Use reagent troughs with multichannel adjustable pipettes. It is advisable to use dedicated multichannel pipettes for dispensing enzyme-conjugated antibodies.
- (iv) The reliability of E-ELISA in quantifying Eca contamination depends on the value of the lower detection level of the method (10^2 cells ml⁻¹) not being affected by peel extract microflora and potato cultivar. However, occasionally a higher detection level of 10^3 cells ml⁻¹ may be found which lowers sensitivity of the method by a factor of 10.
- (v) Fewer peel extract dilutions need be tested if precise quantification of Eca populations is not required, e.g. 1/2, 1/20 and 1/200.
- (vi) Confirmation of results may be done on enriched peel extract by (a) streak-plating on CVP followed by identification of presumptive *Erwinia* colonies formed using traditional biochemical tests as in Section 6 or (b) PCR assay as in Section 5 after diluting by 1/100 to avoid PCR inhibition.

Trouble shooting notes

Colour fails to develop

One step has been omitted; incorrect use of buffers or antibodies or phosphatase substrate: loss of activity; errors in dilutions.

Advice: Use reliable positive controls; pre-test conjugate on substrate.

Erratic well coloration

Incomplete washing; error in well loading sequence; well not completely coated; cross-spillage between wells.

Conjugate or substrate concentration too high; incomplete coating of well walls resulting in adsorption of conjugate.

Advice: Use reliable negative controls, preferably randomly placed, in each plate; increase washing cycles; handle plates carefully with lids on; use pre-determined loading pattern.

Check shelf life of alkaline phosphatase substrate and substrate buffer.

Lack of reproducibility

Incorrect loading or use of controls; reading at wrong wavelength or malfunction of plate reader.

Advice: Check pipettes are being used properly; check plate reader.

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Appendix.

IVIA/REAL-DURVIZ kit: (1000 tests per kit)

Double strength pectate enrichment medium (D-PEM; Meneley & Stanghellini, 1976)

MgSO ₄	0.64 g
(NH ₄) ₂ SO ₄	2.16 g
K ₂ HPO ₄	2.16 g
Sodium polypectate (Slendid type 440; M. Bulger Enterprises)	3.4 g
Distilled water	to 1000 ml

1. Dissolve the first three salts separately in 300 ml distilled water. Heat if necessary.
2. Mix together in the order of the recipe (a precipitate may appear if this is not done) and make up to 1000 ml with distilled water.
3. Suspend 3.4 g of the polypectate in 5 ml of absolute ethanol and add to the salts solution, mixing well using a magnetic stirrer.
4. Steam until the polypectate is completely dissolved before adjusting the pH to 7.2, if necessary.
5. Prepare small aliquots (e.g. 50 ml) and sterilise by autoclaving at 120 °C for 15 min. Store at 4 °C. Once open, do not re-use to avoid contamination.

Antibodies

Included in IVIA/REAL-DURVIZ kit

Coating Eca polyclonal antiserum¹.

Eca monoclonal antibody IVIA-4G4².

Alkaline phosphatase-conjugated goat anti-mouse polyclonal antibodies³.

Working dilutions: ¹ 1 in 100, diluted in carbonate buffer.

² 1 in 1000, diluted in PBS-BSA.

³ 1 in 1000, diluted in PBS.

Alternative antibodies:

Any suitable Eca-specific polyclonal antiserum.

Eca monoclonal antibody 4F6 from Agdia (LINARIS GMBH).

Alkaline phosphatase-conjugated goat anti-mouse antibodies: Sigma, A 3562; Boehringer Mannheim, 1 198 661.

Working dilutions: These should be determined in each case.

Carbonate buffer pH 9.6

Na ₂ CO ₃	1.59 g
NaHCO ₃	2.93 g
NaN ₃	0.20 g
Distilled water	to 1000 ml

Dissolve salts in distilled water and store at 4 °C.

Note: The addition of sodium azide (NaN₃) to increase shelf life is optional. If omitted, prepare buffer fresh each time. Sodium azide is a toxic substance and can cause an explosion when dry; take care to dispose of all spent buffer safely. Use recommended safety precautions when weighing and handling.

Phosphate buffered saline (PBS) stock solution: (10x concentrated)

As in Section 1: Appendix. When the buffer is required, dilute 10 times in distilled water.

Washing buffer (PBS-T)

Add Tween 20 to PBS buffer to a final concentration of 0.05 %.

Store at room temperature for not more than 2-3 days.

Dilution buffers

PBS Tween (PBS-Ta)

As in Section 3: Appendix.

PBS-DIECA

Add antioxidant sodium diethyldithiocarbamate (DIECA) to PBS buffer to a final concentration of 0.02 %. Store at 4°C for about one week.

PBS-BSA

Add bovine serum albumin (Sigma, A 7030) to PBS buffer to a final concentration of 0.5 %. Prepare a fresh solution for each use.

Alkaline phosphatase substrate buffer

Diethanolamine	97.0 ml
NaN ₃	0.5 g
Distilled water	to 1000 ml

Mix the diethanolamine with 600 ml distilled water.

Adjust pH to 9.8 with 5 M HCl and make up to 1000 ml with distilled water.

The addition of sodium azide (NaN₃) at 0.05 % to increase shelf life is optional.

Store at 4 °C.

Note: Prepare fresh for each use if sodium azide is not added. See Note on safety precautions for handling sodium azide given for carbonate buffer above.

Alkaline phosphatase substrate solution

Dissolve one 5 mg *p*-nitrophenyl phosphate tablet (Sigma, 104-105) in 5 ml of alkaline phosphatase substrate buffer. Prepare a fresh solution for each use.

Equipment

ELISA microtitre plates (Nunc, Polysorp F96, Life Technologies)

For anaerobic incubation (follow manufacturers instructions):

BDH Airtight bag: Vacuum bag sealer (235/0461/00). and polythene sleeve (235/0461/50); or gas jar.

Oxoid anaerobic gas system (AN 25A). and anaerobic indicator (BR 55).

Automatic adjustable pipettes, multichannel pipette and tips:

Gilson (Anachem) or Finpipette (Life Sciences).

Microcentrifuge tubes: 0.5 ml and 1.5 ml: Fisher Scientific (TUL-150-010G and TUL-150-150N)

Microtitre plate reader (Labsystems, Multiskan MS, 51118037).

Microtitre plate washer:

Labsystems (Wellwash 4 Mk. 2, WW006, or Cellwash, CW018).

Microtitre plate sealing film (Anachem, 100-SEAL-PLT).

Reagent reservoirs (Anachem, 175-RBAS-010).

DNA amplification by polymerase chain reaction (PCR)

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Introduction

The method is based on the use of *E. c.* subsp. *atroseptica* (Eca)-specific DNA primers to amplify a targeted Eca DNA fragment enzymatically by polymerase chain reaction (PCR) to facilitate detection of both live and dead cells (Hensen & French, 1993). Millions of copies of the target DNA sequence are synthesised in less than 2 h through the activity of a heat stable enzyme (*e.g.* *Taq* polymerase) able to copy DNA in a series of cycles each consisting of three steps: (i) separation (denaturation) of the double stranded template DNA of the target organism by heating; (ii) annealing (hybridisation) of each primer DNA to a homologous region of single stranded DNA by cooling and (iii) extension when new DNA strands complementary to the template sequence are synthesised through DNA- polymerase activity (Fig. 5.1). The newly synthesised DNA strands become further templates in subsequent cycles resulting in an exponential amplification of specific DNA fragments which can then be readily detected by different methods.

The PCR method is the most rapid of the four detection methods described here and, more importantly, allows the detection of Eca regardless of serogroup. Recently a PCR-based method (Q-PCR) has been developed to estimate the number of Eca cells in potato tuber peel extract by relating the amount of Eca DNA to that of a known number of *Escherichia coli* cells which had been added to the peel extract (Hyman *et al.*, 2000). It is at present the method of choice for assessing Eca potato seed contamination. However, sensitivity of the PCR method for the detection of Eca in peel extract without prior DNA purification is poor; at least 10^6 cells ml⁻¹ are required, partly because of the small volume of test material used in a PCR reaction (1-5 µl), but mainly because of the presence of PCR inhibitory substances in peel extract (Van der Wolf *et al.*, 1996). It is, therefore, essential to remove these substances prior to PCR testing, taking the opportunity to concentrate the target DNA at the same time. This can be achieved in different ways (López *et al.*, 1997), three of which are described below: one involving Eca enrichment which does not allow quantification and two based on total DNA extraction.

Three stages are involved in the PCR method for Eca detection and quantification in peel extract: (i) removal of PCR inhibitors in peel extract and concentration of target DNA, (ii) PCR DNA amplification and (iii) detection and quantification of the amplified DNA. The detection limits obtained with the PCR method depend on the procedures used for the three stages and range from 10^1 to 10^3

Eca cells ml⁻¹, which is about the same as that obtained by the three immunological methods described here. The three stages are described below.

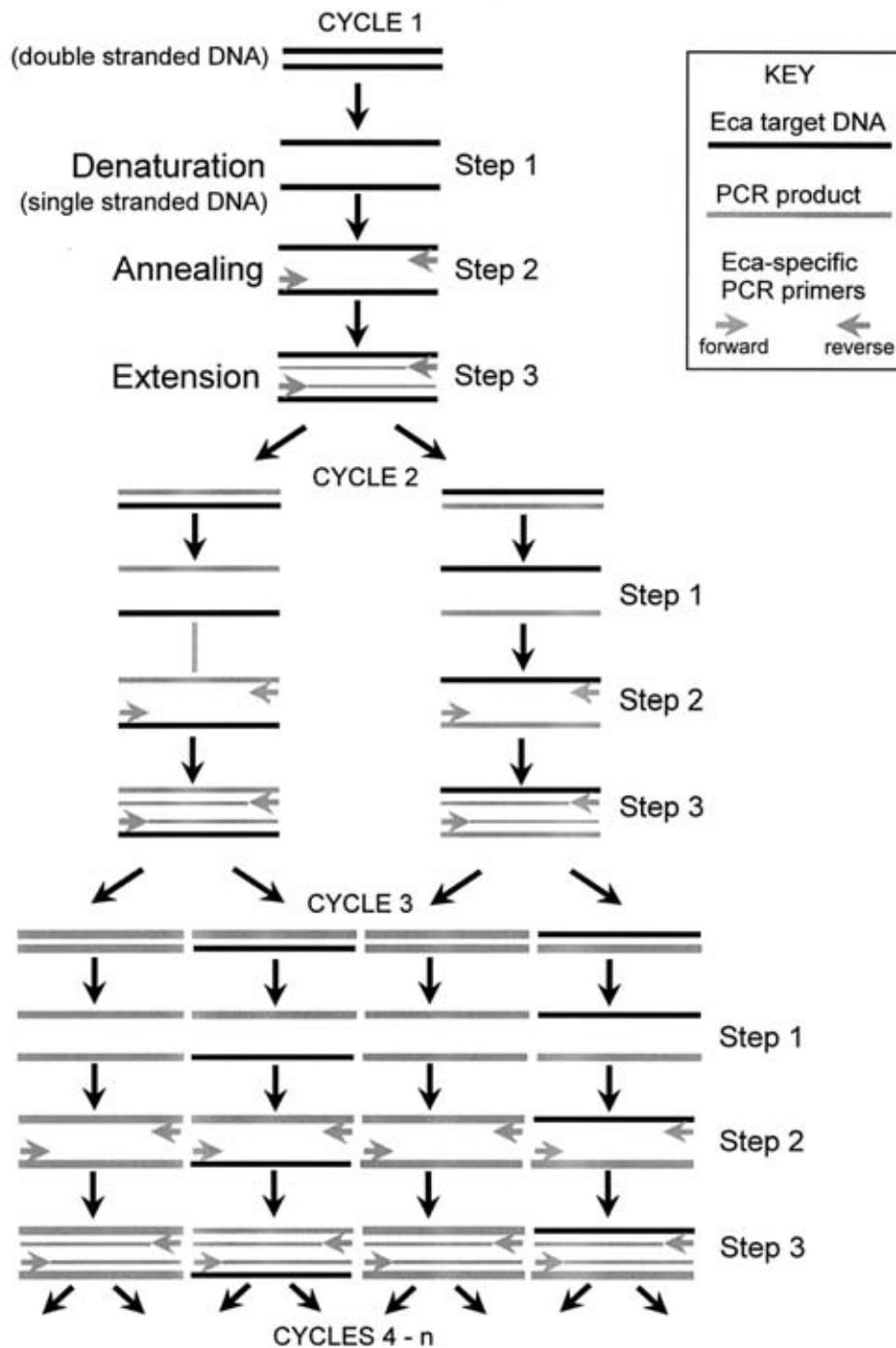


Fig. 5.1 Diagram of DNA amplification by PCR.

A. Peel extract treatment

(a) *E. c. atroseptica* enrichment on CVP (Hyman *et al.*, 1997).

Background

Eca is enriched by a factor of 10^5 when peel extract is inoculated on to a selective medium, CVP, and incubated overnight at 27 °C allowing PCR inhibitory substances (phenolics, carbohydrates, humic acids, etc.) in the peel extract inoculum to be absorbed into the medium. Aqueous suspensions of bacteria from the resulting micro-colonies are diluted and washed by centrifugation to remove PCR inhibitory substances released from the medium before PCR testing. Growth of Eca is not inhibited by overcrowding by other bacteria present in peel extract, as distinct micro-colonies are formed in the short incubation time. The sensitivity obtained, using De Boer & Ward (1995) Eca primers and amplified DNA detection by gel electrophoresis, permits detection of 10^1 - 10^2 cells ml⁻¹.

The method is simple and requires little equipment and reagents. Moreover, only live Eca cells are detected. Although hands on time may be short, an over-night incubation period is necessary. However, it cannot be used readily to quantify Eca contamination in tuber peel extract. This difficulty can be overcome, as in the case of the Enrichment ELISA method (Section 4), by applying PCR assay to bacterial suspensions recovered from CVP plates inoculated with serial dilutions of the peel extract in order to determine the lowest negative dilution.

Protocol

Materials

1. CVP plates*.
2. Sterilised ultra-pure water*.
3. Sterilised microcentrifuge tubes 1.5 ml*.
4. Automatic adjustable pipettes (2-20 µl, 20-200 µl, 200-1000 µl) and tips*.
5. Disposable gloves.
6. Incubator (27 °C).
7. Microcentrifuge.
8. Vortex mixer.

* Details given in Appendix.

Procedures

1. Plate 100 µl aliquots of undiluted peel extract with antioxidant DTT (see Section 1) on two replicate CVP plates and incubate at 27 °C for 20 h.
2. Suspend the bacterial micro-colonies from each plate in 500 µl sterile ultra-pure water using a glass rod spreader and pipette ca. 300 µl into a microcentrifuge tube on ice.
3. Dilute serially 1 in 10 and 1 in 100 by mixing 100 µl in 900 µl sterile ultra-pure water.
4. Wash 200 µl of the dilutions once by centrifugation at 7000 rpm for 8 min and resuspend in the same volume of sterile ultra-pure water.
5. Test material is ready for PCR testing.

(b) Total DNA extraction from peel extract

Background

A more rapid method to remove PCR toxic substances from peel extract, and at the same time concentrate the target material, is to extract the total DNA from the peel extract. Three methods are described here, the first two (i) and (ii) have been adapted from Fréchon *et al.* (1998) and from Cullen *et al.* (2001) and the third (iii) uses a commercial DNA extraction kit. The choice of method depends on circumstances. The kit is particularly useful when testing is not done routinely, and yields pure DNA, but is more expensive than the other two methods, whilst Fréchon *et al.* method tends to yield a purer DNA than Cullen *et al.* method, but takes marginally longer to perform. Regardless of the method used, it is essential to centrifuge the test sample to obtain a pellet and discard the supernatant to remove the bulk of PCR-inhibitory substances present. The pellet can be tested immediately or frozen (-20 °C) for testing at a later date. A sensitivity level of 10^2 Eca cells ml⁻¹ has been obtained using Y45 and Y46 and De Boer & Ward Eca primers.

(i) Fréchon *et al.* (1999) method

Protocol

Materials

1. Positive and negative controls: see Comments below.
2. Sterilised microcentrifuge tubes: 1.5 ml*.
3. Automatic adjustable pipettes and sterile tips with filters or positive displacement pipettes (0.5-2 µl, 2-20 µl, 20-200 µl, 200-1000 µl)*.
4. Microcentrifuge.
5. Vortex mixer*.
6. Water-bath or heating block (60 °C).
7. Sterilised ultra-pure water*.
8. Disposable gloves.
9. Microcentrifuge tube opener*.
10. Crushed ice.
11. TE buffer*.
12. TE/SDS buffer*.
13. Potassium acetate solution (3 mol l⁻¹)*.
14. Sodium iodide solution (6 mol l⁻¹)*.
15. Sodium chloride (5 mol l⁻¹)*.
16. Silica solution*.
17. Wash solution*.

* Details given in Appendix.

Procedure

1. Place two replicate 1 or 0.5 ml volumes of test peel extract without antioxidant on ice for 15-30 min to precipitate starch and debris.

2. Transfer the supernatant to a new microcentrifuge tube.
3. Centrifuge at 7000 rpm for 8 min to precipitate the bacteria and discard the supernatant. If required, freeze the pellet immediately at -20 °C.
4. Resuspend the fresh or thawed frozen pellet in 300 µl of TE/SDS and incubate at 50°C for 15 min.
5. Add 150 µl of 3 mol l⁻¹ potassium acetate, mix well on the vortex mixer, and stand on ice for 5 min.
6. Centrifuge at 13000 rpm for 5 min.
7. Pipette the supernatant into a new tube, add 700 µl of 6 mol l⁻¹ sodium iodide or 5 mol l⁻¹ sodium chloride and mix well.
8. Add 10 µl of the silica solution and mix well.
9. Leave at room temperature for 10 min with occasional shaking.
10. Centrifuge at 13000 rpm for 2 min.
11. Discard the supernatant, add 500 µl of wash solution and mix well.
12. Centrifuge at 13000 rpm for 2 min.
13. Discard the supernatant, taking care to remove most of the liquid.
14. Dry the DNA pellet at room temperature or 50 °C for up to 15 min in order to remove the last traces of ethanol.
15. Resuspend the pellet in 50 µl of TE pH 8.0 buffer and incubate at 50°C for 5 min.
16. Centrifuge at 13000 rpm for 2 min and transfer 45 µl supernatant to a new tube.
17. Store the DNA solution on ice if it is to be assayed the same day; if not, store at -20 °C.

(ii) Cullen *et al.* (2001) method (modified)

Protocol

Materials

1. Positive and negative controls: see Comments below.
2. Sterilised microcentrifuge tubes: 1.5 ml, 2.0 ml*.
3. Microcentrifuge tube opener*.
4. Automatic adjustable pipettes and sterile tips with filters or positive displacement pipettes (0.5-2 µl, 2-20 µl, 20-200 µl, 200-1000 µl)*.
5. Microcentrifuge.

6. Vortex mixer*.
7. Water-bath or heating block (60 °C).
8. Sterilised ultra-pure water*.
9. Disposable gloves.
10. Crushed ice.
11. Extraction buffer*.
12. Chloroform*.
13. Sodium acetate (3 mol l⁻¹, pH 5.2)*.
14. Isopropanol*.
15. Ethanol (70 %).

* Details given in Appendix.

Procedure

1. Place two replicate 1 or 0.5 ml volumes of test peel extract without antioxidant on ice for 15-30 min to precipitate starch and debris.
2. Transfer the supernatant to a new microcentrifuge tube.
3. Centrifuge at 7000 rpm for 8 min to precipitate the bacteria and discard the supernatant. If required, freeze the pellet immediately at -20 °C.
4. Resuspend the pellet in 1 ml extraction buffer.
5. Incubate at 65 °C for 10 min, shaking three to four times during this period.
6. Centrifuge at 6000 rpm for 5 min and transfer the supernatant (1 ml) to a new 2 ml tube.
7. Add an equal volume of chloroform (1 ml) and mix .
8. Centrifuge at 13000 rpm for 5 min and transfer the supernatant to a new 2 ml tube.
9. Add 1/10 volume (90 µl) of 3 mol l⁻¹ sodium acetate (pH 5.2) and an equal volume (990 µl) of isopropanol and incubate for 1 h at room temperature, shaking gently.
10. Centrifuge at 13000 rpm for 5 min to pellet the DNA and carefully remove the supernatant.
11. Add 100 µl 70 % ethanol to wash the pellet and centrifuge at 13000 rpm for 5 min.
12. Remove the ethanol and air dry at room temperature or in a laminar flow for 15 min.
13. Add 50 µl of sterilised ultra-pure water preheated to 50°C to dissolve the DNA pellet.
14. Store at -20 °C until required.

(iii) Qiagen DNeasy Plant Mini kit

Background

Kits which can be used for DNA extraction from plant tissues are becoming increasingly available. Although they tend to be expensive, a considerable saving in time may be achieved as most of the reagents needed in the DNA extraction are provided in a ready to use form. This is particularly useful when PCR testing is done only occasionally. The protocol involving the use of one kit found suitable, Qiagen DNeasy Plant Mini kit, is given below.

Protocol

Materials

Qiagen DNeasy Plant Mini kit

Included in kit:

1. All buffers for DNA extraction.
2. RNase A stock solution.
3. QIAshredder and DNeasy spin columns.
4. Collection tubes.

Not included in kit:

1. Ethanol (96-100 %).
2. Sterilised microcentrifuge tubes (1.5 ml, 2.0 ml)*.
3. Automatic adjustable pipettes and sterile filter tips (0.5-2 μ l, 2-20 μ l, 20-200 μ l, 200-1000 μ l)*.
4. Microcentrifuge.
5. Microfuge tube opener*.
6. Platform shaker*.
7. Vortex mixer*.
8. Waterbath (65 °C).

* Details given in Appendix.

Procedure (modified kit protocol).

1. Place two replicate 1 or 0.5 ml volumes of test peel extract without antioxidant on ice for 15-30 min to precipitate starch and debris.
2. Transfer the supernatant to a new microcentrifuge tube.
3. Centrifuge at 7000 rpm for 8 min to precipitate the bacteria and discard the supernatant. If required, freeze the pellet immediately at -20 °C.
4. Resuspend the pellet in 400 μ l of Buffer AP1 and 4 μ l of RNase A stock solution.
5. Follow kit protocol steps 2-11, including the optional step at 4.
6. Follow kit protocol step 12, but elute DNA with 50 μ l Buffer AE. Repeat elution is not necessary.
7. Store at -20 °C until required.

General comments

It is necessary to process positive and negative controls at the same time as the test material.

Great care should be taken to avoid cross-contamination when processing several peel extracts and positive and negative controls simultaneously. It is advisable to wear disposable gloves and use only pipette tips with filters. It helps to label all tubes before starting and use a micro-centrifuge opener to avoid the formation of aerosols when opening the caps.

It is best if sample preparation is done in a different room from that used for PCR and not immediately following a PCR run.

DNA in peel extract is concentrated 20 times in methods (i), (ii) and (iii), 1 ml being reduced to 50 μ l.

(v) Approximately 30 peel extracts may be processed with methods (i), (ii) and (iii) within 4 h by one person.

All three methods can also be used to purify DNA from pure bacterial cultures.

The use of sodium chloride instead of sodium iodide in method (ii) is preferable as the latter is subject to oxidation.

Method (ii) utilises no harmful chemicals and has been extensively tested on different potato cultivars.

B. PCR assay

Background

Different PCR protocols have been developed for use with the two recommended Eca-specific primers (see Section 1: Molecular probes) and are described below. In contrast to the protocol using De Boer & Ward (1995) primers, that using Fréchon *et al* (1995) primers can be used for research purposes only unless clearance has been obtained with the patent holders (see Section 1). PCR results may be affected by small variations in the prescribed experimental conditions, such as use of a different DNA thermocycler model or a different thermostable DNA polymerase. It is, therefore, essential to adhere closely to the protocol and, if a different thermocycler model or DNA polymerase source is used, specificity for Eca would need to be checked and, if necessary, the protocol modified.

(a) PCR test protocol using De Boer & Ward primers

Protocol

Materials

1. Test material: see "A. Peel extract treatment" above.
2. *Taq* DNA polymerase*.
3. PCR buffer (10x)*.
4. $MgCl_2$ solution (25 mmol l^{-1})*.
5. dNTPs mix (1 mmol l^{-1})*.
6. Primers ECA1f and ECA2r (15 μ mol l^{-1})*.
7. Sterilised ultra-pure water*.
8. Sterilised Tween 20 (1 in 10 diluted in ultra-pure water)*.

9. DNA thermocycler*.
10. Sterilised PCR microtubes*.
11. PCR station or laminar flow cabinet with UV light.
12. Sterilised microcentrifuge tubes (0.5, 1.5 ml)*.
13. Automatic adjustable pipettes with sterile filter tips (0.5-2 μl , 2-20 μl , 20-200 μl , 200-1000 μl)*.
14. Disposable gloves.

* Details given in Appendix.

Procedures

1. Programme the DNA thermocycler as follows:
 Step 1: DNA denaturation at 95 °C for 5 min.
 Step 2: 36 cycles, each consisting of:
 DNA denaturation at 94 °C for 30 s.
 DNA annealing at 62 °C for 45 s.
 DNA extension at 72 °C for 45 s.
 Step 3: DNA extension at 72 °C for 8 min.
 Step 4: Hold at 4 °C.
2. Label each PCR microtube and arrange in microtube holder.

3. Prepare master mix

Volume of reagents per PCR tube (48 μl):

AmpliTaq DNA polymerase ^a	0.2 μl
PCR buffer (10x) ^a	5.0 μl
MgCl ₂ (25 mmol l ⁻¹) ^a	4.0 μl
dNTPs mix (1 mmol l ⁻¹)	5.0 μl
Primer ECA1f (15 $\mu\text{mol l}^{-1}$)	1.0 μl
Primer ECA2r (15 $\mu\text{mol l}^{-1}$)	1.0 μl
Tween 20 (1/10 dilution)	0.5 μl
Ultra-pure water	31.3 μl

^a Perkin Elmer reagents

Prepare enough for the expected number of PCR tests to be done including controls, with two extra to take into account the volume loss due to surface tension retention.

If *Taq* DNA polymerase from Invitrogen is used, use their 10x PCR buffer and MgCl₂ (50 mmol l⁻¹) at 2.0 μl of MgCl₂ per PCR tube, and increase the volume of water to 33.3 μl .

4. Mix well by vortexing.
5. Aliquot 48 μl to each microtube and close all lids.
6. Add 2 μl of test material and the positive and negative controls to each microtube just before amplification, using pipette tips with filters, opening and closing one tube at a time.
7. Place tubes in thermocycler, start the pre-set DNA amplification programme and close thermocycler lid.

8. Remove amplified DNA at the end of programme for testing. If this cannot be done immediately, either programme the thermocycler to hold the tubes at 4 °C at the end of the programme, for example when amplification is done overnight, or store at °C (short term) or -20 °C (long term) until required.

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4

(b) PCR test protocol using Fréchon *et al.* primers

Protocol

Materials

1. Test material: see “A. Peel extract treatment” above.
2. *Taq* DNA polymerase*.
3. PCR buffer (10x)*.
4. MgCl₂ solution (25 mmol l⁻¹)*.
5. dNTPs mix (1.25 mmol l⁻¹)*.
6. Primers Y45 & Y46 (25 µmol l⁻¹)*.
7. Sterilised ultra-pure water*.
8. DNA thermocycler*.
9. Sterilised PCR microtubes*.
10. PCR station or laminar flow cabinet with UV light.
11. Sterilised microcentrifuge tubes (0.5, 1.5 ml)*.
12. Automatic adjustable pipettes with sterile filter tips (0.5-2 µl, 2-20 µl, 20-200 µl, 200-1000 µl)*.
13. Disposable gloves.

* Details given in Appendix.

Procedures

1. Programme the DNA thermocycler as follows:
Step 1: DNA denaturation at 94 °C for 5 min.
Step 2: 35 cycles, each consisting of:
 DNA denaturation at 94 °C for 30 s.
 DNA annealing at 70 °C for 30 s.
 DNA extension at 72 °C for 45 s.
Step 3: Hold at 4 °C.
2. Label each PCR microtube and arrange in microtube holder.
3. Prepare master mix
Volume of reagents per PCR tube (48 µl):

AmpliTaq DNA polymerase ^a	0.2 µl
PCR buffer (10x) ^a	5.0 µl
MgCl ₂ (25 mmol l ⁻¹) ^a	5.0 µl
dNTPs mix (1.25 mmol l ⁻¹)	4.0 µl
Primer Y45 (25 µmol l ⁻¹)	1.0 µl
Primer Y46 (25 µmol l ⁻¹)	1.0 µl

Ultra-pure water
a Perkin Elmer reagents

31.8 μ l

Prepare enough for the expected number of PCR tests to be done including controls, with two extra to take into account the volume loss due to surface tension retention.

4. Mix well by vortexing.
5. Aliquot 48 μ l to each microtube and close all lids.
6. Add 2 μ l of test material and the positive and negative controls to each microtube just before amplification, using pipette tips with filters, opening and closing one tube at a time.
7. Place tubes in thermocycler, start the pre-set DNA amplification programme and close thermocycler lid.
8. Remove amplified DNA at the end of programme for testing. If this cannot be done immediately, either programme the thermocycler to hold the tubes at 4 °C at the end of the programme, for example when amplification is done overnight, or store at 4 °C (short term) or -20 °C (long term) until required.

General comments

- (i) Controls: Because of the sensitivity of the PCR amplification test, there is always the risk of cross-contamination, resulting in misinterpretation of the results. Extensive use of controls is required at all times.

Positive controls: PCR microtube containing 2 μ l of:

- (a) 10^3 , 10^5 , 10^7 Eca cells ml⁻¹ in ultra-pure water to check detection level of PCR assay.
- (b) Either: washed bacterial suspension from CVP plate inoculated with Eca-free peel extract containing 10^5 Eca cells ml⁻¹ (see “A. (a) Peel extract treatment: Eca enrichment on CVP”);
Or: DNA solution from Eca-free peel extract containing 10^7 Eca cells ml⁻¹ purified at the same time as samples (see “A. (b i, ii & iii) Total DNA extraction from peel extract”).

Negative controls: PCR microtube containing 2 μ l of:

- (a) Ultra-pure water.
 - (b) Purified DNA solution from Eca-free peel extract.
- (ii) The number of negative controls depends on the experience of the operator; e.g. one set of controls for every nine test microtubes is recommended for the inexperienced. It is best to load the positive controls before the negative ones.
 - (iii) Avoiding cross contamination: The most important cause of contamination is the carry-over of low molecular weight DNA from previous amplifications. It is advisable to spatially separate certain operations, especially the loading of PCR microtubes. This should be done in a cabinet fitted with UV light to degrade contaminating DNA before and after microtube loading and amplified DNA should not be brought into that area. In addition to the use of dedicated laboratory coats and pipettes for each PCR stage and sterile disposable vials (test material preparation, master mix preparation and amplified DNA revelation), frequent change of disposable

gloves is recommended. Also desirable cleaning and decontamination of PCR workbenches, pipette tip cones, PCR assembly units (Perkin Elmer tray and base) and gloved fingers with 10 % freshly prepared sodium hypochlorite before and after use.

(iv) Further useful, basically good laboratory, practices:

Record batch numbers and dates of receipt of PCR reagents with a limited shelf life and closely follow the producers' storage and preparation recommendations.

On arrival, prepare small aliquotes of labile reagents before storage at -20 °C to avoid repeated freezing and thawing, especially harmful to *Taq* polymerase, primers, dNTPs and DNA stability. Vortex thawed reagents before use.

Long term storage of reagents is at -20 °C and those for immediate use at 4 °C and renewed every week. Pre- and post- PCR reagents and DNA must be stored in separate fridges and freezers.

C. Detection of amplified DNA

Background

Revelation of amplified DNA to determine whether a test is positive or negative usually relies on gel electrophoresis of PCR product followed by staining the DNA band with ethidium bromide. This is then visualised under UV light before recording by photography.

Gel electrophoresis method

Protocol

Materials

1. Amplified DNA (see above).
2. Agarose*.
3. TBE buffer*.
4. Dye mix*.
5. Size marker: 1 Kb or 100 bp ladder*.
6. Ethidium bromide stock solution: 1 mg ml⁻¹*.
7. Horizontal gel electrophoresis apparatus*.
8. Power pack*.
9. Gel combs: for 14, 16 or 20 wells*.
10. Automatic adjustable pipettes and sterile tips (2-20 µl, 20-200 µl)*.
11. Disposable gloves.
12. Platform shaker for gels*.
13. UV transilluminator* or computerised Gel Documentation System*
14. UV protective face shield*.
15. Polaroid camera and film mounted on an adjustable vertical stand* or computerised Gel Documentation System*

* Details given in Appendix.

Procedures

1. Prepare a 1.2 % agarose solution in TBE buffer, allow to cool to ca. 60 °C, pour into the gel-tray of the electrophoresis apparatus containing the combs and allow to set.
2. Place the gel-tray on the electrophoretic apparatus, add TBE buffer, connect the terminals to the power pack, taking care that the positive and negative terminals are correctly positioned, and carefully remove the combs.
3. Add 5 µl of amplified DNA solution to 2 µl of dye mix and load 7 µl into wells, taking care that the contents of adjacent wells do not mix. Load the size marker solution, preferably in the outside wells of the gel.
4. Adjust the voltage on the power pack to 60 V for a mini-gel or 100 V for a standard gel. Electrophorese for 30 min or 2 h for the mini-gel or standard gel respectively, or until the dye front reaches the next row or ca. half way down the gel.
5. Disconnect the power pack and move the gel carefully into a slightly larger shallow tray.
6. Add ethidium bromide working solution (0.5 µg ml⁻¹) and stain while shaking gently on a platform shaker for 20 min, protected from light.
7. Remove the ethidium solution and de-stain the gel in distilled water while shaking gently for 30 to 60 min.
8. Examine the gel under UV light. If DNA amplified by De Boer & Ward or by Fréchon *et al.* primers is present, a fluorescent 690 bp or 438 bp band respectively which is characteristic of Eca, will be visible, its size being estimated in relation to the size of the marker ladder (Fig. 5.2).
9. Record results by photographing the gel on the UV transilluminator using a Polaroid camera or with the computerised Gel Documentation System.

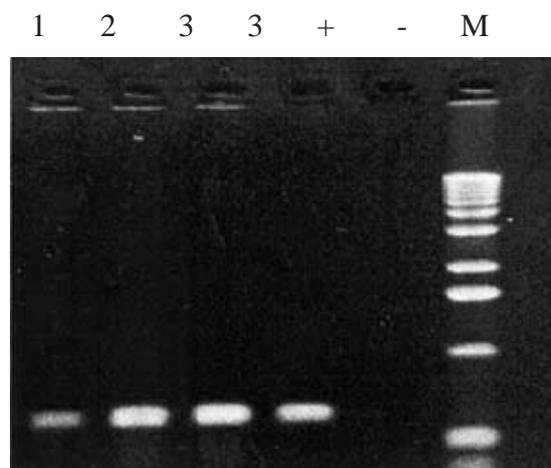


Fig. 5.2 PCR-amplified Eca DNA detection by gel electrophoresis: amplified Eca DNA (samples 1, 2 and 3) using De Boer & Ward (1995) primers visualised under UV light in agarose gel stained with ethidium bromide. Positive and negative controls were Eca cells in water and purified DNA from Eca-free peel extract respectively. A 1 Kb molecular marker ladder (M) was used.

SAFETY:

Ethidium bromide is toxic, mutagenic and carcinogenic.

Precautions must be taken whilst handling it:

Stock solutions must be prepared using pre-weighed tablets and wearing a toxic particle mask to avoid breathing ethidium bromide dust.

Disposable gloves must be worn at all times when handling ethidium bromide solutions and stained gels.

All switches, knobs and surfaces contaminated with ethidium bromide must be cleaned with 70 % ethanol.

A UV protective face shield must be worn when examining and photographing the stained gel if using a transilluminator.

Disposal of spent diluted ethidium solution should be according to current national regulations.

D. Quantitative PCR assay (Hyman *et al.*, 2000)

Background

The approach adopted is to co-amplify the target DNA (Eca) with a competitor PCR template, flanked by the primer-specific sequences of the target DNA, which is usually smaller than the target, to allow direct comparison of amplified products on an agarose gel. The competitor template was derived from the sequenced amplified product using De Boer & Ward primers, using a third primer designed to anneal to an internal sequence of the amplified product as described by Celi *et al.* (1993). The competitor template was cloned into a plasmid, pGEM-T, and transformed into *Escherichia coli* XL2-blue MRF cells to produce strain *E. coli* 4R. When PCR-amplified with De Boer & Ward primers, the *E. coli* 4R strain yielded an amplification product ca. 90 bp smaller than with Eca target DNA. The addition of a known number of the *E. coli* 4R cells to the tuber peel extract prior to DNA extraction allows an estimation of Eca numbers within the range of 10^2 - 10^5 cells ml⁻¹ tuber peel extract by comparing the ratio of products generated from the Eca target DNA and the competitor DNA by PCR in agarose electrophoresis gels. The optimum concentration of *E. coli* 4R cells giving differential ratios of Eca between 10^2 to 10^5 cells ml⁻¹ is 1×10^4 cells ml⁻¹ of sample. This generates an equal ratio of PCR products at an intermediate Eca level of 10^3 - 10^4 cells ml⁻¹, thereby allowing changes in product ratios to reflect Eca concentrations either above or below this midpoint.

Simultaneous DNA extraction of Eca and *E. coli* cells in the peel extract ensures that variation in extraction efficiency does not affect the results and at the same time, *E. coli* 4R provides a DNA extraction control.

Protocol

Materials

DNA extraction

1. As for any of the three methods described above (A. (b) (i, ii & iii)).

2. *E. coli* 4R culture*.
3. Luria Bertani broth supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin (LB Amp)*.
4. Spectrophotometer and 1 ml cuvettes.

PCR assay

As for De Boer & Ward primers protocol described above (B. (a))

Detection and quantification of Eca DNA

1. As for gel electrophoresis method described above (C).
2. Controls: Positive: 1 μl of each of *Eca* and *E.coli* 4R colonies suspended in 500 μl ultra-pure water or 1 μl of each of *Eca* and *E. coli* DNA; Negative: 1 μl ultra-pure water.

* Details given in Appendix.

Procedure

DNA extraction

1. Prepare a culture of *E. coli* 4R grown in a shaking bath at 27 °C for 16h in LB Amp as described in Section 6.
2. Dilute the culture 1 in 10 in LB and adjust the optical density with LB to an absorbance of 0.3 +/- 0.05 at 600 nm which is equivalent to 10^8 cells ml^{-1} .
3. Serially dilute in sterile distilled water to obtain a suspension containing 1×10^6 cells ml^{-1} .
4. Prepare potato peel extract as described in Section 1.
5. Place two replicate 1 or 0.5 ml volumes of test peel extract without antioxidant on ice for 15-30 min to precipitate starch and debris.
6. Transfer the supernatant to a new microcentrifuge tube.
7. Centrifuge at 7000 rpm for 8 min and discard the supernatant.
8. Resuspend the pellet in x ml of extraction buffer, depending which method is used

Add x ml of *E. coli* 4R suspension from step 3 to the resuspended peel extract pellet to give 1 in 100 dilution of *E. coli* 4R equivalent to a final concentration of 10^4 cells ml^{-1} of original sample volume.

e.g. 5 μl of 1×10^6 cells ml^{-1} , to pellet from 500 μl peel extract.

10. Mix well and proceed with one of the three DNA extraction protocols from step 5.

PCR assay

Proceed with PCR protocol using De Boer & Ward primers as described above (B. a)

Detection of Eca DNA

1. Proceed as in the Gel electrophoresis protocol described above in C except run gel for 2.5 h.
2. Controls: as above.

Quantification of *Eca* DNA

An estimation of the number of *Eca* cells present in the peel extract is obtained by comparing visually the ratios of amplification products from the *E. coli* 4R competitor template and *Eca* target DNA, represented by the density of the bands in the gel. *Eca* PCR template band density decreases progressively as cell concentration falls while that for *E. coli* 4R competitor template increases. The density of the two bands is similar only when *Eca* cell concentration lies between 10^3 to 10^4 cells ml^{-1} .

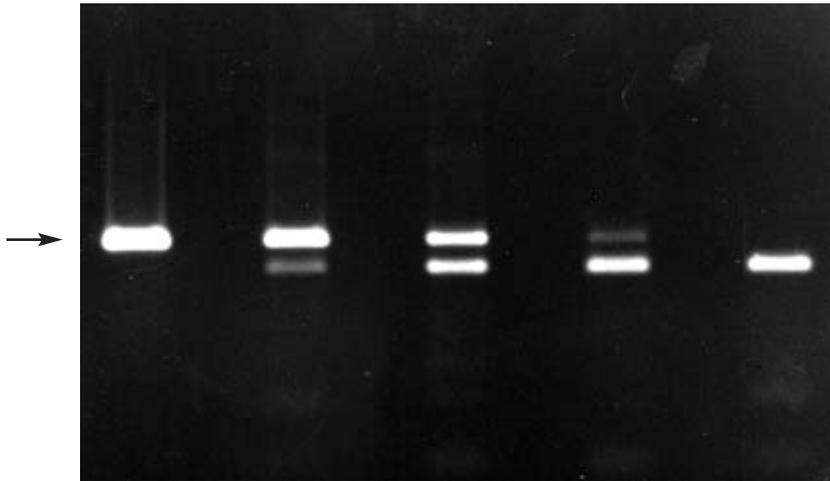


Fig. 5.3. Relationship between PCR product ratios and number of *Eca* cells ml^{-1} potato peel extract. DNA extraction of tuber peel extracts containing *Eca* from 10^6 to 10^1 cells ml^{-1} in the presence of *E. coli* 4R cells at 1×10^4 ml^{-1} . Lane 1, $> 10^5$ *Eca* cells ml^{-1} , lane 2, 10^4 - 10^5 cells ml^{-1} , lane 3, 10^3 - 10^4 *Eca* cells ml^{-1} , lane 4, 10^2 - 10^3 *Eca* cells ml^{-1} , lane 5, $< 10^2$ *Eca* cells ml^{-1} . Arrows indicate 690 bp and 600 bp amplification products from *Eca* and *E. coli* 4R, respectively.

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Appendix

Peel extract treatment

Eca enrichment on CVP

CVP plates: see Section 2: Appendix.

Sterilised ultra-pure water, HPLC-grade: Sigma (27, 073-3).

Sterilised microcentrifuge tubes:

0.5 ml and 1.5 ml: Fisher Scientific (TUL-150-010G and TUL-150-150N)

Automatic adjustable pipettes and tips:

Gilson (Anachem) or Finpipette (Life Sciences).

DNA extraction (Fréchon *et al.* method)

TE buffer pH 8.0

Add 0.12 g Tris (BDH, 10315) and 0.03 g EDTA (Sigma, E 5134) to 80 ml ultra-pure sterile water and dissolve by adjusting pH to 8.0. Make up to 100 ml with ultra-pure sterile water to give 10 mmol l⁻¹ Tris and 1 mmol l⁻¹ EDTA.

TE/SDS buffer

Dissolve 1 g SDS (Sigma, L 4390) in 100 ml TE buffer.

Potassium acetate (3 mol l⁻¹)

1. Dissolve 49.07 g potassium acetate in 100 ml ultra-pure sterile water to give 5 mol l⁻¹
2. Add 28.5 ml ultra-pure water to 60 ml of 5 mol l⁻¹ potassium acetate.
3. Add 11.5 ml glacial acetic acid.

Sodium iodide (6 mol l⁻¹)

1. Dissolve 0.75 g Na₂SO₃ in 40 ml ultra-pure sterile water.
2. Add 45 g NaI.
3. Stir until dissolved and filter (Whatman No.1 paper; BDH 234/0290).
4. Store in dark at 4 °C.

Note: Discard if solution is oxidised (brown) and equilibrate at room temperature before use.

Sodium chloride (5 mol l⁻¹)

Dissolve 29.2 g NaCl in ultra-pure water and make up to 100 ml.

Silica solution

1. Add 60 g silica (Sigma S 5631) to 300 ml ultra-pure sterile water, and make up to 500 ml in a measuring cylinder.
2. Mix well and leave at room temperature for 24 h.
3. Discard 430 ml supernatant.
4. Make up to 500 ml with sterile ultra-pure water.
5. Resuspend silica vigorously and leave for 5 h.
6. Remove 440 ml supernatant.
7. Adjust pH to 2.0 and aliquot into small volumes.

8. Autoclave at 120 °C for 20 min.
9. Store at room temperature, in the dark.

Wash solution

1. Add 0.24 g Tris (BDH 10315), 0.58 g NaCl and 0.03 g EDTA to 90 ml sterile ultra-pure water and dissolve by adjusting pH to 7.5. Make up to 100 ml with sterile ultra-pure water to give 20 mmol l⁻¹ Tris, 1 mmol l⁻¹ EDTA and 100 mmol l⁻¹ NaCl.
2. Add an equal volume absolute alcohol.
3. Store at 4 °C.

Sterilised ultra-pure water: HPLC-grade water: Sigma (27, 073-3).

Sterilised microcentrifuge tubes:

0.5 ml and 1.5 ml: Fisher Scientific (TUL-150-010G and TUL-150-150N)

Microcentrifuge tube opener: Dynal.

Automatic adjustable pipettes and filter tips:

Gilson (Anachem) or Finpipette (Life Sciences).

Microcentrifuge: Philip Harris (MSE Microcentaur C34-101).

DNA extraction (Cullen *et al.* method)

Extraction buffer

di-sodium hydrogen orthophosphate dihydrate	10.685 g
CTAB (hexadecyltrimethyl ammonium bromide)	10 g
Sodium chloride	43.83 g
Distilled water	500 ml

1. Dissolve ingredients in 400 ml distilled water.
2. Adjust pH to 8.0 with either 1 mol l⁻¹ NaOH or HCl and make up to 500 ml with distilled water.
3. Autoclave.

Sodium acetate solution (3 mol l⁻¹, pH 5.2)

1. Dissolve 24.6 g sodium acetate in 100 ml distilled water to obtain a 3 mol l⁻¹ stock solution and adjust pH to 5.2 with either 1 mol l⁻¹ NaOH or HCl.
2. Alternatively buy ready made from Sigma (S 7899).

Chloroform: Sigma (C 2432)

Isopropanol: Sigma (I 9516)

Sterilised microcentrifuge tubes:

1.5 ml and 2.0 ml: Fisher Scientific (TUL-150-150N and TUL-150-290U)

Microcentrifuge tube opener (Dynal).

Automatic adjustable pipettes and filter tips:

Gilson (Anachem) or Finpipette (Life Sciences).

Microcentrifuge: Philip Harris (MSE Microcentaur C34-101).

DNA extraction (Commercial kit)

DNeasy Plant Mini kit: Qiagen (69104).

Sterilised microcentrifuge tubes:

1.5 ml and 2.0 ml: Fisher Scientific (TUL-150-150N and TUL-150-290U).
Microcentrifuge tube opener (Dynal).
Automatic adjustable pipettes and tips:
Gilson (Anachem) or Finpipette (Life Sciences).
Microcentrifuge: Philip Harris (MSE Microcentaur C34-101).
Platform shaker: BDH (330/0114/00).

PCR assay

Protocol using De Boer & Ward primers

Taq DNA polymerase kits:

(i) Perkin Elmer (N808-0038).

AmpliTaq DNA polymerase Stoffel fragment (10 units μl^{-1}).

PCR Stoffel buffer (10x).

MgCl₂ solution (25 mmol l⁻¹).

(ii) Invitrogen (18038-026).

Taq DNA polymerase (5 units μl^{-1}).

PCR buffer (10x).

MgCl₂ solution (50 mmol l⁻¹).

Store at -20°C.

dNTP set: Pharmacia, 100 mmol l⁻¹ (27-2035-01)

1 mmol l⁻¹ stock solution prepared by adding 10 μl of each of the 4 dNTPs to 960 μl sterile ultra-pure water. Store in aliquots at -20 °C.

Primers ECA1f and ECA2r : Pharmacia or MWG (customised order, see Section 1).

Dilute each to 15 $\mu\text{mol l}^{-1}$ with sterile ultra-pure water and store in aliquots at -20 °C.

Sterilized microcentrifuge tubes:

1.5 ml and 2.0 ml: Fisher Scientific (TUL-150-150N and TUL-150-290U)

Sterilised ultra-pure water, HPLC-grade water: Sigma (27, 073-3).

DNA thermocycler: Perkin Elmer (model 9600).

Sterilised PCR microtubes:

Perkin Elmer, 200 μl tubes (N801-0540) or

Bio-Rad 200 μl thin-wall tubes (223-9473).

PCR work station: Jencons (Millenium PCR cabinet, 566-022) or laminar flow cabinet with UV light.

Automatic adjustable pipettes and filter tips:

Gilson (Anachem) or Finpipette (Life Sciences).

Tween 20: Sigma (P2287).

Protocol using Fréchon et al. primers

Taq DNA polymerase kit: Perkin Elmer (N8080161)

AmpliTaq DNA polymerase (5 units μl^{-1}).

PCR buffer II (10x).

MgCl₂ solution (25 mmol l⁻¹).

dNTP set: Pharmacia, 100 mmol l⁻¹, (27-2035-01)

1.25 mmol l⁻¹ stock solution prepared by adding 12.5 µl of each of the 4 dNTPs to 950 µl sterile ultra-pure water. Store in aliquots at -20 °C.

Primers Y45 and Y46 : Genset, or MWG, customised order, see Section 1)

Dilute to 25 µmol l⁻¹ with sterile ultra-pure water and store in aliquots at -20 °C.

Other reagents and equipment as for Protocol using De Boer & Ward primers (see above).

Detection of amplified DNA

Gel electrophoresis

TBE buffer (10x concentrated):

Tris borate	108.0 g
Orthoboric acid	55.0 g
EDTA (pH 8.0)	9.3 g
Distilled water	to 1000 ml

1. Dissolve EDTA: Sigma (E 9884) in 100 ml water and adjust pH to 8.0.

2. Dissolve all reagents in 800 ml distilled water and make up to 1000 ml.

3. Dilute 10x when required.

Agarose: Invitrogen (15510-019) or Bio-Rad (162-0125).

Size marker: 1 Kb or 100 bp ladder : Invitrogen (15615-016 or 15628-019, respectively).

Dye mix

Glycerol	30.0 ml
Bromophenol blue	0.25 g
Xylene cyanole	0.25 g
Ultra-pure water	to 100 ml

Ethidium bromide tablets: Bio-Rad (161-0430) or Sigma (E 4391)

Stock solution: 1 mg ml⁻¹

Working solution: 0.5 µg ml⁻¹

Gel electrophoresis apparatus and combs:

Flowgen (G3-0112 or G3-0202; 10 x 10 cm or 15 x 10 cm trays respectively); or

Bio-Rad (170-4466 or 170-4468; 7 x 10 cm or 15 x 10 cm trays respectively).

Power pack: Flowgen (Model E331, P5-0022) or Bio-Rad (Model 300, 165-5051).

Automatic adjustable pipettes and filter tips:

Gilson (Anachem) or Finpipette (Life Sciences).

Sterilised microcentrifuge tube, 0.5 ml (Fisher Scientific TUL-150-010G).

Platform shaker: BDH (330/0114/00).

UV transilluminator: Flowgen (TFX-35M).

UV protective face shield: Flowgen (U6-0208).

Polaroid camera and hood: Flowgen (P4-0110 and P4-0124, respectively).

Polaroid film: Flowgen (P4-0200).

Computerised Gel Documentation System: Jencons (UVP, GDS-8000)

Quantification PCR

DNA extraction

As for any of the three methods described above.

E. coli 4R strain (see Hyman *et al.* 2000).

Luria Bertani broth (LB): Sigma (L-3522).

Ampicillin: Sigma (A 2804).

PCR assay

As for PCR test protocol using De Boer & Ward primers given above.

Detection and quantification of Eca DNA

As for gel electrophoresis detection method described above.

Isolation and identification

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Introduction

It may sometimes be necessary to confirm the detection of *E. c.* subsp. *atroseptica* (Eca) made by other methods or, more generally, to isolate and identify soft rot erwinias which may be present in affected plant tissues or in the environment, such as soil, irrigation water, harvesters and graders. As the four detection methods rely on the recognition of only one character believed to be specific to Eca, a more reliable identification of Eca would be obtained if more than one character is taken into consideration. This approach takes into account any diversity which may exist within Eca and, at the same time, allows the differentiation of the different erwinias which may be also present. It is notable that identification as well as taxonomy and nomenclature of the bacteria are still largely based on morphological and biochemical characters, although DNA analyses are becoming increasingly to the fore. For example, as a short cut, Eca identity can be confirmed using the PCR primers mentioned in this manual as they have been extensively tested on a wide range of bacterial species.

In this Section, methods for the isolation and identification of the five subspecies of *E. carotovora* with emphasis on the two so far found on potatoes, namely *E. c.* subsp. *carotovora* (Ecc) and Eca, and *E. chrysanthemi* (Table 6.1), are described. Further information on the subject is given in Dye (1969, 1981), Graham (1972) and Schaad (1988). Because the taxonomy and nomenclature of these bacteria are still unsettled, the designations adopted here are based on the approved list of the Judicial Commission of the International Committee on Systematic Bacteriology (Skerman *et al.*, 1980) and on Bergey's Manual of Determinative Bacteriology (1994).

Isolation

Isolation of the two above mentioned *Erwinia* species is readily made on the selective-diagnostic CVP medium (see Section 2). The bacteria form characteristic deep cup-like cavities or pits which are different from those formed by the pectolytic pseudomonads, which are shallower and wider. Preparation of the test material depends on whether infection is active, when isolation can be made directly, or latent, when an enrichment step is advisable prior to isolation.

Isolation from an active infection

1. Wash the diseased organ (rotting tuber or stem) under running water to remove excess soil but avoid breaking the skin.
2. Break or cut open the skin and remove a small amount of tissue (ca. 0.1 g) from the rotting front of the lesion using a sterile scalpel.
3. Cut and tease the tissue in sterile water (ca. 0.2 ml) in a plastic Petri dish. Leave for ca. 5 min to allow the bacteria to diffuse out of the tissue.

4. Streak, with a sterile inoculating needle with a loop at the end, a loopful of the liquid from 3 on to a CVP plate previously dried to remove excess surface moisture (see Section 2: Appendix) to obtain isolated colonies. Streaks should be made in four right angle directions, flaming and cooling the loop after each directional streak.
5. Incubate the CVP plate upside down at 27 °C for 48 h (at higher temperatures, *Eca* may fail to grow and at lower temperatures growth of the other erwinias may be very slow).
6. Select at least two well spaced colonies/cavities per CVP plate and re-streak the bacteria on to a fresh CVP plate. Incubate as in 4 and 5.
7. Select two colonies (cavities), streak each on a nutrient agar (NA)* or Luria broth agar (LBA)* plate previously dried gently in a ventilated incubator or in a laminar flow cabinet long enough to remove excess surface moisture and incubate at 27 °C for about 2 wk to ensure that only erwinia colonies are present. Erwinias form round convex creamy-translucent colonies on NA or LBA.

Isolation from a latent infection or other sources

1. Preparation of test material depends on its nature:
 - tubers: crush peel as in Section 1: Tuber peel extract preparation.
 - soil/debris from farm equipment: remove stones, break up aggregates and cut plant tissues into small pieces.
 - water: use as received, or concentrate the bacteria by centrifugation at 7000 rpm for 10 min and resuspend in a small volume of water.
2. Place the test tissue in single strength enrichment medium (PEM)* (double strength PEM medium for liquid samples) and incubate anaerobically in bags* at 27 °C for 48 h as in Section 4. The amount of test material and PEM used may vary but the ratio to aim at is ca. 1:3 to 1:5 (w/v) tissue: single strength PEM, and 1:1 (v/v) liquid sample: double strength PEM.
3. Carry out steps 4 to 7 as in isolation from active infection.

* Details given in Appendix.

Storage of cultures

Short term storage

E. carotovora isolates may be stored for a few years on the surface of an NA or LBA slope at room temperature (ca. 20 °C) in the dark in a small screw-cap vial, following inoculation with a pure culture from a NA or LBA plate and incubation at 27 °C for 48 h. Survival of *E. chrysanthemi* is restricted to ca. one week under these conditions and the bacterium is best stored at -80 °C, as described below.

Long term storage

1. Inoculate 10 ml of nutrient broth (NB)* or Luria broth (LB)* in a stoppered 150 ml conical

flask with a loopful taken from a single colony of a pure fresh (<72 h) culture growing on NA or LBA and incubate in a shaking water bath at 27 °C for 18 h.

- 2a. Mix 1 ml of the broth culture with 1 ml of a freezing medium*, prepare 0.1 ml aliquots in 0.5 ml microcentrifuge tubes, flash freeze by dipping in liquid nitrogen for 2-3 min and store at -80 °C. Alternatively, coat small glass beads* by dipping in the mixture of bacterial culture and freezing medium, drain, freeze and store at - 80°C.
- 2b. The bacteria may also be stored in water: centrifuge the NB or LB culture at 7000 rpm for 10 min in a sterile tube, pour off the supernatant, resuspend the pellet in 1 ml of sterile distilled water and aliquot 100 µl into 1 ml sterile water in vials. Store at room temperature in the dark for up to 1 year.
3. When fresh cultures are required, thaw the frozen culture in the microcentrifuge tubes slowly and streak on to NA or LBA plates, or scatter some beads on to the plates, or streak-plate a loopful of bacterial suspension in water on to the plates. Incubate at 27° C for 48 h.

* Details given in Appendix.

Preparation of an erwinia suspension

From colonies

1. Suspend one or two loopfuls of the bacteria taken from a 48 h culture of the erwinia on NA or LBA in 2 ml sterile NB or LB and mix thoroughly by vortexing.
2. Adjust the optical density with broth to an absorbance of 0.2 at 600 nm (equivalent to ca. 10^8 cells [colony forming units; cfu] ml⁻¹) on a spectrophotometer.

From broth cultures

Inoculate 10 ml NB or LB in a 150 ml conical flask with a loopful of the bacteria from a 48 h culture of the erwinia on NA or LBA.

Incubate for 18 h in a shaking water bath at 27 °C.

Dilute 1 in 10 with NB or LB and adjust the optical density as above.

Determination of erwinia numbers by dilution plating

1. Prepare serial decimal dilutions from a suspension prepared as above, by adding 100 µl to 900 µl sterile distilled water in a microcentrifuge tube, mixing thoroughly at each step.
2. Spread-plate 100 µl of 10^{-4} and 10^{-5} dilutions on duplicate NA or LBA plates using a glass rod spreader until all the liquid is absorbed and incubate for 48 h at 27 °C.
3. Count the number of colonies per plate within the range of 50-500 colonies per plate and calculate the numbers of cells in the original bacterial suspension, taking into account the dilution factor.

Identification

Soft rot erwinias are facultatively anaerobic, peritrichously flagellated, rod shaped and Gram negative. Although it is desirable to confirm these characters, it is possible to rely on their ability to grow and form cavities on CVP to identify presumptively the more common soft rot erwinias, *E. caro-*

tovora and *E. chrysanthemi*. Only a few tests are necessary to confirm their identity as erwinias. These are listed below:

Test*	<i>Erwinia</i> reaction
Catalase	+
Oxidase	-
Oxidation/fermentation	+/+
Rotting of potato tuber slice	+

* Details given in Appendix.

Identification at species and subspecies level is based on bacterial reaction pattern to a relatively small number of differential tests (Table 6.1). As only three species/subspecies are commonly associated with potatoes, identification may be further simplified by applying a selected number of tests (Table 6.1). For completeness, the characteristics of two less common soft rot erwinias, *E. cypripedii* and *E. rhapontici*, have been included in Table 6.1.

It is essential to use freshly growing pure cultures of the bacteria on NA or LBA (48 h) when performing the tests. The inclusion of bacteria known to give a positive or a negative reaction for a particular test (Table 6.1) is also useful. These control species may be obtained from national culture collections (see Appendix). If necessary, the identity of a bacterium may be confirmed by sending a culture to the National Collection of Plant Pathogenic Bacteria, UK or the International Mycological Institute, UK.

Table 6.1. Physiological and biochemical tests for differentiating the more common soft rot erwinias¹.

Test*	Ecc	Eca	Ecb	Eco	Ecw	Ech	Ecy	Erh
Cavity formation on CVP medium (27 °C, 48 h)	+	+	+	+	+	+	-	-
Growth on NA at 37 °C	+	-	+	+	-	+	+	d
Growth in 5 % NaCl*	+	+	nd	nd	nd	-	d	+
Sensitivity to erythromycin*	-	-	-	nd	-	+	+	+
Production of reducing substances from sucrose**	-	+	+	+	-	-	-	d
Production of: indole**	-	-	-	-	-	+	-	-
phosphatase**	-	-	-	nd	-	+	d	d
Acid production from:								
lactose**	+	+	d	+	+	-	-	+
maltose**	-	+	+	d	nd	-	+	+
a-methyl glucoside**	-	+	+	+	-	-	-	d
trehalose	+	+	+	nd	nd	-	nd	nd
cellobiose	+	+	-	+	nd	d	+	+
sorbitol	-	-	-	+	nd	-	+	+
inulin	-	-	+	-	-	d	nd	nd
raffinose	+	+	-	nd	-	+	nd	nd
palatinose*	-	+	+	+	nd	nd	nd	nd
melibiose	+	+	-	+	-	nd	nd	nd
d-arabitol	-	-	-	+	nd	nd	nd	nd
Utilization of organic acids:								
citrate	+	+	-	+	+	+	nd	nd
malonate*†	-	-	-	-	nd	+	nd	nd

¹ Adapted from Hyman (1995).

d, 21-79 % of strains positive; nd, not done.

Ecc, *E. c. carotovora*; Eca, *E. c. atroseptica*; Ecb, *E. c. betavasculorum*; Eco, *E. c. odorifera*; Ecw, *E. c. wasabiae*; Ech, *E. chrysanthemi*; Ecy, *E. cyripedii*; Erh, *E. rhapontici*.

* Details given in Appendix.

*† Tests to differentiate Ecc, Eca and Ech.

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Appendix

CVP medium

As in Section 2: Appendix.

Nutrient agar

Lab Lemco (Oxoid, L29)	1.0 g
Yeast extract (Oxoid, L21)	2.0 g
Bacto peptone (Oxoid, L37)	5.0 g
NaCl	5.0 g
Agar (BDH, 33004)	15.0 g
Distilled water	to 1000 ml

Adjust to pH 7.5 and autoclave at 120 °C for 20 min.

Note: The medium may also be bought ready made from Oxoid (CM4).

Nutrient broth

As nutrient agar but without the agar or ready made from Oxoid (CM1).

Luria broth agar

Bacto peptone (Oxoid, L37)	10.0 g
Yeast extract (Oxoid, L21)	5.0 g
NaCl	10.0 g
Agar (BDH, 33004)	15.0 g
Distilled water	to 1000 ml

Adjust to pH 7.5 and autoclave at 120 °C for 20 min.

Note: The medium may also be bought ready made from Sigma (L 3147)

Luria broth

As Luria broth agar but without the agar or ready made from Sigma (L 3522)

Single strength PEM medium

As in Section 4: Appendix, except that the concentrations are halved.

Anaerobic bags and gas system

As in Section 4: Appendix.

Freezing medium

K ₂ HPO ₄	12.6 g
KH ₂ PO ₄	3.6 g
Sodium citrate	0.9 g
MgSO ₄ .7H ₂ O	0.18 g
(NH ₄) ₂ SO ₄	0.18 g
Glycerol	88.0 g
Water	to 1000 ml

Glass beads

ca. 1 mm diameter glass beads (any source); sterilise at 120 °C for 20 min before use.

Growth on NA at 37 °C and 39 °C

1. Lightly touch spot-inoculate NA plates with a 48 h culture using a straight inoculating needle. Alternatively, spread-inoculate plates with 100 µl of a 10⁴ cfu ml⁻¹ culture using a glass rod spreader.
2. Incubate at 27, 37 and 39 °C for 48 h and check for growth relative to 27 °C.

Rotting of potato tuber slice

1. Sterilise whole, washed, unblemished, turgid, non-sprouting tubers for 10 min in 5 % sodium hypochlorite solution containing a drop of detergent. Rinse in sterile distilled water and air dry at room temperature.
 2. Cut 7-8 mm thick slices with a large sterile knife and place one in a Petri dish containing a few filter papers soaked with sterile water. Take care that the tuber slice surface does not become wet.
 3. Rub a large loopful of an 48 h NA or LBA culture of the bacterium on to a 5 mm diameter spot at the centre of the tuber slice and incubate at room temperature (ca. 20 °C) for 48 h.
- Probe the tuber surface with a sterile needle to determine whether the slice has rotted beyond the inoculated area.

Note: Tubers of some cultivars rot more rapidly and extensively than those of others. This is not a pathogenicity test as some non-pathogens may rot potato tubers under these conditions. It merely indicates the ability of the bacteria to produce large quantities of certain pectic enzymes.

Biochemical tests:

(a) Catalase test

1. Mix a loopful of a 48 h culture on NA or LBA with a drop of freshly prepared 3 % H₂O₂ on a glass slide or the well of a micro-slide.
2. The formation of gas bubbles indicates a positive reaction.

(b) Oxidase test

1. Rub a small loopful of a 48 h culture on NA or LBA on a filter paper impregnated with a drop of 1 % (w/v) freshly prepared aqueous tetramethyl-*p*-phenylenediamine dihydrochloride solution*.
2. Development of a purple coloration *within 10 s* indicates a positive reaction; colour development within 10-60 s is a delayed positive reaction and if after 60 s, the reaction is negative. The use of a platinum loop is necessary to avoid false positives as iron may catalyse the oxidation of the reagent.

* Dimethyl instead of the tetramethyl form may be used. Commercial oxidase touch sticks are available (Oxoid, BR64).

(c) Oxidation-fermentation test

This test differentiates fermentative from oxidative metabolism of carbohydrates.

O/F medium:

Bacto peptone (Oxoid, L37)	2.0 g
NaCl	5.0 g
K ₂ HPO ₄	0.3 g

Bromothymol blue	0.03 g
Agar	8.0 g
Distilled water	to 1000 ml

Dissolve and melt the ingredients, adjust the pH to 7.0, dispense ca. 4 cm height in test tubes and sterilise at 120 °C for 20 min.

Cool to 45-50 °C and aseptically add filter-sterilised (0.45 µm filter) 10 % glucose solution to each tube to a final concentration of 1 %, mix and allow to cool. It is advisable to use a freshly prepared tube to reduce the risk of oxygen diffusion into the medium.

Test

1. Stab-inoculate two parallel test tubes containing freshly prepared O/F medium with a straight wire inoculating needle smeared at the tip with bacteria from a 48 h culture on NA or LBA (see above).
2. Pour one cm depth of sterile paraffin oil into one tube to induce anaerobic conditions and incubate both tubes at 27 °C for 24-48 h.
3. Colour formation resulting from acid production in both tubes indicates fermentative bacteria (+/+); colour formation only in the tube without the oil cover indicates oxidative bacteria (+/-).
4. As controls, tubes with inoculated medium without glucose and tubes with non-inoculated medium with and without an oil over-layer, should be included for comparison.

(d) *Production of reducing substances from sucrose*

Sucrose medium

Bacto peptone (Oxoid, L37)	10.0 g
NaCl	5.0 g
Sucrose	40.0 g
Distilled water	to 1000 ml

Adjust the pH to 7.3, aliquot 3 ml per test tube and sterilise at 120 °C for 20 min.

Benedict's reagent

Sodium citrate	173.0 g
Na ₂ CO ₃ ·H ₂ O	100.0 g
CuSO ₄ ·5H ₂ O	17.3 g
Distilled water	to 1000 ml

1. Dissolve the first two salts by stirring and heating in 800 ml distilled water.
2. Filter through Whatman paper no. 1 with the aid of a vacuum pump and adjust the filtrate to 850 ml with distilled water.
3. While stirring, add the third salt and, when dissolved, adjust to 1000 ml with water.
4. Store in a brown bottle away from light or cover with aluminium foil.

Test

1. Inoculate sucrose medium with a loopful of a 48 h NA or LBA culture of the bacterium and incubate at 27 °C for 48 h.
2. Add an equal volume (3 ml) of Benedict's reagent and heat in a boiling water bath for 10 min.
3. Production of a yellow-orange/ brown colour (with or without precipitate) indicates a positive reaction. A green colour may be considered doubtful and the test needs to be repeated. A negative reaction is when the initial blue coloration remains.

(e) *Production of indole*

Test medium:

Tryptone (Oxoid, L42)	10.0 g
L-tryptophan	1.0 g
Distilled water	to 1000 ml

Adjust pH to 7.4, dispense 5 ml per test tube and sterilise at 120 °C for 15 min.

Kovac's reagent:

<i>p</i> -dimethylaminobenzaldehyde	5.0 g
Pure amyl, iso-amyl or butyl alcohol	75 ml
HCl (concentrated)	25 ml

Dissolve the aldehyde in alcohol in a water bath at 50-55 °C, cool and slowly add the acid.

Note: The aldehyde should be light in colour. Kovac's reagent should be prepared in small quantities and stored at 5 °C for not more than one week. Alternatively Kovac's reagent may be bought ready made from Fluka (60983)

Test

1. Inoculate the test medium with a loopful of a 48 h culture of the bacterium on NA or LBA and incubate at 27 °C for 2 to 5 days.
2. Add 1 ml of Kovac's reagent, shake gently and allow to stand for ca. 5 min at room temperature.
3. A positive reaction is indicated by the presence of a dark red colour in the surface layer.

(f) *Production of phosphatase*

Test medium

Bacto peptone (Oxoid L37)	10.0 g
Lab-Lemco (Oxoid L29)	5.0 g
Sodium phenolphthalein diphosphate solution (10%)	5 ml
Agar (BDH, 33004)	15 g
Distilled water	to 1000 ml

1. Dissolve the first two ingredients in distilled water by heating and adjust to pH 7.0.
2. Sterilise at 120 °C for 10 min and cool to 45-50 °C.
3. Prepare and sterilise by filtration (0.45 µm filter) a 10 % sodium phenolphthalein diphosphate solution.
4. Add the phenolphthalein solution, mix well and pour in Petri dishes.

Test

1. Spot-inoculate plates dried as in "Isolation" sub-section above with a 48 h culture of the bacterium on NA or LBA and incubate at 27 °C for 48 h.
2. Place 0.1 ml of ammonium hydroxide in the lid of the Petri dish and invert the medium on to it. After ca. 30 seconds at room temperature, phosphatase-positive colonies become bright pink or red. The colour may fade with time.

(g) *Growth in 5 % NaCl*

Test medium:

Bacto peptone (Oxoid, L37)	10 g
NaCl	50 g

Distilled water to 1000 ml

Adjust pH to 7.3, aliquot 3 ml per test tube and sterilise at 120 °C for 15 min.

Test

1. Inoculate with a loopful of a 48 h NA or LBA culture of the bacterium and incubate at 27 °C for up to 7 days.
2. Score daily for turbidity, indicating bacterial growth.

(h) *Acid production from organic compounds*

Test medium

Bacto peptone (Oxoid, L37)	10.0 g
Bromocresol purple indicator solution (1.5 %)	0.7 ml
Organic compound stock (20 %)	50 ml
Distilled water	950 ml

Test

1. Prepare the dye stock solution by dissolving 1.5 g in 100 ml ethanol.
2. Mix and dissolve ingredients, except the test organic compound, in 900 ml water and sterilise at 120 °C for 15 min.
3. Prepare and filter sterilise using a 0.45 µm filter a 10 % stock solution of test organic compound and add aseptically to the sterilised medium.
4. Aseptically dispense 2.5 ml into sterile test tubes and inoculate with 0.1 ml of 10⁸ cells ml⁻¹ water of test bacterium from a 48 h culture on NA or LBA. Incubate at 27 °C for up to 7 days.
5. A change in colour from blue to yellow indicates a positive reaction.

(i) *Utilisation of organic acids*

Yeast extract-dextrose-CaCO₃ (YDC) medium:

Yeast extract (Oxoid, L21)	10.0 g
Dextrose (glucose) 20 % solution	100 ml
CaCO ₃ (finely ground)	20.0 g
Agar (BDH, 33004)	15.0 g
Distilled water	900 ml

Dissolve ingredients, except dextrose, in 900 ml water and sterilise at 120 °C for 1 h. Cool to 50 °C and aseptically add 100 ml of a 20 % filter sterilised (0.45 µm filter) dextrose solution. Mix well to keep the calcium salt in suspension and pour in Petri dishes.

Test medium

NH ₄ H ₂ PO ₄	0.5 g
K ₂ HPO ₄	0.5 g
MgSO ₄ ·7H ₂ O	0.2 g
NaCl	5.0 g
Yeast extract (Oxoid, L21)	0.8 g
Organic acid	2.0 g
Bromothymol blue	0.016 g
Agar (BDH, 33004)	12.0g
Distilled water	to 1000 ml

Dissolve ingredients, adjust pH to 6.8, dispense 10-12 ml in test tubes, sterilise at 120° C for 15 min, slant tubes and allow to set.

Test

Inoculate YDC medium with test bacterium and incubate at 27 °C for 48 h.

Streak-inoculate the slanted surface of the test medium with a loopful of the YDC inoculum and incubate at 27 °C. Observe for growth and the development of a blue coloration (alkaline reaction) at 7, 14 and 21 days. A light blue colour is considered to be a doubtful or negative reaction.

Abbreviations and symbols

bp	Base pair
cfu	Colony forming unit
CVP	Crystal violet pectate
$C_6H_5Na_3O_7 \cdot 2H_2O$	Sodium citrate
$CaCO_3$	Calcium carbonate
$CaCl_2 \cdot 2H_2O$	Calcium chloride
$CuSO_4 \cdot 5H_2O$	Cupric sulphate
D-PEM	Double strength pectate enrichment medium
DASI-ELISA	Double Antibody Sandwich Indirect ELISA
DIECA	Sodium diethyldithiocarbamate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
E-ELISA	Enrichment ELISA
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
Eca	<i>Erwinia carotovora</i> subsp. <i>atroseptica</i>
Ecc	<i>Erwinia carotovora</i> subsp. <i>carotovora</i>
Ech	<i>Erwinia chrysanthemi</i>
FITC	Fluorescein isothiocyanate
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
HRP	Horse radish peroxidase
IFC	Immunofluorescence colony staining
IMS	Immunomagnetic separation
IMS-CVP	Immunomagnetic separation + crystal violet pectate
IgG	Immunoglobulin
kb	Kilo base
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen orthophosphate
$MgCl_2$	Magnesium chloride
$MgSO_4 \cdot 6H_2O$	Magnesium sulphate
NA	Nutrient agar
Na_2CO_3	Sodium carbonate
$Na_2HPO_4 \cdot 12H_2O$	Di-sodium hydrogen orthophosphate
Na_2SO_3	Sodium thiosulphate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaI	Sodium iodide
NaN_3	Sodium azide

NaNO ₃	Sodium nitrate
NaOH	Sodium hydroxide
(NH ₄) ₂ SO ₄	Ammonium sulphate
PBS	Phosphate buffered saline
PBS-BSA	PBS buffer + bovine serum albumin
PBS-DIECA	PBS buffer + sodium diethyldithiocarbamate
PBS-T	PBS buffer + Tween 20
PBS-Ta	PBS buffer + Tween 20
PBS-Tb	PBS buffer + Tween 20
PCR	Polymerase chain reaction
PEM medium	Pectate enrichment medium
PT	Polygalacturonic acid/Tryptone medium
RTB	Ringer solution/Tween/bovine serum albumin
SCRI	Scottish Crop Research Institute
SDS	Sodium dodecyl sulphate
TBE	Tris-Borate-EDTA buffer
TE	Tris-EDTA buffer
TE/SDS	Tris-EDTA buffer + sodium dodecyl sulphate
TMB	Tetramethylbenzidine dihydrochloride
<i>Taq</i>	DNA polymerase from <i>Thermus aquaticus</i>
Tris	Tris-methylaminomethane
UV	Ultra-violet
mol l ⁻¹	moles per litre, molar (M)
mmol l ⁻¹	milli moles per litre (10 ⁻³ moles per litre), 10 ⁻³ M
μmol l ⁻¹	micro moles per litre (10 ⁻⁶ moles per litre), 10 ⁻⁶ M
g	gram/gravitational force
mg	milligram (10 ⁻³ gram)
μg	microgram (10 ⁻⁶ gram)
ml	millilitre (10 ⁻³ litre)
μl	microlitre (10 ⁻⁶ litre)
μm	micrometre (10 ⁻⁶ metre)

Section 8

Sources of materials

Equipment and reagents

Only a restricted range of materials has been quoted in the Manual as a guide; their sources, mostly British for simplicity, are listed below. National addresses of international firms may be obtained by contacting the addresses given here. Alternative sources for most of the materials are available.

Adgen, SAC, Watson Peat Building, Auchincruive, Ayr, KA6 5HW, Scotland, UK

Avanced Magnetics

Anachem Ltd (Gilson), 20 Charles Street, Luton, Bedfordshire, LU2 0EB, England, UK

BDH (Corning), Merck Ltd, Merck House, Poole, Dorset, BH15 1TD, England, UK

Bio-Rad Laboratories Ltd, Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire HP2 7TD, England, UK

Boehringer Mannheim, Sandhoer Strasse 116, D-68298 Mannheim, Germany.

Dynal UK Ltd, 10 Thursly Road, Croft Business Park, Bromborough, Wirral, Merseyside, L62 3PW, England, UK

Fisher Scientific, Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG, UK.

Fluka Chemicals, The Old brickyard-New road, Gillingam, Dorset SP8 4JL, UK.

Genset, 1 rue Delaunay, 75011 Paris, France.

ICN Biomedicals Ltd (Flow Laboratories, Nalgene, Titertek), Unit 18, Thame Park Business Centre, Wennum Road, Thames, Oxfordshire OX9 3XA, England, UK

Imperial Machine Company (Peelers) Ltd, Croxley Green, England, UK

International Mycological Institute, Bakeham Lane, Englefield Green, Egham, Surrey TW20 9TY, England, UK

Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF, Scotland, UK

Jencons Scientific Ltd, Cherrycourt Way Ind. Est., Stanbridge Road, Leighton Buzzard, Bedfordshire, LU7 8UA, England, UK

Leica AG, Heerbrugg CH-9435, Switzerland.

Life Sciences International UK Ltd (Finpipette, Labsystems, Forma IFC, Hybaid, Spectronic,

Denley, Luckham, Shandon), Unit 5, The Ringway Centre, Edison Road, Basingstoke, Hampshire, RG21 2BR, Engla

LINARIS GMBH (Agdia), Biologische Produkte, D-978577 Bettingen am Main, Germany.

M. Burger Enterprises, 2225 Eton Ridge, Madison, Wisconsin 53705, USA.

Meku (Pollähne), Eric Pollähne, Am Weingarten 14, 3015 Wennigsen am Deister, Germany.

Metachem Diagnostics LTD (Advanced Magnetics), 29 Forest Road, Piddington, Northampton, NN7 2DA, England, UK

MWG-Biotech Ltd, Waterside House, Peartree Bridge, Milton Keynes MK6 3BY, England, UK.

Nalgene, Techmate Ltd., 10 Bridgeturn Avenue, Old Wolverton, Milton Keynes MK12 5QL, UK.

Nikon Europe B.V., P.O. Box 222, 1170 AE Badhoevedorp, The Netherlands.

Nunc Plastic ware, Life Technologies Ltd., 3 Fountain Drive, Inchinan Business Park, Paisley PA4 9RF, UK.

Olympus Diagnostic Systems, Olympus House, 7 West Links, Tollgate, Eastleigh, Hants., SO53 3TG, England, UK

Oxoid, Unipath Ltd., Wade Road, Basingstoke, Hampshire RG24 8PW, UK.

Perkin Elmer, Kelvin Close, Birchwood Science Park North, Warrington, Cheshire, WA3 7PB, England, UK

Pharmacia Biotech, 23 Grosvenor Road, St Albans, Herts., AL1 3AW, England, UK

Philip Harris (Flowgen), Lynn Lane, Shenstone, Lichfield, Staffordshire, WS14 0EE, England, UK

Plant Research International, P. O. Box 16, 6700 AA Wageningen, The Netherlands (Dr J. M. van der Wolf).

REAL-DURVIZ S.L., Marqués de San Juan, 15-17, 46015 Valencia, Spain.

Sigma-Aldrich Company Ltd (Sigma), Fancy Road, Poole, Dorset, BH12 4QH, England, UK

Stuart Scientific Co. Ltd, Holmethorpe Industrial Estate, Redhill, Surrey, RH1 2NB, England, UK

Unipath Ltd (Oxoid), Wade Road, Basingstoke, Hampshire RG24 8PW, England, UK

Vindon Scientific Ltd, Diggle, Oldham, England, UK

Whatman International Ltd, St Leonard's Road, 20/20 Maidstone, Kent ME16 0LS, England, UK.

Zeiss, Carl Zeiss, Geschäftsbereich Mikroskopie, D-73446 Oberkochen, Germany.

National culture collections

France: Collection Nationale de Bactéries Phytopathogènes, INRA, Station de Pathologie Végétale et Phytobactériologie (CNBP), B.P. 2011, route de Saint-Clément, Beaucouzé, 49000 Angers.

The Netherlands: Culture Collection of Plant Protection Service, PO Box 9102, 6700 HC Wageningen.

United Kingdom: The National Collection of Plant Pathogenic Bacteria (NCPBP), Central Science Laboratory, Sand Hutton, York YO4 1LZ, England.

USA: American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852.