Fire Blight

Diagnostic Manual

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1. DIAGNOSIS OF FIRE BLIGHT

The main purpose of the diagnostic protocol is to confirm the identity of *Erwinia amylovora* in first samples from a suspect outbreak of fire blight in Australia, and in subsequent samples from national surveys to define the extent of the outbreak.

Plant bacteriologists met in 1997 at the Institute for Horticultural Development Knoxfield to consider and refine the previous protocol. Dr Klaus Geider and Ms Susanne Jock, Max Planck Institute, Germany also attended this meeting.

Experts were asked to identify the preferred suite of tests to isolate and identify *E. amylovora* and to recommend the order or sequence of commissioning these tests in a live situation. The estimated time taken for completion of each test was determined and the critical point identified at which sufficient information is available to justify quarantine action.

In addition, subsequent activities have focussed on the assembly of a representative gallery of images that can be used to assist in the identification of symptoms of fire blight on host plants.

The fire blight diagnostic manual was developed at three national meetings. The first in 1994 concerned the development of the contingency plan for fire blight, at which Australian plant bacteriologists formulated the diagnostic protocol. This was published in the Draft Contingency Plan for Fire Blight 1996. The second meeting was organised during the national response to the report of fire blight in the Royal Botanic Gardens, Melbourne in 1997. A national workshop held in September 2004 at the Department of Primary Industries (DPI) – Knoxfield was the third meeting; plant pathologists from all Australian states were in attendance. Feedback provided during the workshop identified a number of improvements, and these changes have subsequently been made to the diagnostic manual, where practical.

At the time of writing (2004), the diagnostic protocol is considered to represent best practice for the isolation and identification of *E. amylovora,* however it is recommended that the methods be periodically reviewed to ensure the protocol remains a relevant and contemporary document.

1.1. Laboratories

Laboratories dedicated for diagnosis of suspect samples of fire blight are to be registered as quarantine areas by AQIS, with controls imposed on the movement of personnel, equipment, plant material and cultures.

After registration, appropriate signage is posted on buildings and doors advising of quarantine controls and restrictions on movement. Two categories of laboratories are foreshadowed:

- **A Field Laboratory**, which is relatively close to the restricted area and used for receipt, initial examination and repackaging of samples to the Diagnostic Laboratory. The Field Laboratory should house all necessary equipment for initial examination and microbiological isolation and culturing, *eg*. microscopes, incubators, fridges, laminar flow cabinets and access to an autoclave.

- **A Diagnostic Laboratory** with equipment and expertise to apply a range of serological, biochemical, molecular and pathogenicity tests to confirm identity of *E. amylovora*. The Diagnostic Laboratory must also have access to secure quarantine approved and registered plant growth facilities for conduct of pathogenicity tests on seedling host plants.

Diagnostic laboratories at DPI – Knoxfield and the Elizabeth Macarthur Agricultural Institute (EMAI) - NSW were endorsed by the "National Workshop on Fire Blight" in 1997, as having the resources and expertise to conduct confirmatory diagnoses for *E. amylovora*.

Note: National standards are being developed for the diagnosis of exotics such as *E. amylovora,* an outcome can be that diagnostic scientists in other laboratories can be trained and recognised as centres for diagnosis of particular exotic pests and pathogens.

2. DIAGNOSTIC PROTOCOL

Accuracy and speed are essential elements of diagnosis where an outbreak of fire blight is suspected, and diagnosis can be partitioned into six elements:

1. examination of symptoms;
2. isolation of cultures of suspect bacteria (48 h);
3. quick tests (48 h), the results from which will be used to justify increased security at the infected site through the establishment of interim quarantine
4. confirmatory tests (6 to 7 days) which, if positive, provide sufficient information for establishment of official quarantine
5. comprehensive tests (12 days) which absolutely confirm the identity of *E amylovora* as the causal organism
6. tests applied to samples from field surveys, after fire blight is confirmed, and where a quick turn around time (24 to 48 h) is required to enable a rapid 'field' response.

Collection and dispatch of samples:

Where possible samples should be collected on the same day as dispatch, alternatively they should be stored in a cool place (preferably not a fridge, because the low temperatures can cause problems in isolating *E amylovora*) until arrangements are made for mailing. Samples should be selected to include the junction between healthy and diseased tissues. They should be wrapped in newspaper and consigned in a bubble pack envelope.

Additional information including details of the sample date, location and site should be recorded on an accompanying sheet, together with any other relevant notes. This information should be placed in a plastic bag, on which is also written the summary details of the sample and the address. This is also included in the bubble pack.

The bubble pack envelope should be clearly addressed with the name and address of the consignee together with appropriate "urgent" signage.

Transport by express post or courier is preferred.

Important: Prior to dispatch the Manager of the laboratory to which the sample is being consigned should be advised by telephone (not email) of the expected arrival date. Special arrangements may need to be made for weekends.

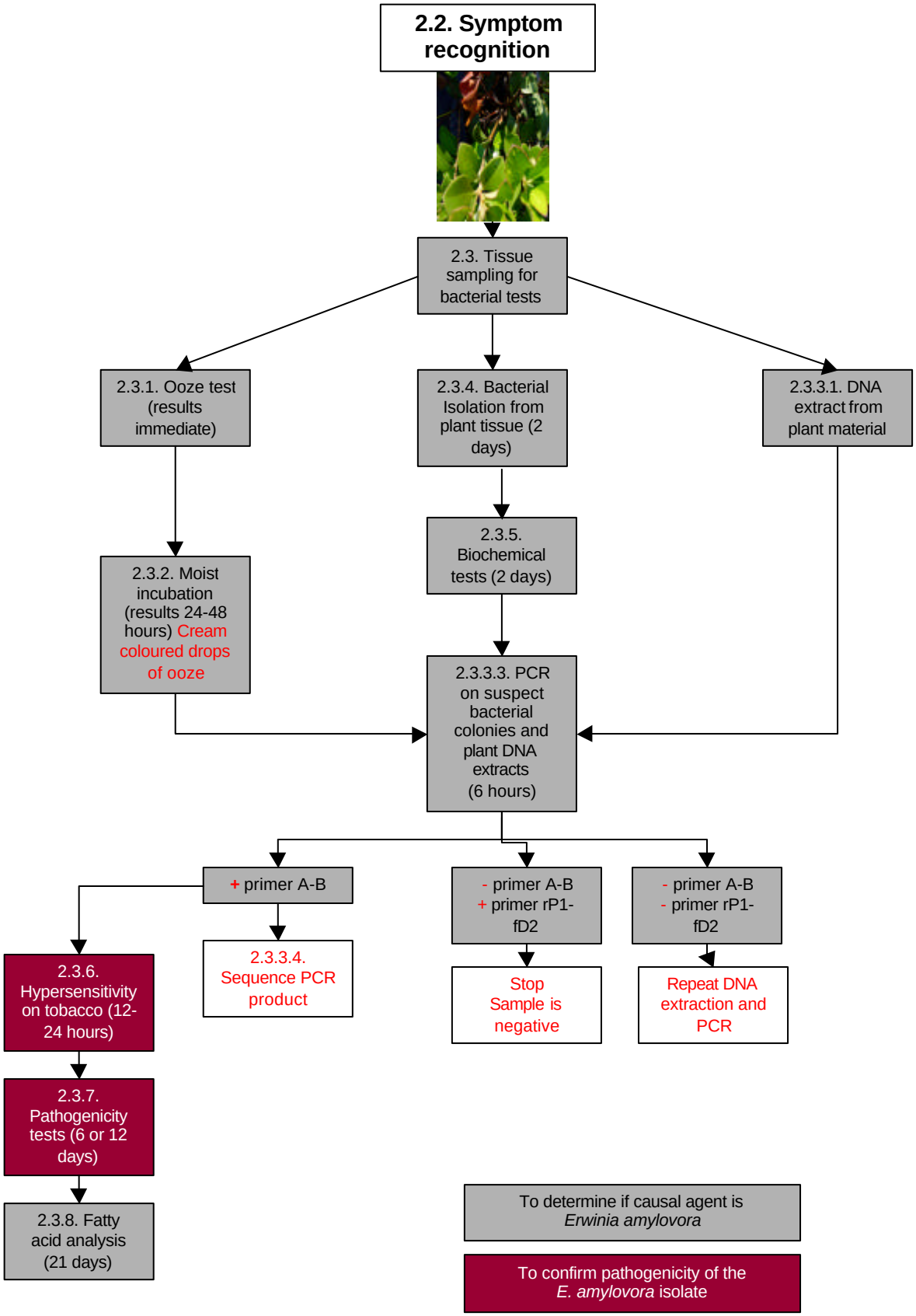
At the time of writing two laboratories have been identified for confirming the diagnosis of fire blight caused by *E amylovora.* Samples should be addressed to:

Manager, Crop Health Services
DPI - Knoxfield
Private Bag 15
Ferntree Gully Delivery Centre VIC 3176
Telephone: 03 9210 9222
Fax: 03 9800 3521

Manager, Pest and Disease Diagnostics
Elizabeth Macarthur Agricultural Institute
Woodbridge Rd
Menangle NSW 2568
PMB 8, Camden NSW 2570
Telephone: 02 4640 6333
Fax: 02 4640 6415

Dedicated facilities and expertise for identification of *E amylovora* by fatty acid analysis are available through NSW Agriculture, Orange Agricultural Institute, Forest Rd, Orange, NSW, 2800. Tel: 02 63913978 Fax 02 6391 3899.

2.1. Diagnostic Flow Chart



2.2. Symptoms

Survey teams will be provided with pocket field guides (Fig. 1) that illustrate the characteristic symptoms of fire blight, which include:

- dark brown to black blight of blossoms and fruitlets
- glassy, water soaked, “off green” appearance of extension shoots and leaves which later turn brown to black – the dead leaves remain attached to the tree
- bacterial ooze may be present on shoot tips, blossoms and at the margins of cankers, especially during warm humid conditions
- characteristic shepherd's crook shape to the tips of diseased shoots
- red to reddish brown streaks in the sap wood beneath the bark
- after leaf fall dark brown blossoms and leaves affected by fire blight remain attached to the tree
- slightly sunken areas (cankers) on larger branches, sometimes with a water soaked or blistered margin

Symptoms typical of fire blight on apple, pear and some ornamental rosaceous hosts are presented in figures 2 to 14.

2.3. Diagnostic Tests

Listed below are the tests, recommended by the National Workshop on Contingency Planning (Knoxfield, September 2004) for fire blight, which are considered the most reliable for identification of *E amylovora*.

Pseudomonas syringae, frequently the cause of shoot blight in apples and pears, can be confused with symptoms caused by *E amylovora.* These organisms can be distinguished by ooze and culture tests on Kings medium, which are described below.

An attenuated strain of *E. amylovora* (*Ea* 322) is recommended for use as a positive control in culturing or molecular tests, but not for pathogenicity testing. Type cultures originally from Dr Steve Beer (Cornell University, USA), are held at DPI Knoxfield and EMAI Camden.

Confirmatory tests for *Erwinia amylovora*

Type of Test	**Time taken for completion**
From Plant Samples:	
1. ooze test	Immediate
2. moist incubation	24 to 48 hours
3. polymerase chain reaction (PCR)*	6 hours
4. isolation of colonies	48 hours
* recommended for ooze or active cankers – confirmation from dormant cankers can be difficult	
On Bacterial Cultures:	
5. biochemical tests	2 days
6. pear slice inoculation	2 days
7. PCR test on bacterial colonies	6 hours
8. sequence of PCR product	2 to 3 days
9. hypersensitivity test on tobacco	12, 24, 36 and 48 hours
10. pathogenicity tests	6 to 21 days
11. analysis of fatty acids	21 days

Details of the methods, together with a list of ingredients and suppliers, are described below.

2.3.1. OOZE TEST
(results available immediately)

Cut thin sections from the plant sample at the interface between diseased and healthy tissue. Mount in water on a microscope slide and examine under low magnification (×40 to ×50) for bacteria oozing from the diseased tissue.

Note: Experience overseas has shown that *E. amylovora* may be present, but the ooze test can still be negative either because symptoms are too old or conditions too dry.

2.3.2. MOIST INCUBATION
(results available within 24 to 48 hours)

Place suspect plant sample and a moist (but not saturated) pad of cotton wool or paper towel in a sealed plastic bag and incubate for 24 h at room temperature.
Clear to cream drops of ooze indicates the presence of *E. amylovora.*

Note: Ooze is not usually produced by *Pseudomonas syringae,* which is quite commonly associated with shoot tip and leaf blight in pome fruit in Victoria.

2.3.3. POLYMERASE CHAIN REACTION (PCR)
(results available in 6 hours)

PCR is a quick test that can be applied directly to bacterial ooze on plant samples, to the suspect plant samples, or to bacterial colonies isolated from samples.

Specialised equipment and chemicals are required, including a PCR thermal cycler, gel electrophoresis apparatus, and reagents including PCR primers specific for *E. amylovora.*

2.3.3.1. DNA preparation from plant tissue*

Reagents

Modified SCP

	For 1000 ml	Final Concentration
Disodium succinate $C_4H_4Na_2O_7$	1g	3.7mM
Trisodium citrate $C_6H_5Na_3O_7$	1g	3.9mM
Monobasic potassium phosphate K_2HPO_4	1.5g	8.6mM
Dibasic potassium phosphate KH_2PO_4	1g	7.3mM
PVP40 (polyvinyl pyrrolidone 40)	50g	1.25mM

Mix and make up to 1000 ml with distilled H_2O.
Autoclave.
Add ascorbic acid (0.02M) and adjust to pH 7 just prior to use.
The stock buffer, without ascorbic acid, can be stored frozen (-20C) for up to 6 months. The buffer with ascorbic acid can be stored at room temperature.

PBS/BSA

a) 10X PBS	For 1000 ml	Final Concentration
Sodium chloride NaCl	80g	1.4M
Dibasic potassium phosphate KH_2PO_4	2 g	14.7mM
Disodium phosphate Na_2HPO_4	11.5 g	81.0mM
Potassium chloride KCl	2g	26.8mM

Mix and make up to 1000 ml with distilled H_2O. Autoclave. Store at room temperature.

b) PBS/BSA
1x PBS plus 0.2% BSA (bovine serum albumin). Store at 4^{o}C.

CTAB buffer + 0.2% mercaptoethanol

For 100 ml

1M Tris, pH 7.5 $H_2NC(CH_2OH)_3$	20 ml
5M NaCl	28 ml
500mM EDTA, pH 8.0 $[CH_2.N(CH_2.COOH).CH_2COON_9]_2.2H_2O$	4 ml
CTAB $C_{19}H_{42}NBr$	2 g
β-Mercaptoethanol	200 µl

Mix and make up to 100 ml with distilled H_2O. Store at room temperature.

Choloroform:isoamyl alcohol

24:1 mix of choloroform to isoamyl alcohol. Store at room temperature.

Isopropanol

100% isopropanol stored at 4°C.

Ethanol

80% ethanol. Store at room temperature.

Water

Sterile distilled H_2O.

Method

1. Place CTAB buffer in 60°C water bath
2. Under sterile conditions select approximately 700 mg of diseased tissue (preferably close to the interface of diseased and healthy tissue)
3. Homogenise in 5 ml of modified SCP grinding buffer with autoclaved mortar and pestle and 0.1g sterile sand
4. Strain homogenate through sterile cheesecloth and transfer 500 µl to a sterile 2 ml centrifuge tube
5. Centrifuge at 12000 rpm for 5 minutes
6. Discard supernatant and re-suspend the pellet in 500 µl of PBS/BSA with pipette
7. Immediately add 800 µl pre-warmed (60°C) CTAB buffer + 0.2% mercaptoethanol
8. Vortex and incubate the centrifuge tube at 60°C for 20 minutes, with occasional mixing
9. Add 600 µl chloroform:isoamyl alcohol (24:1) and vortex vigorously
10. Centrifuge at 12000 rpm for 5 minutes
11. Transfer supernatant to a sterile 2 ml centrifuge tube
12. Add equal volume of cold isopropanol, mix well and leave on ice (or in freezer) for 10 minutes
13. Centrifuge at 12000 rpm for 8 minutes.
14. Rinse pellet with 500 µl 80% ethanol
15. Centrifuge at 12000 rpm for 5 minutes and remove all ethanol with pipette
16. Air dry pellet by placing tube on its side
17. Re-suspend pellet in 200 µl sterile dH_2O

* An alternate method for DNA extraction from plant tissue is presented in Appendix 1.

2.3.3.2. DNA preparation from colonies
From colonies isolated in section 2.3.4.

1. Grow the colony overnight in 5 ml of Luria-Bertani broth.
2. Remove 500 µl of culture and spin for 5 min at 13,000 rpm.
3. Decant the supernatant and resuspend the pellet in 100 µl of sterile distilled water. Add 1 µl of Tween 20 (final concentration is 1%).
4. Heat the resuspension at 60°C for 10 min.
5. Use 1 µl of the resuspension (template) for each 25 µl PCR reaction.

2.3.3.3. Polymerase chain reaction

(1) Primer sequences (current world standard) (Bereswill *et al*., 1992) are:
Primer A 5' CGG TTT TTA ACG CTG GG 3'
Primer B 5' GGG CAA ATA CTC GGA TT 3'

(2) In each PCR run include a positive (template containing *E. amylovora* DNA) control, and two negative (i, sdH_2O and ii, template containing healthy plant-host DNA) controls, to be added in the following order:
i) Negative control,
ii) Sample DNA(s),
iii) Negative control,
iv) Positive control.

(3) Place in thermal cycler using the following program:

Cycle 1	step 1	94°C	5 min
Cycles 2 to 37	step 1	94°C	1 min
	step 2	52°C	2 min
	step 3	72°C	2 min
Cycle 38	step 1	72°C	15 min
	step 2	15°C	1 min
Cycle 39	hold at 15°C		

(4) When the PCR run is finished, place samples onto ice or freeze until ready to electrophorese.
(5) Analyse PCR products by electrophoresis on a 1.5 % agar gel at 110 volts for 40 min, and concurrently run DNA markers.
(6) **Note**: The expected size of the amplified product generated by primer pair A-B is 0.9kb. Stain gel using ethidium bromide (concentration 2.5µl/ml), or similar, and illuminate with UV transilluminator to visualise bands.
(7) Record results.

Note: Bands from test samples which are at the same position on gels as the positive control (*Ea* 322) indicate the presence of *E. amylovora* (Fig. 15, lanes 3 and 5).

Internal PCR control using "Housekeeping Genes" to Bacterial 16s Ribosomal DNA

It is important to use housekeeping genes such as ribosomal DNA as a check to ensure the DNA template does not contain PCR inhibitors. This reduces the possibility of a false negative result. The recommended primer set is published by Weisburg *et al.* (1991), which target the 16sRNA gene of bacteria. The primer names and sequences are; fD2 (5'-AGA GTT TGA TCA TGG CTC AG-3') and rP1 (5'-ACG GTT ACC TTG TTA CGA CTT-3'), and the expected amplicon size is approximately 1.5 kb. The annealing temperature for the PCR cycling is optimal at 55°C. However, this primer pair can be multiplexed with the fire blight specific primers A & B, using the annealing temperature of 52°C (Fig. 15, lanes 3, 4, 7 and 8).

Note: A number of other *E. amylovora*-specific PCR tests can be used to confirm results. These include: **Nested**: McManus & Jones (1995); **AMS**: Bereswill *et al*. (1995).; **NZ**: Hale & Clarke (1990).

2.3.3.4. Sequencing the PCR product

The PCR product should be sequenced to confirm the identity of *E. amylovora*.

PCR products are purified from gels using the QIAquick Spin kit (Qiagen) as per manufacturer's instructions. The cleaned PCR products are prepared for sequencing with ABI Big Dye (Applied Biosystems/Roche), as per the manufacturer's instructions. Sequencing is outsourced. The raw sequences are compared against all sequences posted on the GenBank database using the program BlastN (Altschul *et al*., 1997).

2.3.4. INITIAL ISOLATION OF BACTERIA FROM PLANT SAMPLES
(results available in 2 days)

1. Under sterile conditions, select 100 mg of diseased tissue (preferably close to the interface of diseased and healthy tissue), place into 1 ml of sterile distilled water and coarsely macerate the tissue.
2. The macerate is left for 30 min at room temperature.
3. Add 100 µl of neat macerate and 100 µl at 10^{-2} dilution to KB* and LB5** agar plates.
4. Incubate at 28°C for 2 days.
5. Select small white colonies (Fig. 16) for biochemical tests on selective media (see below) or PCR (see above). Please note that the *E. amylovora* strain depicted in figure 16 is visually representative of *E. amylovora*, there may be slight variations in colour and growth rate amongst other strains.

* Kings B medium

** Luria-Bertani medium with 5 % sucrose

2.3.5. BIOCHEMICAL TESTS ON SELECTIVE MEDIA
(results available in 2 days)

Six selective media are used in combination to identify *E. amylovora*:

1. King's B Medium (KB)
2. Luria-Bertani Medium (LB)
3. Luria-Bertani Medium with 5% sucrose (LB5)
4. Minimal Media 1 with Copper (MM1)
5. Minimal Media 2 with Copper (MM2)
6. CCT Medium (CCT)

Selection of pure cultures is not necessary at this stage. A series of six petri dishes each containing a different selective medium are divided into 25 sectors (Fig. 17) with a 12 o'clock reference point marked on the bottom part of the dish.

Sterile toothpicks are used to pick off individual colonies from the serial dilution plates, which have characteristics similar to *E. amylovora*. *Ea* 322 colonies are used as positive controls. The toothpick bearing bacteria from each colony is used to inoculate the agar at the same numbered point in each of the six dishes and then discarded. A new toothpick is used to select a second colony and inoculated at a second point on the six plates. The process can be repeated until all 25 sections on each petri dish have been inoculated.

Plates are incubated at 28°C for 2 days and examined for colony characteristics typical of *E. amylovora* (Fig. 18), with results recorded on the table in appendix 2.

A summary of the expected colony morphology for typical strains of *Erwinia amylovora* on various selective media:

Selective medium	Colony morphology
King's B Medium	Non fluorescent small white colonies with entire margin
Luria-Bertani Medium	Dull white flat colonies with entire margin
Luria Bertani Medium with 5% Sucrose	Domed dull white colonies due to levan production
Minimal Media 1 with copper	*E. amylovora* colonies should not grow on this medium
Minimal Media 2 with copper	Shiny yellow colonies that become mucoid after 5-6 days
CCT Medium	Domed colonies (levan production) with a slightly clear margin and a slightly darker centre. Striations from the centre of the colony outwards may appear.

1. King's B Medium

Used to distinguish between *Pseudomonas* species, which fluoresce green/blue under UV light, and *Erwinia amylovora*, which does not respond to UV.

Proteose peptone		20 g	Oxoid
Glycerol	MW = 92.09	10 g	BDH
K_2HPO_4	MW = 174.18	1.5 g	BDH
$MgSO_4.7H_2O$	MW = 246.48	1.5 g	BDH
Agar		15 g	Oxoid

Distilled water	1 litre (final volume)

Weigh ingredients, mix and make up to 1 litre with distilled H_2O.
To make a homogenous solution, place in autoclave and bring to pressure for 3 minutes, or heat in a microwave.
Dispense into 500ml bottles, ensuring that the caps are not too over-tight.
Return to autoclave and sterilise at 121^{o}C for 20 minutes.

2. Luria-Bertani Medium

Erwinia amylovora colonies are dull white on this medium.

Tryptone	10 g	Oxoid
Yeast extract	5 g	Oxoid
NaCl (MW = 58.44)	5 g	Sigma
Agar	15 g	Oxoid

Weigh ingredients, mix and make up to 1 litre with distilled H_2O.
Adjust pH to 7.0 with NaOH and sterilise at 121°C for 20 min.

3. Luria-Bertani + 5% Sucrose Medium

Erwinia amylovora colonies are very domed on this medium due to the production of the polysaccharide levan.

As for LB Medium, with the addition of 5% (50 g) sucrose (BDH).

4. Minimal Media 1 with Copper

Erwinia amylovora colonies should not grow on MM1.

L-Asparagine ($C_4H_8N_2O_3 \cdot H_2O$)	MW = 150.14	1.5 g	Merck
K_2HPO_4	MW = 174.18	3.5 g	BDH
KH_2PO_4	MW = 136.09	1.5 g	BDH
$(NH_4)_2SO_4$	MW = 132.14	1.0 g	Merck
$MgSO_4.7H_2O$	MW = 246.48	5 mg	BDH
Sodium citrate.$2H_2O$	MW = 294.10	0.25 g	Sigma
Sorbitol	MW = 182.17	10 g	Sigma
$CuSO_4.5H_2O$	MW = 249.68	0.48 g	Sigma
Agar		15 g	Oxoid

Weigh ingredients, mix and make up to 1 litre with distilled H_2O.
After the autoclaved agar has cooled to approx. 60°C, add the following filter sterilised ingredients:

Nicotinic acid (2% w/v aqueous solution)	12.5 ml	Sigma
Thiamin hydrochloride (2% w/v aqueous solution)	10 ml	Sigma

Note. Store both 2 % solutions at 4^{o}C.

Poured plates can be kept at room temperature for up to 1 month.

5. Minimal Media 2 with Copper (MM2)

Erwinia amylovora colonies are shiny and yellow on this medium and become mucoid after 5-6 days.

L-Asparagine ($C_4H_8N_2O_3 \cdot H_2O$)	MW = 150.14	4 g	Merck
K_2HPO_4	MW = 174.18	2 g	BDH
$MgSO_4.7H_2O$	MW = 246.48	0.2 g	BDH
Sorbitol	MW = 182.17	10 g	Sigma
NaCl	MW = 58.44	3 g	Sigma
$CuSO_4.5H_2O$	MW = 249.68	0.48 g	Sigma
Agar		15 g	Oxoid

Weigh ingredients, mix and make up to 1 litre with distilled H_2O.
After the autoclaved agar has cooled to approximately 60°C, add the following filter sterilised ingredients:

Nicotinic acid (2% w/v aqueous solution)	10 ml	Sigma
Thiamin hydrochloride (2% w/v aqueous solution)	10 ml	Sigma
Note. Store both 2 % solutions at 4°C.		

Poured plates can be kept at room temperature for up to 1 month

6. CCT Medium (CCT)

After 48-72h growth on CCT medium, *Erwinia amylovora* colonies are domed (levan production) with a slightly clear margin, darker centre, and striations emanating from the centre of the colony outwards to the interface between the clear and the dark region of the colony.

Sucrose	MW = 342.30	100 g	BDH
Sorbitol	MW = 182.17	10 g	Sigma
Tergitol 7 (anionic) (1% w/v aqueous solution)		30 ml	Sigma
Crystal violet (0.1% w/v in absolute ethanol)		2 ml	BDH
Nutrient agar		23 g	Oxoid

Mix and make up to 1000 ml with distilled water in a flask.
Sterilise at 121°C for 20 min. When the medium has cooled add:

Thallium (I) nitrate (1% w/v aqueous solution)*	2 ml	BDH
Cycloheximide (1% w/v aqueous solution)	5 ml	Sigma
Note. Store both 2 % solutions at 4°C.		

CCT plates should be stored in the dark in the refrigerator.

***Thallium (I) nitrate is a highly toxic chemical and should be handled with care**.

2.3.6. HYPERSENSITIVITY ASSAY
(results available in 12, 24, 36 and 48 hours)

The optimal tissue to use to induce a hypersensitive response is young tobacco (*Nicotiana tabacum*) plant leaves at least 7 cm in length.

The following 'infiltration' method has several advantages over the traditional method of direct injection into veins.
- It is quicker and less skill is required for successful infiltration to occur.
- Several different inoculations can be made on individual leaves;
- And more than one leaf per plant can be used;

1. The colony to be tested is grown overnight at 28°C in 5 ml of LB broth.
2. Take 500 µl of liquid culture and centrifuge at 13,000 rpm for 5 min to obtain a pellet.
3. Decant the supernatant and resuspend the pellet in sterile distilled water to obtain a concentration of approx. 10^8 cells.
4. Injure the tobacco leaf in the centre of an interveinal region with a fine needle
5. Wearing gloves, draw up 300 µl of cell suspension in a 1 ml syringe.
6. Support the back of the tobacco leaf with one hand/finger directly behind the needle injury point.
7. Place the syringe opening (no needle attached) over the needle injury point on the upper surface of the leaf, and press gently but firmly.
8. Slowly expel the 300µl inoculum into the leaf.
9. Infiltration of the mesophyll cells should occur via the needle injury point.
10. Label the leaf segment with self-adhesive labels
11. Maintain plants at 20 – 25°C and avoid conditions of high humidity as this can adversely affect the development of the hypersensitive reaction
12. Check plants after 12, 24, 36 and 48 hours for hypersensitive reaction
13. To determine bacterial concentration in inoculum, please refer to the colony counting technique outlined in appendix 4.

2.3.7. PATHOGENICITY TESTS
(results available in 6 to 21 days)

2.3.7.1. Pear fruitlets
(results usually available in 6 days)

Pear fruitlets are picked green when they are approximately 5 cm long. Suitable cultivars are either 'Williams' ('Bartlett') or 'Packham's Triumph'. They should be stored under Controlled Atmosphere (0°C, 2½% O_2, 2% CO_2) for up to 1 year.

Note: Laboratories with a commitment to diagnose fire blight should ensure annual supplies of pear fruitlets.

Pear fruitlets should be washed in sodium hypochlorite (0.5%) and rinsed in sterile water. Transverse slices (1 cm thick) of fruitlets are placed on filter paper moistened with sterile distilled water in small petri dishes (Fig. 19) and inoculated by a toothpick soaked in a suspension of the suspect bacteria (10^7 - 10^8 cells/ml). The toothpick inoculation can also be done by dipping the end of the toothpick into a bacterial culture (*eg*. from a selective media

plate) and stabbing three times into different areas of the fruitlet slice. Containers are sealed with parafilm and examined daily for a rot of the fruitlet tissue and production of a clear to cream ooze, which is characteristic of *E. amylovora*. Ooze droplets are either subcultured and identified, or tested directly using PCR for identification of *E. amylovora*.

Note: Mature pear fruit can be substituted in the absence of available fruitlets. However, the use of mature fruit is considered to give less satisfactory results.

2.3.7.2. Seedling host plants
(results usually available in 12 days)

Pathogenicity testing requires access to apple and/or pear seedlings and seedlings of other known hosts (appendix 3; hosts of fire blight) from which *E. amylovora* was isolated. These plants should be grown under strict quarantine in a glasshouse or growth-room which has been sterilised if previously occupied by host plants affected by fire blight.

Note: The minimum time required to produce pear and apple seedlings is approximately 12 weeks. The seed needs to be vernalised for a minimum of eight weeks at 4°C in moist vermiculite and then planted under warm growth conditions. True leaves should then be produced within three to four weeks.

Pathogenicity should ideally be tested on apple and pear seedlings, and on host plants from which *E. amylovora* was isolated. Koch's postulates should be confirmed by checking that the inoculation of host plants with the test organism causes disease, and that the same organism can be isolated from diseased tissues.

A young, fully expanded leaf is cut transversely at its widest point and then dipped for 30 seconds into a suspension of the bacterium at 10^7 - 10^8 cells/ml. The inoculated leaf/branch is covered with a plastic bag containing cotton wool moistened with sterile distilled water. After 12 hours at 20-25°C, bags are removed and plants are examined daily for symptoms of brown to black veins and shoot tip blight.

Disease tissue is sampled and the diagnostic tests (described above) are applied to tissue to check for the presence of bacteria and confirm their identity as *E. amylovora.*

Notes: 1) Overseas information indicates that detached shoots of susceptible pear cultivars can be substituted for seedlings providing that turgor is maintained throughout the pathogenicity test. 2) To determine bacterial concentration in inoculum, please refer to the colony counting technique outlined in appendix 4.

2.3.8. ANALYSIS OF FATTY ACIDS
(results available in 21 days)

Special equipment and experience are required for these tests.

For fatty acid analysis, pack moist samples, not wet, between newspaper and enclose in plastic bags. The samples should comprise both early and late stages of symptom development. Pure bacterial cultures can also be consigned on either slopes or fresh agar plates. The slopes/plates need to be clearly labelled and bubble wrapped for protection. The plant samples or cultures should be appropriately labelled and forwarded by express courier to: Orange Agricultural Institute - NSW Agriculture, Forest Rd, Orange, 2800, Tel: 02 6391 3978, Fax: 02 6391 3899.

2.3.9. DISPOSAL OF CULTURES AND PLANT SAMPLES FROM THE DIAGNOSTIC TESTING LABORATORIES AND GLASSHOUSES

With the exception of material that is being used to confirm diagnosis, all other cultures and plant samples should be destroyed as soon as work on them has been completed and recorded.

Cultures should be autoclaved and disposed of in the industrial waste system. Plant samples from the field and pathogenicity tests should bagged and either autoclaved or burnt.

2.3.10. ADDRESSES OF CHEMICAL SUPPLIERS

BDH *(for media chemicals)*
C/o Crown Scientific Pty Ltd
P O Box 2450
ROWVILLE VIC 3178
Ph. 1800 134 175
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Figure 1: Pocket field guide for survey teams

Copies of this guide can be obtained by contacting the relevant State agencies or Industry Development Officers

Figure 2: Shoot tip blight on apple

Figure 3: Stem canker on apples

Figure 4: Rootstock blight of apples

Figure 5: Foliar symptoms of fire blight on apple

Figure 6: Bacterial ooze on apple shoot due to fire blight infection

Figure 7: Foliar symptoms of fire blight on pear

Figure 8: Blossom blight and ooze of pear flowers

Figure 9: Foliar symptoms of fire blight on *Cotoneaster* sp.

Figure 10: Foliar symptoms of fire blight *Cotoneaster* sp.

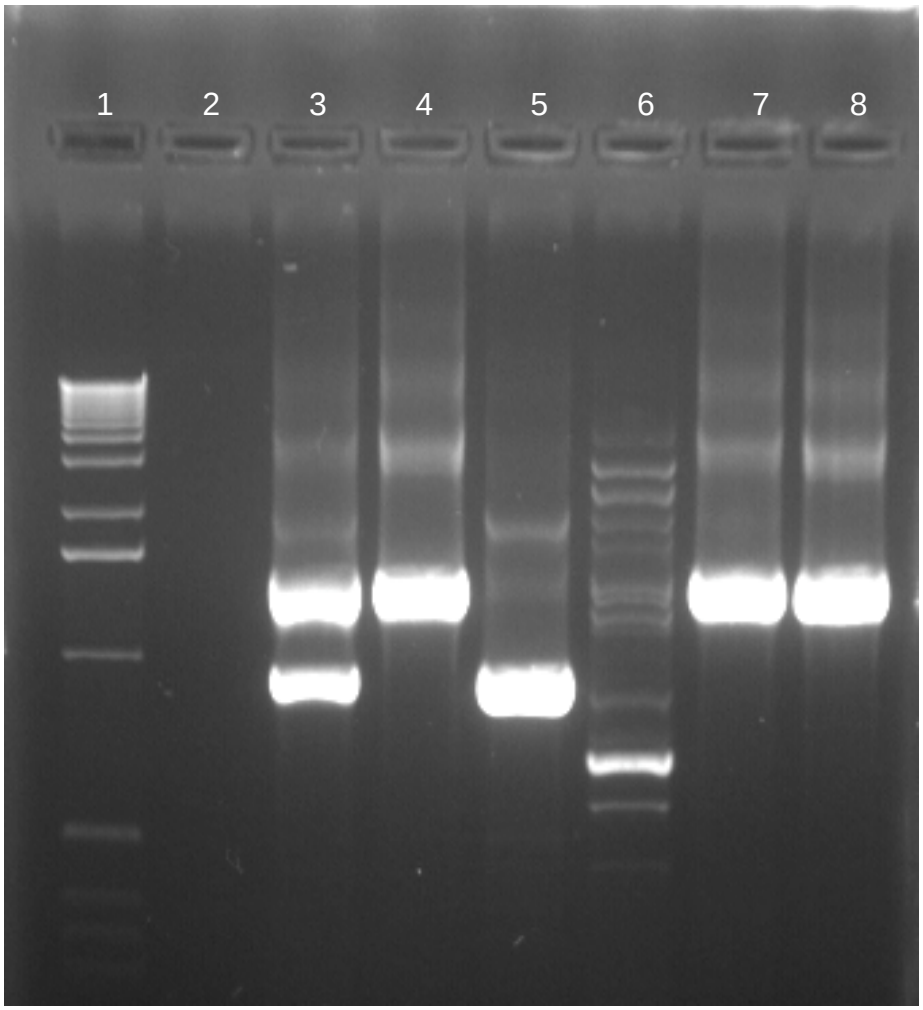
Figure 11: Foliar symptoms of fire blight on Hawthorn (*Crataegus* sp.)

Figure 12: Twig canker on *Crataegus* sp. due to fire blight infection

Figure 13: Stem canker on *Rubus* sp.

Figure 14: Foliar symptoms of fire blight on wild blackberry

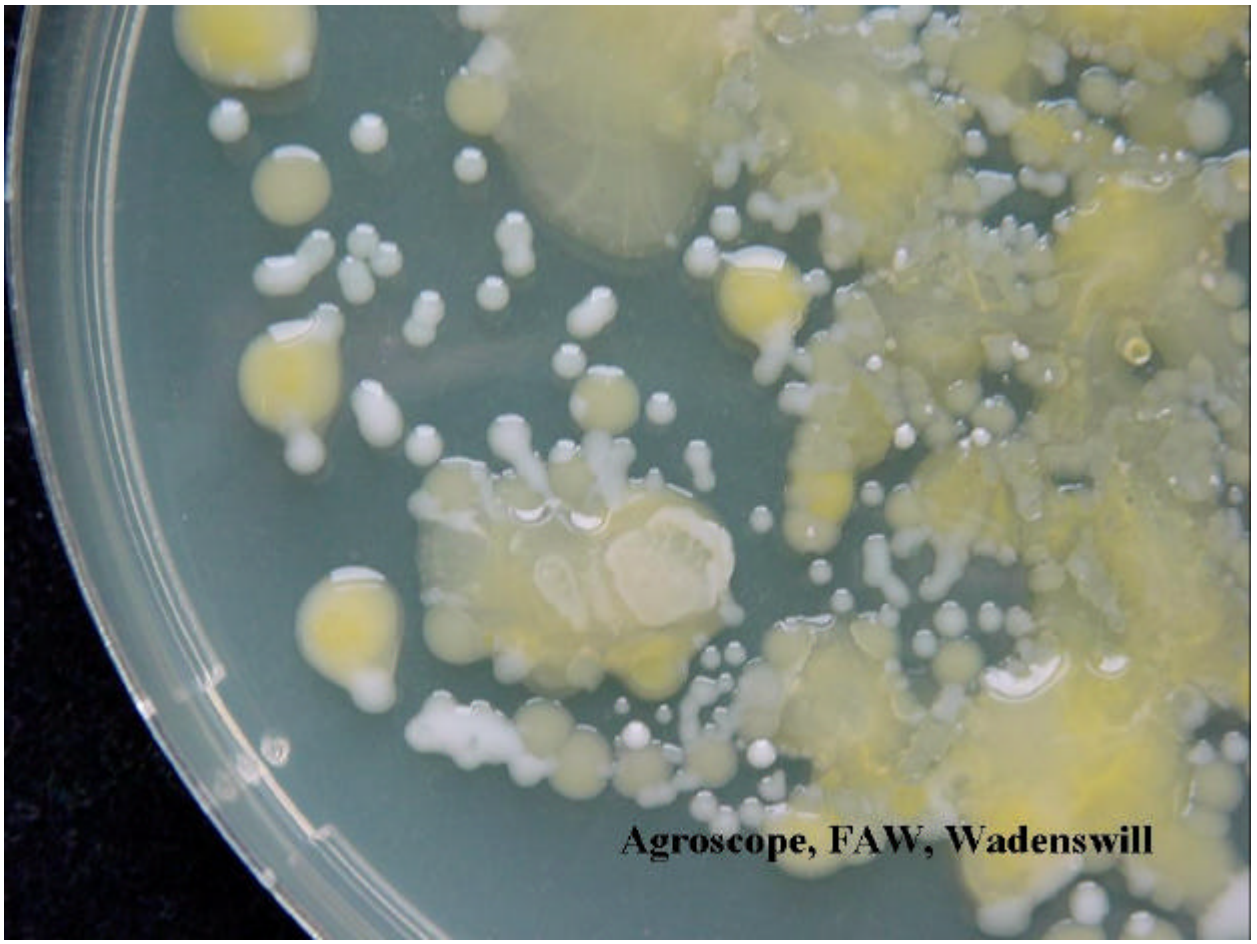
Figure 15: PCR using A-B (*Erwinia amylovora* specific) and rP1- fD2 (house-keeping; for the detection of all known bacteria) primer pairs



Electrophoretic gel showing bands generated by the *E. amylovora* specific A-B primer set (Bereswill *et al*., 1992) that produces a 0.9 kb amplicon, and the house-keeping rP1-fD2 primer set (Weisburg *et al.*, 1991) that detects all known bacteria and produces a ~1.5 kb amplicon;

Lane 1: DNA molecular weight marker X, 0.07-12.2
Lane 2: Buffer control multiplex with primers A-B and rP1-fD2
Lane 3: *E. amylovora* positive control Ea322 multiplex with primers A-B and rP1-fD2
Lane 4: Unknown bacteria isolated from apple multiplex with primers A-B and rP1-fD2
Lane 5: Ea322 with primers A-B
Lane 6: Unknown bacteria isolated from apple with primers A-B
Lane 7: Ea322 multiplex with rP1-fD2
Lane 8: Unknown bacteria isolated from apple with primers rP1-fD2

Figure 16: Colonies of *Erwinia amylovora* (small white colonies with pearly lustre) in a mixed bacterial culture on King's medium B



Courtesy: Chin Gook, DPI Victoria.

Figure 17: Template for grid formation of biochemical tests (selective media)

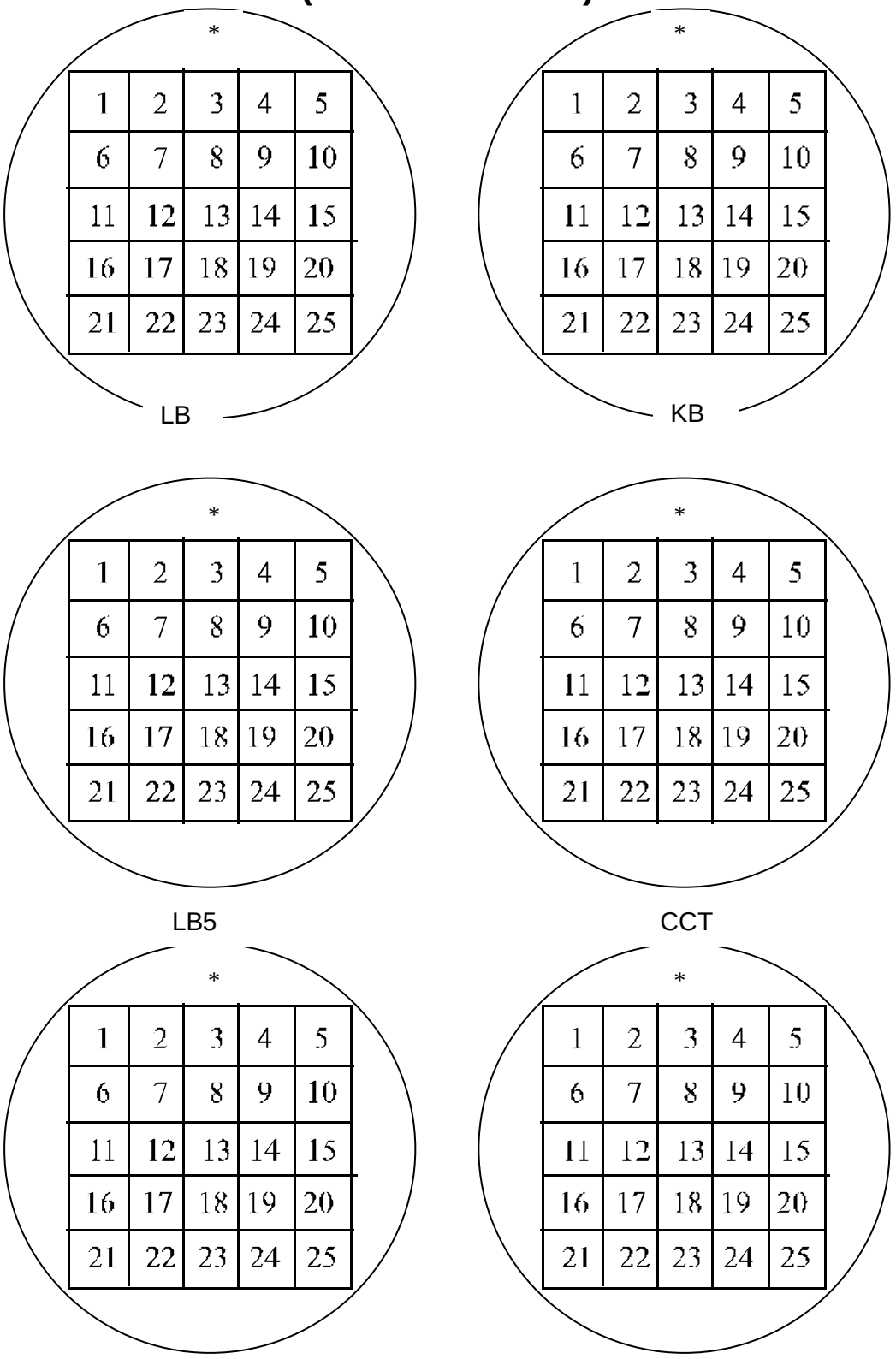
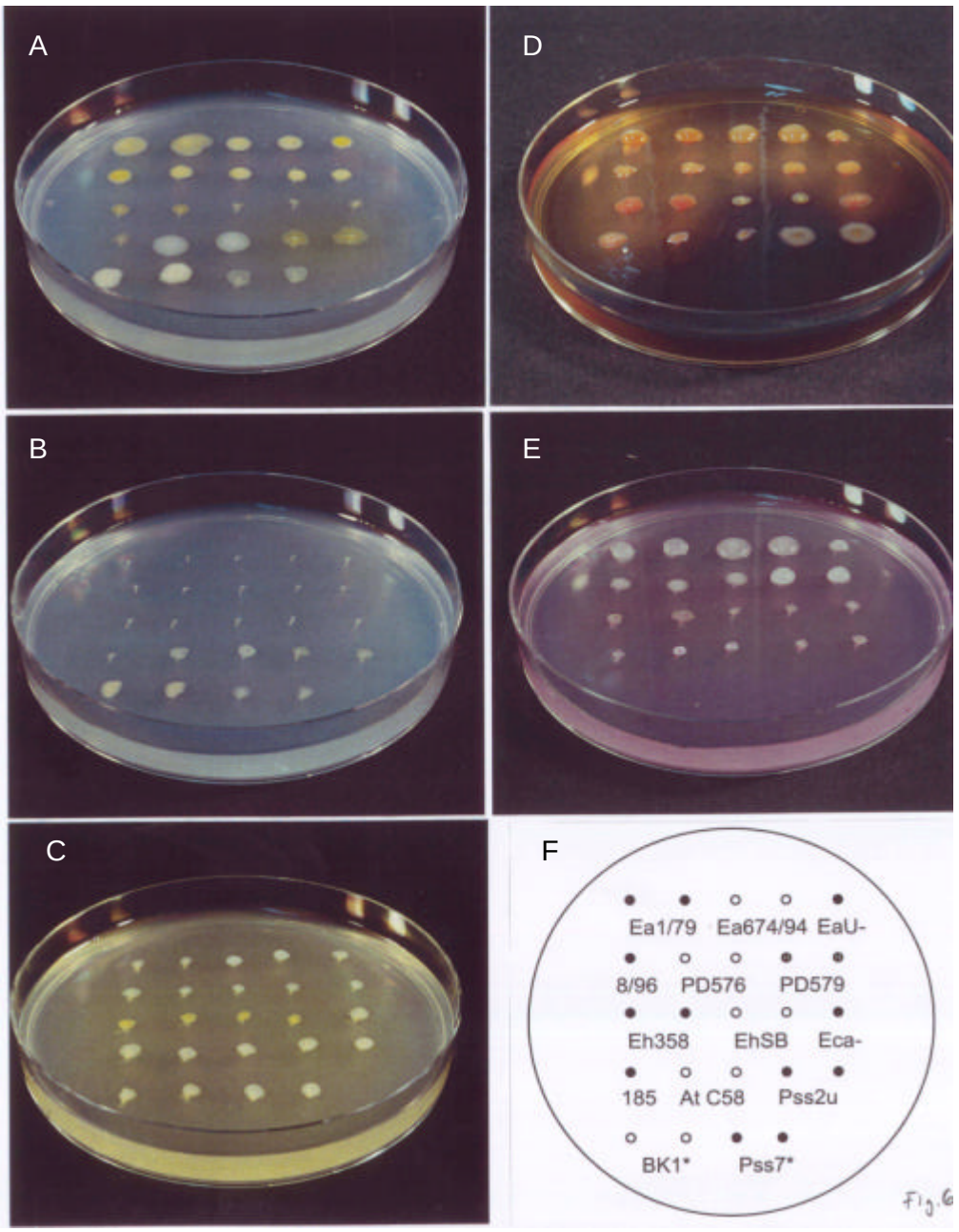
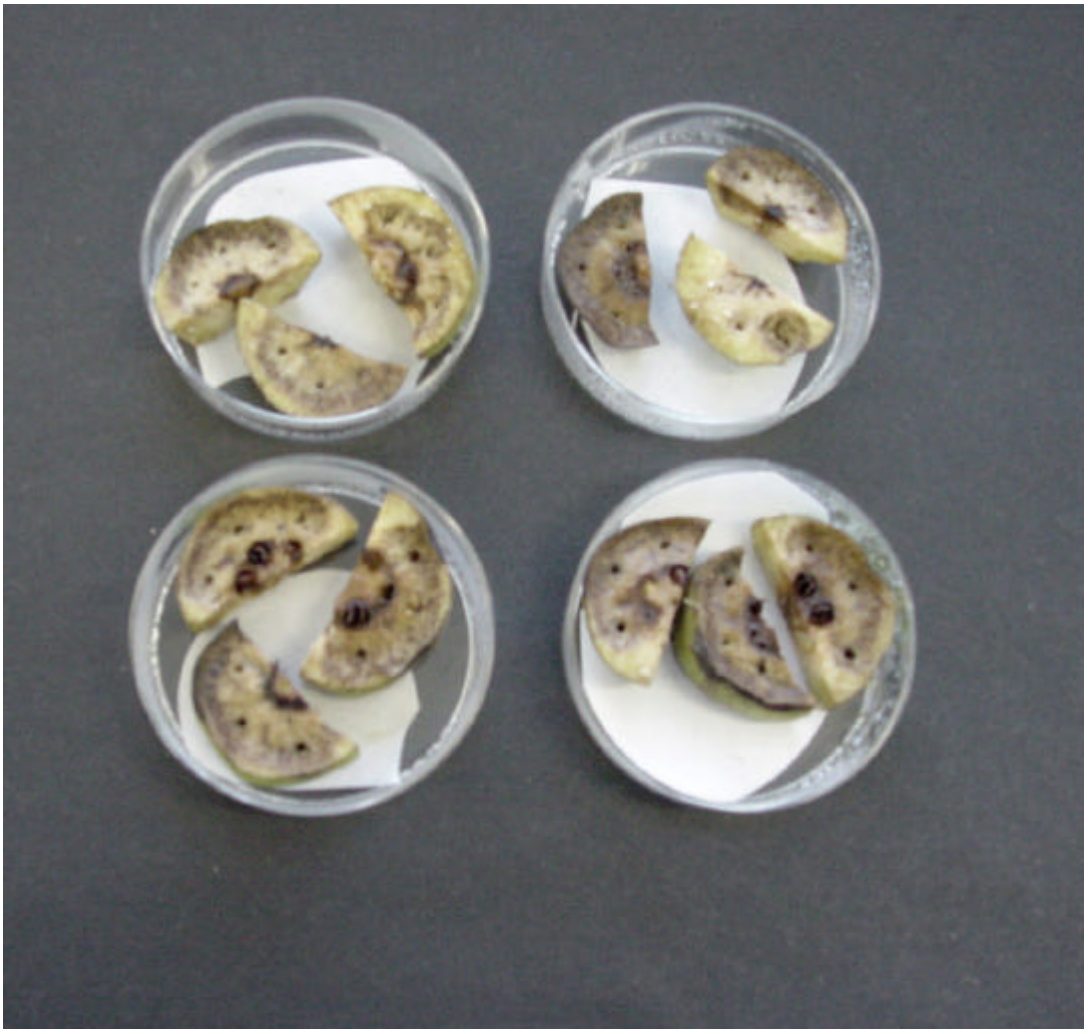


Figure 18: Selective media used to detect *Erwinia amylovora*



Growth of *Erwinia amylovora* and other plant associated bacteria on various solidified media: (A) LB; (B) MM2Cu; (C) MM1Cu; (D) NSA (this media is not recommended by this manual); and (E) CCT. (F) Scheme for strains applied: Ea and PD, *E. amylovora*, Ea1/79 (Germany), Ea674/94 (Austria), EaU8/96 (Utah, United States), PD576 and PD579 (levan-deficient strains from the Netherlands); Eh, *E. herbicola*; Eca, *E. carotovora* subsp. *atroseptica*; At, *A. tumefaciens*; Pss, *P. syringae* pv. *Syringae*; BK1, unidentified bacterium from a pear tree. The marked strains (*) are high in levan production and were omitted from plates with sucrose-containing agar (NSA and CCT) (Bereswill *et al.*, 1998).
Figure courtesy of Prof. Klaus Geider, Max Planck Institute, Germany.

Figure 19: Pear slice inoculations



Transverse sections (1 cm thick) of green pear fruitlets are placed on moistened filter paper in small petri dishes and inoculated by a toothpick soaked in a suspension of bacteria (10^7 – 10^8 cells/ml). Containers are sealed with Parafilm and examined daily for a rot of the fruitlet tissue and production of a clear to cream ooze.

BIBLIOGRAPY

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, **25**, 3389-3402, Oxford: Oxford University Press.

Bereswill, S., Bugert, P., Bruchmuller, I., Geider, K. (1995). Identification of the fire blight pathogen, *Erwinia amylovora*, by PCR assays with chromosomal DNA., *Applied and environmental Microbiology*, 61, 2636-2642.

Bereswill, S., Jock, S., Bellemann, P. & Geider, K. (1998). Identification of *Erwinia amylovora* by growth morphology on agar containing copper sulfate and by capsule staining with lectin, *Plant Disease*, **82**, 158-164.

Bereswill, S., Pahl, A., Bellemann, P., Zeller, W. & Geider, K. (1992). Sensitive and species-specific detection of *Erwinia amylovora* by polymerase chain reaction analysis, *Applied and environmental Biology*, **58**, 3522-3526.

Fay, P. & Penrose, L.J. (1980). Fire blight, *Erwinia amylovora. In* Guidelines for the eradication of some exotic plant pests and diseases. Plant Quarantine Branch, Canberra.

Hale, C.N. & Clarke, R.G. (1990). Detection of *Erwinia* amylovora from apple tissue by DNA hybridisation, *Acta Horticulturae* **273**, 51-55.

McManus, P.S & Jones, A.L. (1995). Detection of *Erwinia amylovora* by nested PCR and PCR-dot-blot and reverse-blot hybridizations. *Phytopathology* **85**, 618-623.

Vanesste, J., Lex, S., Vermeulen, M. & Berger, F. (2001). Isolation of *Erwinia amylovora*-like organisms from blighted plums (*Prunus domestica*) and potato roses (*Rosa rugosa*), Proc. 9th International Workshop on Fire Blight, Napier, New Zealand.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. & Lane, D.J (1991). 16S ribosomal DNA amplification for phylogenetic study, *Journal of Bacteriology* **173**, 697-703.

APPENDIX 1: DNA EXTRACTION FROM PLANT TISSUE

1. Under sterile conditions, select 100 mg of diseased tissue (preferably close to the interface of diseased and healthy tissue), place into 1 ml of sterile distilled water and coarsely macerate the tissue.
2. Leave the macerate for 30 min at room temperature.
3. Spin a 0.5 ml aliquot of the macerate for 5 min at 13,000 rpm; decant the supernatant and resuspend the pellet in 0.1 ml of sterile distilled water. Add 1 µl of Tween 20 (final concentration is 1%).
4. Heat the resuspension at 60°C for 10 min.
5. Use 1 µl of the resuspension (template) for each 25 µl PCR reaction.

Note: This alternative method of DNA extraction is less effective at removing PCR inhibitors, particularly from mature cankers. This method may best be used if extracting DNA from bacterial ooze or fresh active cankers.

APPENDIX 2: RECORD TABLE FOR SELECTIVE MEDIA

No.	Sample	KB	CCT	MM1	MM2	LB	LB + 5%	Comments
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								
18								
19								
20								
21								
22								
23								
24								
25								

KB: + /- = positive growth/No flor, **(Ea-like)**;
- = no growth; +/+ = florescent

CCT: +/+ =positive growth/ Levan positive, opaque **(Ea-like)**;
+/- = positive growth/ no levan; - = no growth or no levan

MM1: - = no growth **(Ea-like)**; + = colony growth

MM2: +/+ = positive growth/yellow **(Ea-like)**;
+/- = positive growth/ not yellow; - = no growth

LB: +/+ = **Ea-like colony**; +/- = positive growth/ not Ea-like

LB + 5%: +/+ = positive growth/ Levan positive and Ea-like colour **(Ea-like)**;
+/- = positive growth, no levan

Comments:

APPENDIX 3: HOSTS OF FIRE BLIGHT.

Genus	Common Name	Flowering Time
Amelanchier	Service berry, June berry	Spring
Cotoneaster	Cotoneaster	Late Spring to Summer
Crataegus	Hawthorn	Spring
Cydonia	Quince	Spring
Eribobotrya	Loquat	Autumn to early Winter
Malus	Apple	Late Winter to Early Spring
Mespilus	Medlar	Spring
Pyracantha	Fire Thorn	Spring
Prunus salicina	Japanese plum	Spring
Pyrus	Pear	Spring
Rubus ideus	Raspberry	Spring
Rubus	Thornless Blackberry	Spring
Sorbus	Mountain Ash	Spring
Stransvaesia	-	Spring and Summer

Note: Blackberry has recently been confirmed as a host of *E. amylovora* and flowers during November to February.

Flowering times of hosts of fire blight will change with latitude and altitude and there is currently insufficient information available from different parts of Australia.

APPENDIX 4: COLONY COUNTING TECHNIQUE

(Courtesy: Chin Gook, DPI Victoria.)

Aim:

To utilise dilution series, spread plate and drop plate methods for counting live bacterial colonies.

Materials:

Suspect bacterial colony (SBC) (36-48 hour culture on KB)
Loop/ cell scraper
King's medium B plates (KB), dried.
PBS (phosphate buffered saline), sterile
Dispenser, sterilised
Test tubes (10 ml), sterilised, 10
Pipette tips (1000 µl), sterilised, set at 1 ml
Pipette tips (10 –100 µl), sterilised, set at 100 µl
Test tube rack
Vortex Mixer

Methods:

Day 1:

1. Subculture SBC on KB, incubate culture at 27^{o}C for 36-48 hours.

Day 1-2:

1. Prepare/sterilise required materials.
2. King's medium B plates may be dried in a laminar flow cabinet, upside down with lids removed, for 30-60 min.

Day 3:

Preparation of bacterial stock suspension (SBC Stock)

1. Dispense 5 ml of PBS into test tube.
2. Use a loop and streak across colonies and pick up a loopful of bacteria.
3. Disperse bacteria in PBS.
4. Vortex tube well until no lumps seen. Should get a cloudy suspension.

10-fold Dilution series

1. Line up 10 test tubes in a rack.
2. Label tubes 1, 2, 3, etc.
3. Dispense 9 ml of PBS into each tube.
4. Vortex SBC Stock, add 1 ml of stock suspension in tube No. 1 (use 1ml pipette tip)
5. Vortex tube 1, use a new pipette tip, transfer 1 ml of mixed suspension in tube No. 1 to tube No. 2.
6. Repeat process from tube No. 2 to tube No. 3 etc.

Spread Plate Method

1. Label dried KB plates on bottom of plates: name of SBC, date, tube number, replicate number, Drop or Spread. Label for tube numbers 5-10 only. Vortex tube No. 10, use a 10-100 µl pipette, draw up liquid, expel, repeat draw/expel 2-3 times, then add 100 µl of liquid on agar.
2. Spread the liquid immediately on agar surface using a sterilised hockey stick. Rotate plate while spreading until the surface appears dry.
3. Use a new pipette tip, repeat process for tube No. 9, and so on, using a new plate for each tube.
4. Incubate plates at 27°C. Check growth for the next two days.
5. Count colonies on plates once they become discrete colonies (approx. in 2-3 days).
6. Count plates with colony range 0 – 500.
7. Refer separate sheet on calculation of number of colony forming units per ml (CFU/ml).

Drop Plate method

1. This is suitable when the CFU/ 10 µl are likely to be low (0 – 30).
2. Using tubes No. 5 –10. Label KB plates as above.
3. Vortex tube as in spread plate. Use a pipette, draw and expel as before, then draw up 20 µl of liquid, spot the liquid on the agar surface. Without disturbing the drop, add four other drops from the same tube on the agar, make sure the drops are spaced out.
4. Leave the plates to dry completely on the bench before transferring to 27°C for incubation.
5. Check colony growth in the next 2 days, count as soon as colonies are countable, before they merge.

Dilution Series Calculation

Tube No.	Stock	1	2	3	4
Dilution Factor	0	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Number / ml In tube					
Number / ml Spread Plate					
Number / 20 µl drop					
Mean No. / 20 µl drop					
Number /ml Drop Plate					

Tube No.	5	6	7	8	9	10
Dilution Factor	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Number /ml in tube						
Number / ml Spread Plate						
Number / 20 µl drop						
Number / ml Drop Plate						