

13 The diagnostic laboratory and greenhouse

13.1 The diagnostic laboratory

The following recommendations are based on existing diagnostic laboratories established in Quang Nam Plant Protection Sub-department (PPSD), Hue PPSD, Nghe An PPSD and the School of Agriculture and Forestry at Hue University through ACIAR funding (Diseases of Crops in the Central Provinces of Vietnam: Diagnosis, Extension and Control, CP/2002/115). These laboratories were established to assist mainly with the laboratory diagnosis of fungal diseases. However, the facilities are also suitable for the isolation of common bacterial plant pathogens. Before working in any laboratory potential safety issues and health risks must be considered. Appendix 2, health and safety, outlines common risks encountered in a diagnostic plant pathology laboratory, however please consult the laboratory supervisor before entering an unfamiliar laboratory.

13.1.1 Location of the laboratory

The diagnostic laboratory should be in a building with walls protected from rain. In tropical regions fungi commonly grow on the inside of walls exposed to rain. Such fungal growth can produce spores which contaminate cultures. Ideally, the laboratory should be located on the second level of the building. This reduces problems with rats and other pests such as ants. It is recommended that the laboratory consist of two large rooms, a preparation room and a clean room.

We also recommend that a room or covered area be used for initially examining field samples and removing soil from root samples by washing. In this area small plant samples should be selected for later isolation of fungal or bacterial pathogens in the clean room. This area can also be used to extract plant parasitic nematodes from soil.



Figure 13.1 Typical arrangement of equipment in a diagnostic laboratory (laboratory in Nghe An PPSD): (a) and (b) two views of clean room, (c) and (d) two views of preparation room.

13.1.2 Preparation room

The preparation room is used for preparing media, including sterilising items in the autoclave, sterilising Petri dishes in an oven, washing glassware and storing glassware, chemicals and other basic items. This room should have an exhaust fan to remove hot air produced by the autoclave and the oven.

13.1.3 Clean room

The clean room is used for isolating fungi and bacteria from cleaned subsamples of diseased plant tissue into pure cultures. It is also used for growing cultures under clean conditions. The microscopes are located in this room for examining cultures and fungal structures.

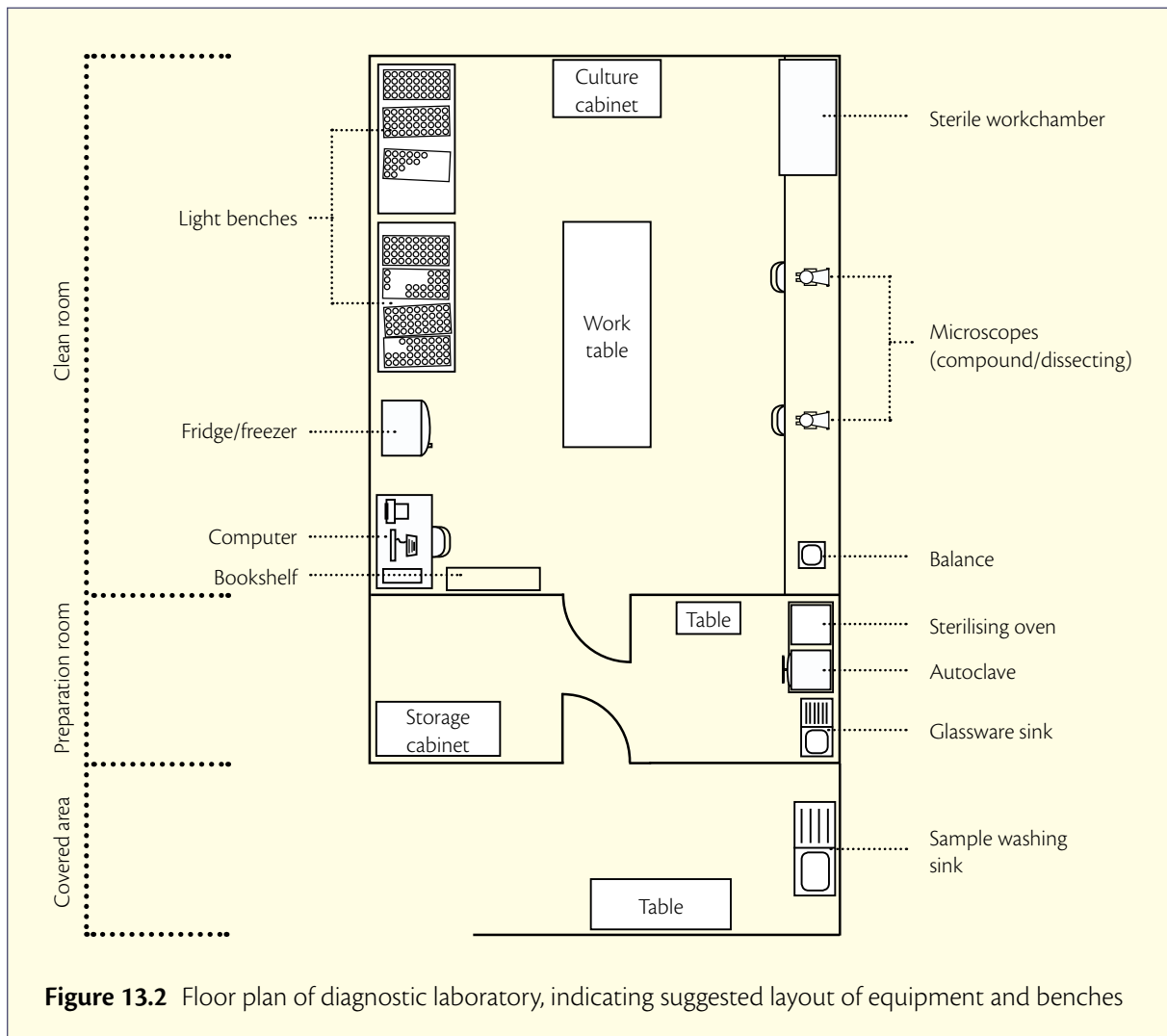


Do not examine large plants in the clean room. Isolate from small plant samples that have been washed free of dust or soil outside the laboratory.

This room should be air-conditioned, if possible, to protect equipment and cultures. It should also be kept free from dust and insects. However, do not have an airtight clean room or humidity will be too high and fungus (mould) will develop on walls and equipment. A dehumidifier is useful in this room. No soil is allowed in the clean room as soil is a source of fungus-eating mites that can contaminate cultures.

13.2 Laboratory layout

When designing a laboratory there are many aspects to consider. It is important that work can be carried out in a logical order and that particular parts of the diagnostic protocol are separated from one another. The following is a layout of a diagnostic laboratory (Figure 13.2), mainly concerned with the diagnosis of fungal plant pathogens.



13.3 Laboratory equipment

13.3.1 Equipment for the clean room

Essential items of equipment for this room are listed below and shown in Figure 13.2:

- A compound microscope fitted with $\times 10$, $\times 20$, $\times 40$ and $\times 100$ (oil immersion) objective lenses. A basic student-grade microscope is sufficient for most diagnostic work. If funds are available the microscope can be fitted with a $\times 20$ metallurgical lens with a long working distance. This lens is ideal for examining fungal structures *in situ* in cultures, as it has a long depth of field (see Section 6.2.2).
- A dissecting microscope for examining diseased plant samples for fungal structures. This is especially important for many leaf infecting pathogens which cannot be grown in artificial media. It is also used for transferring germinated single spores or hyphal tips for purifying cultures, and for studies of plant pathogenic nematodes (see Section 6.2.1).
- A sterile work chamber for pouring media and isolating fungi from plant tissues. The tropical climate of Vietnam means that there are many fungal spores in the air. These spores contaminate media while pouring it, plating tissue or performing culture transfers, unless a sterile work chamber is used.
- A bench with overhead fluorescent lights for stimulating sporulation and pigment production of many fungal species, either in culture or on leaves in moist chambers. Ideally there should be one bench for clean cultures and one bench for plates with diseased tissues. A culture cupboard is useful for incubating cultures in the dark. This is necessary for cultures on media containing antibiotics that are affected by light (e.g. *Phytophthora* selective medium).
- A refrigerator for storing media in bottles, Petri dishes with media (in plastic bags or foil to stop the media drying out), as well as antibiotics, cultures and small tissue samples.
- An electronic balance with an accuracy of 0.001 g is recommended for weighing small amounts of antibiotics or chemicals.
- Large work benches, one for microscopes and the electronic balance, and one for general isolation and cultural work.
- Comfortable chairs for sitting at work benches.
- Accession books, for recording details of each diagnosis and for recording a list of stored cultures.

- A bookshelf containing a wide range of printed information on diseases:
 - textbooks
 - manuals
 - compendia of disease
 - research papers.
- At least one computer with internet access and printer for:
 - database work
 - searching for information
 - access to picture libraries
 - communication via email.
- Small instruments for isolation and cultural work, including:
 - fine forceps
 - inoculating needles
 - surgical scalpel handles
 - transfer loops (for bacterial work)
 - surgical scalpel blades
 - marker pens
 - small knives
 - ethyl alcohol
 - transfer needles (flat tip)
 - tissues
 - cutting boards
 - microscope slides and coverslips
 - filter paper.

Check the walls and equipment regularly for fungal growth.



The floor of the clean room should be mopped regularly to remove any dust particles from the area. Fans should also be turned off and windows closed whilst culturing, to reduce the movement of air in the laboratory. Critical work should be carried out in a laminar flow cabinet which has been wiped down with 70% alcohol.

Surface disinfect as necessary.





Figure 13.3 Essential instruments for isolation, subculturing, purification and identification of fungal and bacterial plant pathogens



Sterilise the bench and wash hands before working with any pure cultures to reduce the chance of contamination.

13.3.2 Equipment for the preparation room

Essential items of equipment for the preparation room are listed below and shown in Figure 13.1:

- An oven for sterilising glass Petri dishes, which should be wrapped in newspaper or in paper bags.
- A small autoclave suitable for sterilising volumes of 1–2 litres of media or water in flasks or Schott bottles. The autoclave is also used to sterilise media or water in glass test tubes or McCartney bottles, pipettes and other glassware wrapped in paper or aluminium foil.

- A pressure cooker to sterilise small amounts of media and water. This can be purchased at most large markets.
- A balance (0.1 g accuracy) for weighing chemicals, potatoes, carrots and so on for media preparation.
- An electric hot plate for boiling potatoes and carrots for media.
- A bench for media preparation.
- A sink for washing Petri dishes and other glassware.
- A storage cabinet.

13.4 Greenhouse for plant disease studies

A greenhouse is an important part of a diagnostic laboratory as it is required for pathogenicity testing, evaluating fungicides and other disease control methods. The design should allow good plant growth and prevent cross contamination in pathogenicity tests and other experiments (Figure 13.4).

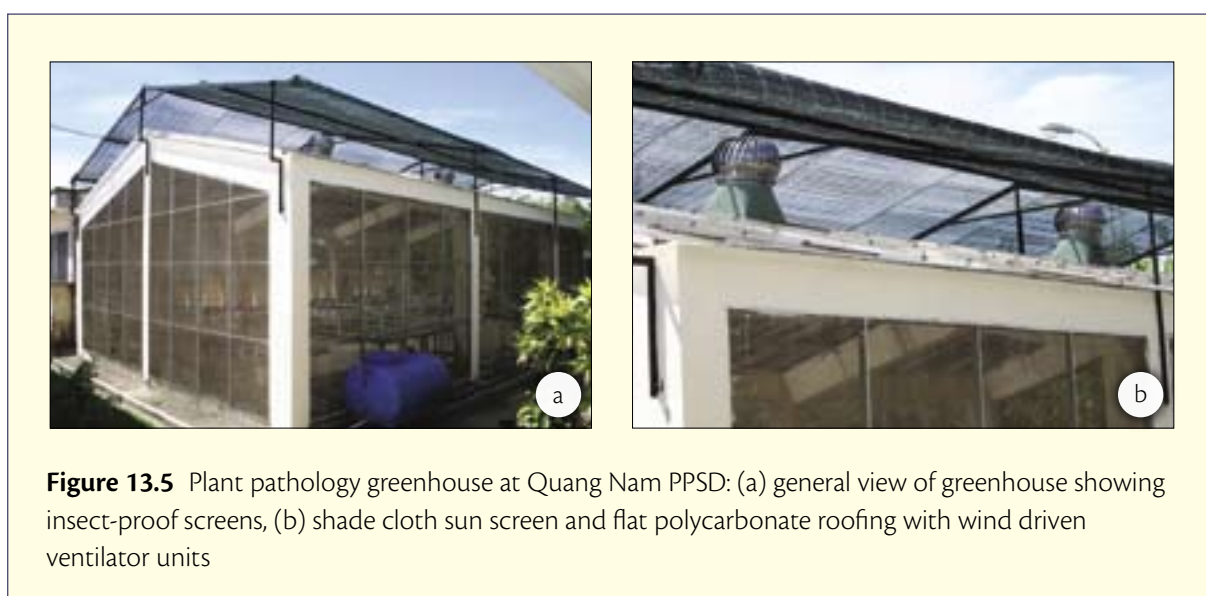
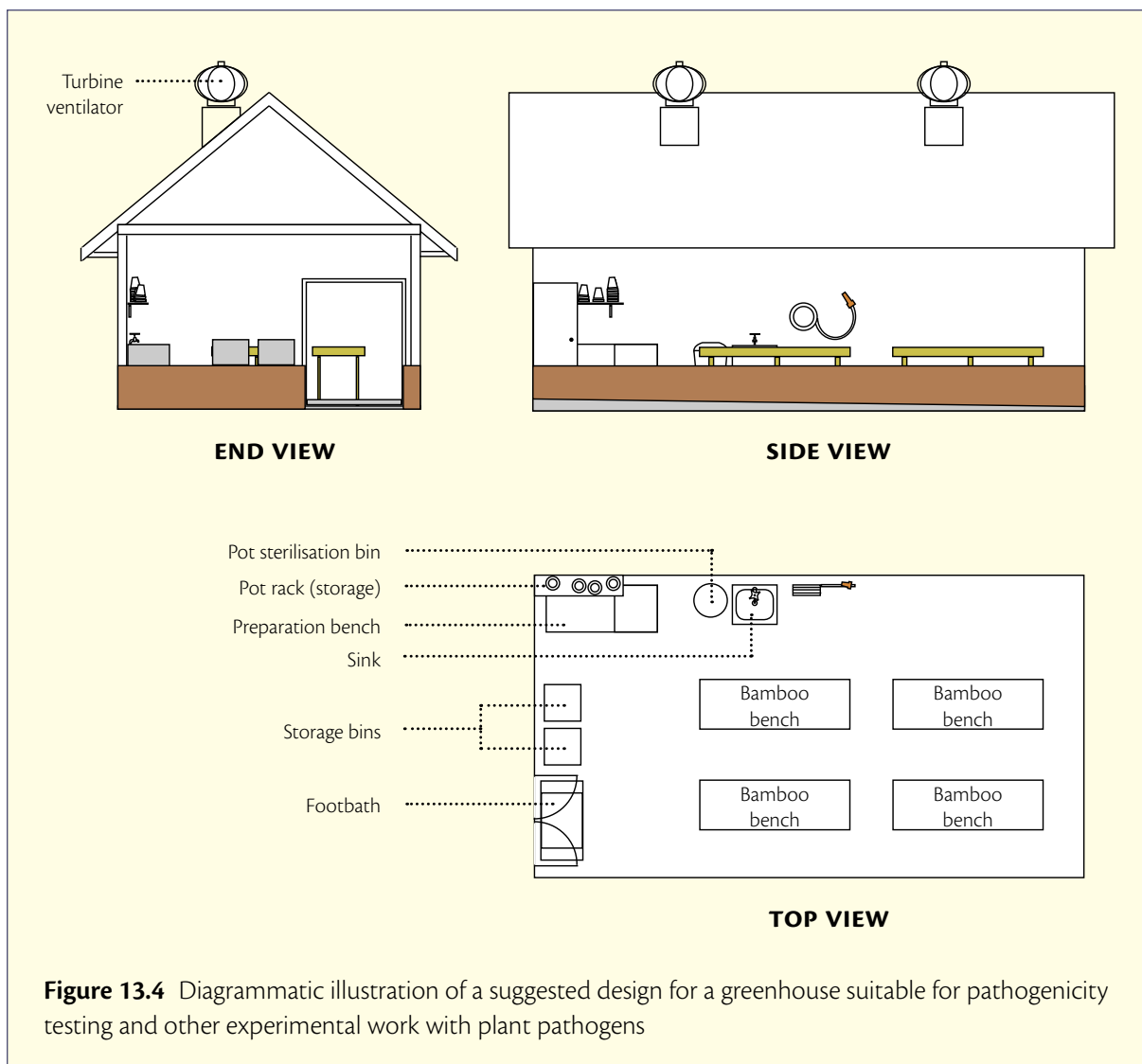
A basic greenhouse should include:

- a transparent roof
- a sloping concrete floor with good drainage
- good ventilation for hot weather (wind driven exhaust fans are very effective) (Figure 13.5)
- a rat-proof design
- a good water supply
- benches (Figure 13.5)
- a preparation area within or near the greenhouse.

The transparent roof should allow at least 75% transmission of sunlight. A corrugated polycarbonate material is ideal for the roof as it is ultraviolet resistant, very durable and easy to attach to a steel or timber roof frame.

Plastic sheeting can also be used for roofing material, but will only last for 1–2 years. Glass roofs are not suitable for regions affected by typhoons or hail. Ideally the roofing should be attached so as to provide eaves that are boxed in (for typhoon protection). Shade cloth can be used in mid-summer to decrease the temperature in the greenhouse (Figure 13.5).

A sloping concrete floor drains well and can be kept clean by hosing with water.



The sides of the greenhouse can be brick walls (height approximately 1 m). Wire netting (such as B40 wire netting) or galvanised wire mesh (with holes approximately 1 cm diameter) can be fixed between pillars, the low brick wall and the roof supports. The wire netting or mesh allows good ventilation and helps to stop rats and birds entering the greenhouse. Insect-proof mesh is expensive, but is important because it prevents insect pests from entering the greenhouse.

A good water supply is needed to keep the greenhouse floor clean and to provide pathogen-free water for the plants. The hose should be hung on the wall so the nozzle is never in contact with the floor of the greenhouse.

Electricity for lighting and for instruments is useful.

Rust-proof steel benches for pots of plants should be approximately 1 m high and 2–3 m long. This height minimises the risk of contamination from the floor. The benches should be portable, so that they can be removed easily if the greenhouse is needed for tall plants, vines on trellises or young fruit trees in large pots. Bamboo benches can be used but must be treated with copper fungicide to inhibit mould growth.

A 10 kg pan balance should be located in the greenhouse for use in weighing pots to monitor the water content of the potting mixture.

13.4.1 Preparation area

The preparation area can be located in the greenhouse or in a nearby building. It should contain storage well above the floor level for all pots and equipment. It should also contain a facility for storing pathogen-free potting soil (mixtures) or sand, coconut fibre, composted saw dust, or other materials used for growing plants for pathogenicity tests. A bench is needed for preparing pots of plants, inoculating soil and other activities. The bench should have a top suitable for easy surface sterilisation, such as stainless steel or marble.

13.4.2 Potting mixture

Pathogen-free potting mixture is essential for pathogenicity tests and many experiments. It is also essential for producing pathogen-free seedlings and cuttings for transplanting for field experiments.

There are many types of potting mixtures. The main features of a good potting mixture are that it has a good water holding capacity and it drains easily. Several types of potting mixture are used in Vietnam. Common materials include composted sawdust, coconut fibre, sand, peat and pelleted chicken manure. Some of these components may be contaminated with plant pathogens; sand may be contaminated with pathogens such as *Pythium* and *Phytophthora*. Coconut fibre and sawdust are usually free of contamination.

Field soil usually contains many plant pathogens. These pathogens should be killed by fumigation or heat treatment (pasteurisation with steam/air mixture at 60 °C for 30 minutes) before the soil can be used for pathogenicity tests. Untreated field soil should not be brought into a greenhouse as the pathogens in the soil can contaminate the greenhouse.

Sawdust compost potting mixture can be made by mixing sawdust, sand and pelleted chicken manure (70:28:2 by volume) and composting it for 4–6 months. Initially this potting mixture should heat up to approximately 50 °C for an extended period which will kill any plant pathogens in the mixture. The potting mixture should be composted in large bins. It is essential that it is kept free from contact with field soil or diseased plants. Coconut fibre can also be a valuable component of potting mixes. Potting mixes can also be pasteurised with steam/air mixture if not composted.

Potting mixtures can be mixed in an electric concrete mixer that has been disinfected. Granular fertiliser can be added during this mixing process.

13.4.3 Greenhouse hygiene

It is essential to have strict rules for staff using the greenhouse to avoid contamination of pathogenicity tests or other studies with plant pathogens in field soil. Equipment and procedures to follow include:

- installing a footbath at the entrance (doorway)
- having rubber boots or sandals for use only in the greenhouse
- not taking field soil or diseased plants into the greenhouse
- removing experimental plants and soil immediately after an experiment is completed and burning diseased plants
- using pathogen-free water
- always keeping the end of the hose away from the floor
- hosing the floor regularly
- staff not entering the greenhouse directly after visiting the field, but showering and putting on clean clothing before using the greenhouse
- sterilising all pots with a strong disinfectant, such as 1% sodium hypochlorite in water for 24 hours, after use in experiments
- storing sterilised pots on shelves well above the floor
- treating bamboo benches with copper fungicide.

13.4.4 Plant management and nutrition

Growing plants in pots for pathogenicity tests and other studies requires careful management of plant nutrition.

It is recommended that pots have holes in the base for good drainage. Small stones can also be placed in the bottom of the pot to allow for good drainage. The aim is to prevent water-logging (saturation) of the material in which the roots grow. Weigh pots regularly to maintain uniform moisture in the potting mixture and prevent water-logging. Soil should only be wet to field capacity.

Plants should be grown in pathogen-free potting mixture. The choice of potting mixture depends on the plants involved, the availability of materials and the nature of the experiment. Nutrition should be adequate for normal plant growth. Granular fertiliser may need to be added to the potting medium before planting. Usually a liquid fertiliser such as Hoagland's solution or a commercial product is applied every 1–2 weeks to maintain normal growth (Figure 13.6). Regular applications of liquid fertiliser are particularly necessary if larger plants are grown in relatively small pots for long periods of time. Commercial liquid N–P–K concentrate and micronutrient liquid concentrate are readily available in Vietnam in small packets.



Figure 13.6 Preparation of commercial fertiliser for greenhouse use

Alternatively, Hoagland's solution can be used (see formula in Box 13.1). This is particularly helpful if the nutrient status needs to be monitored closely or particular nutrients are being left out as part of a nutritional study.

Box 13.1 Hoagland's solution

This solution contains all essential nutrients for good plant growth and development. Hoagland's solution is made up from a range of pre-made stock solutions, which are mixed with water before being used.

For each litre of water add:

- 5 mL potassium nitrate solution
- 5 mL calcium nitrate solution
- 1 mL potassium acid phosphate solution
- 2 mL magnesium sulfate
- 1 mL micronutrient stock solution
- 10 mL iron-EDDHA stock solution.

Stock solutions:

- | | | | |
|-------|--|--------------------------|---------------------------|
| • 1 M | KNO_3 | potassium nitrate | (approx. 101 g in 1 L) |
| • 1 M | $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ | calcium nitrate | (approx. 236 g in 1 L) |
| • 1 M | KH_2PO_4 | potassium acid phosphate | (approx. 136 g in 1 L) |
| • 1 M | $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ | magnesium sulfate | (approx. 246.5 g in 1 L). |

Micronutrient stock:

- | | | | |
|-----------|---|---------------------|-------------------------|
| • 0.046 M | H_3BO_3 | boric acid | (approx. 2.86 g in 1 L) |
| • 0.009 M | $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ | magnesium chloride | (approx. 1.81 g in 1 L) |
| • 0.765mM | $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ | zinc sulfate | (approx. 0.22 g in 1 L) |
| • 0.320mM | $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ | copper sulfate | (approx. 0.08 g in 1 L) |
| • 0.111mM | $\text{H}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$ | molybdic acid (85%) | (approx. 0.02 g in 1 L) |

Iron-EDDHA stock

- | | | | |
|--------|----------------------------|------------|-------------------------|
| • 10mM | $\text{Fe}(\text{NO}_3)_3$ | iron-EDDHA | (approx. 2.45 g in 1 L) |
|--------|----------------------------|------------|-------------------------|

Appendix 1

Making a flat transfer needle

The flat transfer needle is one of the most important tools in the laboratory. NiChrome (Nickel and Chromium Alloy, 80:20) 1 mm diameter wire, commonly used for heating hair driers, has been found to be the most suitable material (Figure A1.1).

1. Cut a 60 mm length of wire.
2. Flatten one end of the wire to approximately three times the original width.
3. Trim the flattened wire to a point using sidecutters or heavy-duty scissors.
4. File off the rough edges of the flat area.
5. Mount the needle in a handle.
6. Completed flat transfer needle.



Figure A1.1 A step-by-step guide to making a flat transfer needle

Appendix 2

Health and safety

In the field

- Take care to follow all recommended safety precautions when applying pesticides, particularly those used for insects (insecticides). Only use registered chemicals.
- Wash hands carefully before eating meals, especially when soil has been handled.
- Drink adequate water on hot days in the field.
- Take care with machetes so that you do not cut yourself or other people.

In the laboratory

- Check the safety aspects of all chemicals before use. Such information can be found on the product packaging or on the internet. Major chemical companies supply links to the chemical Material Safety Data Sheets that correspond with their products.
- Use gloves where appropriate.
- Ethyl alcohol is highly flammable. Do not wipe benches near a flame.
- Keep a fire blanket in the laboratory to put out clothing fires.
- Wear shoes in the laboratory to protect feet from sharp instruments dropped accidentally. Closed shoes also protect feet from broken glass and chemicals.
- Do not open the autoclave until the internal air pressure reaches atmospheric pressure (reading 0 on the dial). Always use heavy duty material gloves when removing any material from the autoclave or oven.
- Take care when opening the oven. High temperatures and steam can cause serious burns.

Appendix 3

Media, sterilisation and preservation of cultures

The media section includes the recipes for a number of commonly used media. There are many more types of media which have been developed for specific fungi or experimental procedures. These are described in scientific literature, particularly journal articles.

It is important to understand the basic principles of heat sterilisation of media, glassware and other equipment. Treatment times need to be adjusted to correspond to the volume and nature of the material being sterilised. Treatment times also differ significantly between wet heat (autoclave) and dry heat (oven).

There are many types of preservation techniques to preserve living cultures of fungi. A few common methods are outlined in this section and many others have been documented in other literature.

A large number of media have been developed for culturing fungi. Many of these are general purpose media, such as water agar (WA) and potato dextrose agar (PDA), suitable for growing most fungi. Other media, such as *Phytophthora* selective medium (PSM) and peptone pentachloronitrobenzene agar (PPA) are selective and are used for the isolation of particular fungi from plants or soil.

Synthetic media, those made entirely from defined chemical compounds, are by nature uniform as their chemical composition is standard. Natural media, for example PDA or potato carrot agar (PCA), are inexpensive and encourage good growth of fungi. However, natural media (those made from natural material, usually plant extracts) are variable depending on the extract from the plant. If using natural media for distinguishing morphological characters or growth rate studies it is important that the same batch of media is used across all isolates. Some natural media such as PDA have high carbohydrate levels, giving rapid growth of fungi, with abundant aerial mycelium. Repeated subculturing on these types of media can lead to rapid degeneration of the culture and loss of virulence. Therefore, low nutrient media are preferred for maintenance of cultures.

Remember when making media to loosely screw on lids of bottles during autoclaving and tighten afterwards. This will prevent bottles from exploding under pressure and a lot of clean-up work.



We recommend that glass Petri plates be used in small diagnostic laboratories in tropical areas. Our experience indicates that there is less contamination from airborne fungal spores of media in glass plates than the contamination of plastic plates.

A3.1 Comments on some components of media

Water

Tap water is suitable for use for most media, as it contains trace elements which may be missing from distilled water. However in some areas tap water may contain substances which are toxic to fungi. One of the most significant is copper, which is inhibitory to many fungal species. In these cases distilled water is preferred.

Agar

Agar is an extract from algae, and its quality can vary depending on its source. It is available as a powder, or in a block or flake form. Many powdered agars dissolve readily during autoclaving; the recipes given below are for agar of this type.

Use a good grade of agar so that media such as water agar (WA) are transparent. It is essential that WA used for isolation studies, single sporing, hyphal tipping and identification be transparent. This allows hyphae and spores to be seen clearly under the dissecting microscope.



Use a lower quality agar only for media for which transparency is not as important, such as PDA and PCA. However it is best to avoid lower quality agar if possible.

Water agar is the most useful general purpose isolation medium. Do not use PDA for isolating fungi from plant material. Use PDA only to grow cultures to determine colony morphology and pigmentation. Use other media for encouraging reproduction and sporulation, such as sterile leaf or stem pieces in water agar, or sterile bean pods in water agar. Selective media are very useful for isolating fungi from roots or severely diseased tissue with contaminating saprophytic fungi and bacteria.

Antibiotics

Antibiotics may be added to fungal isolation media to prevent the growth of bacteria or unwanted fungi (Table A3.1). Most antibiotics (except chloramphenicol; see below) are unstable if heated and need to be added to the medium after autoclaving. These antibiotics are dissolved in a small quantity of sterile distilled water, according to the recipe. For most purposes this may be added directly to the medium but, for critical work the antibiotic solution should be filter-sterilised before use.

Table A3.1 Commonly used antibiotics

Antibiotic	Active against	Solubility
Penicillins	Gram-positive bacteria	Water soluble
Streptomycin	Gram-negative bacteria	Water soluble
Neomycin	Gram-positive bacteria	Water soluble
Chloramphenicol	Gram-positive and negative bacteria	Ethanol soluble

Chloramphenicol may be added to the medium before autoclaving. Chloramphenicol is a suspected carcinogen, and it and all other antibiotics must be handled with care.

Fungicides are frequently used in selective media. For example, *Fusarium* species are relatively tolerant to pentachloronitrobenzene (PCNB; Terrachlor® or Quitozone) and dichloronitroaniline (DCNA; Allisan®) and these fungicides are added to media selective for *Fusarium*.

Rose Bengal is added to some media used for isolating fungi from soil. It inhibits the growth of all fungi, and is added to prevent fast-growing species from overgrowing colonies of slow growing fungi. Rose Bengal becomes more toxic on exposure to light. Plates of Rose Bengal media should be stored and incubated in the dark.



When adding antibiotics to media it is important that they are completely dissolved in 10 mL of sterile water to ensure an even spread of antibiotics throughout the media. Once poured into the medium (at 55 °C) the antibiotics should be mixed into the medium by swirling carefully to avoid making excess air bubbles.

Often a range of different media are used in the laboratory at the same time. It is a good idea to devise a system for marking the edges of Petri dishes with dashes made with different coloured permanent markers so that different media can be easily differentiated. The system can be then posted on the wall of the laboratory to avoid any confusion.



A3.2 General purpose media for fungi

Water agar (WA)

WA (2%) consists of 20 g agar in 1 L of water and is recommended as the substrate for the germination of conidia used to initiate single spore cultures. Hyphal growth is sparse on this medium so it is suitable for cultures from which single hyphal tips are to be taken for the initiation of new colonies. Sparse growth on WA also facilitates the isolation of fungi from plant material, particularly roots.

For single sporing and hyphal tipping it is suggested that plates be poured when the medium is still quite hot so that thin plates can be produced—this restricts fungal growth and makes it easier to cut out the spores or hyphal tips.

WA (0.05%), 0.5 g agar in 1 L of water, is used in the preparation of soil dilution series. The small quantity of agar slightly retards sedimentation rates of fungal propagules. The agar is dissolved in water before being dispensed into McCartney bottles. Bottles are capped loosely during sterilisation and caps are tightened when sterilisation is complete.

Carnation leaf-piece agar (CLA) or other natural plant substrate agar

CLA is a natural substrate medium (Fisher et al. 1982) prepared by placing sterile carnation leaf pieces (approximately 1 piece per 2 mL agar) in a Petri plate and then adding sterile 2% WA.

The carnation leaf pieces are prepared from fresh carnation leaves free from fungicide or insecticide residue. Immediately after collection the leaves are cut into 5–8 mm pieces and dried in a forced-air oven at approximately 70 °C for 3–4 hours until brittle. Leaf pieces can also be dried in a microwave oven. The dried leaf pieces are packaged in aluminium or polycarbonate containers and sterilised by gamma irradiation (25 kilograys). Sterilised leaf pieces can be stored at 2–5 °C for up to 12 months before use.

Many species sporulate on CLA in 6–10 days. On this medium, conidial shapes are more uniform than when using carbohydrate rich media such as PDA. Macroconidia of *Fusarium* are formed mainly in sporodochia, which usually develop on the leaf pieces. Macroconidia formed in sporodochia are preferred in identification, as they are more consistent in shape and length than macroconidia formed from solitary monophialides on hyphae on the agar. Microconidia are more common on hyphae growing on the agar, often away from the leaf pieces. The mode of formation of microconidia, the presence of chains of microconidia, and the presence of chlamydospores can be determined by direct examination with a compound microscope when small plates of CLA (5 cm diameter) are used for routine identification of *Fusarium* cultures. CLA is also suitable for producing large numbers of conidia for experimental work.



A variety of plant parts such as green rice stem pieces and bean pod pieces can be substituted for carnation leaf pieces. If necessary sterilise these plant pieces by autoclaving. You should experiment to find the most suitable plant pieces for your laboratory.

Potato dextrose agar (PDA)

PDA is a carbohydrate rich medium which contains 20 g dextrose, 20 g agar and the broth from 250 g white potatoes made up to 1 L with tap water. The potatoes are unpeeled, but are washed and diced before boiling until just soft. The boiled potatoes are filtered through cheesecloth, leaving some sediment in the broth.

Conidia formed on PDA are usually variable in shape and size, and so are less reliable for use in identification. However, colony morphology, pigmentation and growth rates of many fungal species on PDA are reasonably consistent, as long as the medium is prepared carefully and the cultures are initiated from standard inocula and incubated under standard conditions. These colony characteristics are useful secondary criteria for identification. Although PDA is used for the isolation of some fungal pathogens, many saprophytic fungi and bacteria also grow rapidly on PDA and may inhibit the recovery of the pathogen. We do not recommend using PDA for isolation studies. Do not use PDA for isolation from roots.

It is recommended that one quarter strength PDA be used for isolation purposes, emended with antibiotics when isolating from stem or leaf tissue.

Spezieller Nährstoffarmer agar (SNA)

SNA is a weak nutrient agar which can be used for the identification and maintenance of *Fusarium* and *Cylindrocarpon* isolates (Nirenberg 1976). In addition to limiting cultural degeneration, this medium promotes uniform sporulation of microconidia in particular. SNA is prepared by autoclaving, in 1 L distilled water:

Agar	20 g
KH ₂ PO ₄	1 g
KNO ₃	1 g
MgSO ₄ ·7H ₂ O	0.5 g
KCl	0.5 g
Glucose	0.2 g
Sucrose	0.2 g

Two pieces of sterile filter paper (1 cm square), placed on the agar surface when set, assist in stimulating sporulation.

Because SNA is transparent, cultures can be viewed by direct examination under the microscope or small blocks can be mounted on a slide with a drop of water and cover slip for observation. A liquid broth made from this medium, but with no addition of agar, is often used for preparing mycelium for DNA extraction.

Potato carrot agar (PCA)

Carrot puree	20 g
Potato puree	20 g (made from peeled potatoes)
Agar	20 g

Peel the potato and dice the potato and carrot into small pieces. Place in a beaker containing approximately 200 mL of distilled water and gently boil for 30 minutes on low heat. The vegetables can then be forced through a fine sieve or blended to create a puree. Add agar and then distilled water to make up to 1 litre. Mix and autoclave. When pouring the media occasionally swirl to keep the carrot/potato mix in suspension.

The carrot puree is rich in sterols, which are essential for oogonial production in Oomycete species. PCA is an important medium for stimulating the production of oogonia in *Pythium* and *Phytophthora*.

A3.3 Selective media for specific fungi

Phytophthora selective medium (PSM)

This recipe includes penicillin and was originally recommended for use in Vietnam to the authors by Mr Nguyen Vinh Truong.

Agar	8 g
Carrot puree	20 mL (recipe below)
Potato puree	80 mL (recipe below)

Make up to 1 L with distilled water, autoclave and when cooled to 55 °C, add:

Hymexazol	3.7 mL of stock solution in water
Pimaricin	400 µL
Penicillin	200 mg

Wrap the plates in plastic wrap and store them in the fridge out of the light. Discard after a month. To make a medium selective for both *Phytophthora* and *Pythium*, do not add Hymexazol.

Carrot puree

Wash and dice 400 g carrots and autoclave for 10 minutes in 400 mL distilled water. Puree the mix, then add an additional 500 mL water. This can be measured out and frozen in plastic containers until needed.

Potato puree

Dice 200 g potato and boil in 500 mL tap water until tender. Puree and make up to a total of 800 mL with additional water. Store as above.

Hymexazol stock solution

Add 0.3 g pure hymexazol to 20 mL sterile water.

Pimaricin

Pimaricin can be added directly to the molten agar. Shake well before dispensing. Store wrapped in foil in the fridge.

Peptone PCNB agar (PPA / Nash–Snyder medium)

PPA is comprised of a basal medium to which antibiotics and fungicides are added, and it enables the selective isolation of *Fusarium* species from soil dilutions (Nash and Snyder 1962) or plant material. It is highly inhibitory to most other fungi and bacteria, but allows slow growth of *Fusarium*, which form small colonies of 5–10 mm diameter after 5–7 days.

Basal medium in 1 L water:

Agar	20 g
Peptone	15 g
KH ₂ PO ₄	1 g
MgSO ₄ ·7H ₂ O	0.5 g
Terrachlor®	1 g (contains PCNB 75% w/w)

Autoclave basal medium and cool to 55 °C before adding, in 10 mL sterile water:

Streptomycin sulfate	1 g
Neomycin sulfate	0.12 g

The prepared plates should be allowed to ‘dry’ in a cool dark place before use so that the water used for the soil suspension is rapidly absorbed. Most species of *Fusarium* do not form distinctive colonies on PPA; sporulation is poor and conidial morphology abnormal. Colonies must be subcultured and purified for identification. *Fusarium* cultures should not be maintained on PPA, because the metabolism of peptone leads to the accumulation of toxic ammonia.

Quarter-strength PDA with antibiotics

This medium is designed primarily for routine isolation of *Fusarium* species from plant tissue, such as stems infected with *F. oxysporum* wilt pathogens. It can also be used with a range of other pathogens, but test it before use in an important experiment. It is a useful medium for diagnostic studies.

Do not use this medium for isolating *Fusarium* or other fungi from soil.



In 1 L of water, mix:

Potato extract	Broth from 62.5 g of cooked potato
Agar	20 g
Dextrose	5 g
PCNB (Terrachlor®)	0.1 g

Autoclave basal medium and cool to 55 °C before adding, in 10 mL sterile water:

Streptomycin sulfate	0.16 g
Neomycin sulfate	0.06 g

Dichloran chloramphenicol peptone agar (DCPA)

DCPA was developed for the selective isolation of *Fusarium* species and dematiaceous hyphomycetes from cereal grains (Andrews and Pitt 1986). The basal medium, made up with 1 L distilled water, contains:

Agar	20 g
Peptone	15 g
K ₂ HPO ₄	1 g
MgSO ₄ ·7H ₂ O	0.5 g
Chloramphenicol	0.2 g (broad spectrum antibiotic—can be autoclaved)

After autoclaving add, in 10 mL ethanol:

Dichloran	0.002 g
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DCPA should not be used as a maintenance medium because the metabolism of peptone leads to accumulation of ammonia to toxic levels. Mucoraceous fungi are suppressed by dichloran, and the absence of a carbohydrate source is selective against *Aspergillus* and *Penicillium* species.

Rice leaf (or grass leaf) medium for *Pythium*

This medium is useful for stimulating and observing the formation of sporangia and oogonia by many *Pythium* species. The sporangia and oogonia form in mycelium growing on the surface of the water near the leaf pieces. This medium can be prepared by floating pieces of sterile rice or grass leaves in Petri plates of water:

1. Cut rice leaves into pieces about 3 cm long.
2. Autoclave and place 4–5 pieces into large Petri plates containing 15 mL sterile water.
3. Inoculate with a plug from an agar culture.

The fungus will colonise the grass pieces, and mycelium will grow over the surface of the water. To mount the fungus for microscopic observation:

1. Place a coverslip under the surface of the water.
2. Carefully tease off some of the mycelium and draw the material onto the coverslip.
3. Remove the coverslip from the culture, invert, and place onto a drop of water on a microscope slide.

A3.4 Media for use with bacteria

King's B medium (KBM)

Agar	15 g
Proteose peptone No. 3	20 g
Glycerol, C.P.	10 mL
K ₂ HPO ₄	1.5 g
MgSO ₄	1.5 g
Distilled water	1 litre

Combine all ingredients except MgSO₄. Adjust pH to 7.2 ± 0.2. Add MgSO₄ slowly and mix. Autoclave and pour into 90 mm Petri dishes.

Sucrose peptone agar (SPA)

Sucrose	20 g
Peptone	5 g
K ₂ HPO ₄	0.5 g
MgSO ₄ ·7H ₂ O	0.25 g
Agar	20 g
Distilled water	1 L

Combine all ingredients. Adjust pH to 7.2 ± 0.2 . Autoclave and pour into 90 mm Petri dishes.

Tetrazolium medium

This medium (Kelman 1954) can be used to differentiate between mutant and wild type colonies of *Ralstonia solanacearum*. Mutants commonly form round, butyrous, deep red colonies with a narrow bluish border. Wild type colonies are irregularly round, fluidal white colonies with a pink centre.

Peptone	10 g
Casein hydrolysate	1 g
Glucose	5 g
Agar	17 g
Triphenyl tetrazolium chloride	0.05 g
Distilled water	1 L

Combine all ingredients. Autoclave and pour into 90 mm Petri dishes.

A3.5 Sterilisation

Sterilisation is the process of killing all living organisms in a culture medium or on the surface of glassware used for sterile work, such as glass Petri dishes.

Heat sterilisation

The temperature and time required for killing are inversely related. Table A3.2 shows the minimum times required for effective sterilisation at the temperatures given for both moist and dry heat:

Table A3.2 Required times for sterilisation using moist and dry heat over a range of temperatures

Temperature	Moist heat	Dry heat
100 °C	20 hours	
110 °C	2.5 hours	
121 °C	15 minutes	8.0 hours
130 °C	2.5 minutes	
140 °C		2.5 hours

These times do not guarantee sterility. They are times calculated from experience and are based on normal levels of contamination with heat resistant organisms.

The species, strain and spore forming ability of a microbe greatly affects its susceptibility to heat. In moist heat the vegetative forms of most bacteria, yeasts and fungi and most animal viruses, are killed in 10 minutes by temperatures between 50 °C and 60 °C. However bacterial spores require 15 minutes at temperatures ranging from 100 °C to 121 °C. In dry heat bacterial spores require 1 hour at 160 °C.

The nature of the material in which the organisms are heated is also an important factor. A high content of organic substances generally tends to protect spores and vegetative organisms against the lethal action of heat. Proteins, gelatin, sugars, starch, nucleic acids, fats and oils all act in this way. The effect of fats and oils is greatest in moist heat as it prevents access of moisture to the microbes. The pH is also very important. The heat resistance of bacterial spores is greatest at neutral pH and decreases with increasing acidity or alkalinity.

Dry heat sterilisation

Dry heat kills microbes by oxidation. The dry heat process is the best method for the sterilisation of dry glassware such as test tubes, glass Petri dishes, flasks, pipettes, all glass syringes and instruments such as forceps, scalpels and scissors.



Glassware should be packed so as to allow proper penetration of the hot air throughout the load. This is aided by the fan. The holding period required for sterilisation is 160 °C for 1 hour. However most ovens, particularly if packed, will take 2 to 3 hours to reach temperature. Thus 4 hours at 160 °C would be the minimum for a big load. Four hours at 170 °C allows a safety margin.



Ovens must not be opened during their cycle, as one opening for a few seconds may drop the temperature by up to 70 °C, which takes the oven perhaps an hour to recover. This leads to the non-sterilisation of that load.

Moist heat sterilisation

Moist heat kills microorganisms, probably by coagulating and denaturing their enzymes and structural proteins, a process in which water participates. All culture media therefore are sterilised by moist heat.



Autoclaving at temperatures greater than 100 °C is the most reliable method and widely used for the sterilisation of culture media. Most autoclaves and pressure cookers operate at 121 °C, at which the minimum holding period for sterilisation is 15 minutes. It is essential that all air is expelled from the autoclave, otherwise it will not reach the correct temperature. Many large autoclaves do this automatically.

If using a pressure cooker or a manual autoclave, allow steam to hiss from the outlet for 2–3 minutes before closing the valve or placing on the cap. Baskets and not tins should be used for autoclaving and pipettes should not be autoclaved in canisters as localised air pockets will make for inefficient sterilisation. Temperature and NOT pressure is the true criterion for sterilisation procedures.

The autoclave should be adjusted so that the chamber pressure does not fall too rapidly as this results in media boiling over and wetting plugs. Media should be left in the autoclave for about 5 minutes after it has returned to atmospheric pressure, as sometimes solutions remain superheated and, when disturbed, spray boiling medium or agar over the operator, resulting in nasty burns. If left in the autoclave for longer periods, excessive loss of volume will occur as a vacuum builds up in the autoclave.

An effort should be made to avoid sterilising large and small volumes of media in one load as time must be allowed for large volumes to reach the required holding temperature, and this will result in small volumes receiving too much heat.

Table A3.3 provides a rough guide to the extra time that must be added to reach holding temperature:

Table A3.3 Suggested times for sterilisation of different volumes of liquid

Volume of liquid	Extra time (minutes)	Total time at 121 °C (minutes)
100 mL bottle	10	25
250 mL bottle	12	27
500 mL bottle	18	33
1000 mL bottle	22	37
2000 mL bottle	27	42

Sterilisation of instruments

Forceps, inoculating needles and other instruments must be sterilised before contact with a culture to avoid cross-contamination. Inoculating needles are best sterilised by heating to red heat in a flame.

The needle must be allowed to cool to room temperature again before being used. Hot needles are the most common cause of failure of subculturing, hyphal tipping and single sporing.



Forceps and scalpels are sterilised by dipping in alcohol. Before use, the alcohol is burnt off by passing the forceps through a flame to ignite it. Do not hold the instrument in the flame, since this will heat it up too much. Be very careful not to place hot or flaming instruments in or near alcohol, since this is a fire hazard.

Sterilisation of work surfaces

Trays, benches and other surfaces may be sterilised with a liquid disinfectant. Alcohol is the most commonly used. Alcohol works best as a sterilant if it contains some water, and a solution of 70% ethyl alcohol is suitable. Methylated spirits is also suitable.

A3.6 Preservation of cultures

Preservation of living cultures

Living cultures are stored for use as reference cultures, or for later use in pathogenicity tests or other experiments. Cultures are stored in national culture collections as part of reference materials that support a national database of plant pathogens.

Storage in sterile water—*Pythium* and *Phytophthora*

This is a low-cost, simple method that is particularly suitable for *Pythium* and *Phytophthora*. A sterile work chamber should be used for this procedure. Agar blocks 1 cm square are cut from the margin of a young, actively growing fungal colony. These are placed in sterile water in a McCartney bottle and the cap is screwed down. The bottles are stored under cool conditions. Do not store in a refrigerator as some species are killed at low temperatures. Cultures can be stored between 6 months to 2 years, depending on the species. Cultures are revived by removing a block of agar from the bottle and placing mycelium side down on fresh medium. It is essential to ensure that the water and agar blocks are not contaminated by bacteria—the presence of bacteria will lead to rapid death of the fungus.

Storage of sclerotia

Sclerotia can be stored for long periods under cool dry conditions in a small screw cap glass bottle or ampoule. This is a suitable technique for storage of species such as *Sclerotium rolfsii*, *Sclerotinia sclerotiorum*, *Rhizoctonia* spp. (sclerotial forming isolates).

In tropical regions it is best to store sclerotia on sterile paper tissue over blue silica gel in a McCartney bottle (or similar screw cap bottle) to ensure very low humidity for storage.

Storage as colonised pieces of plant stem or leaves

Cultures are grown on sterile WA containing pieces of sterile plant tissue or seeds. The colonised pieces are air dried and then stored in a small glass tube. Alternatively they can be stored on a sealed container on sterile paper above blue silica gel to ensure very dry storage conditions.

For more in-depth information regarding the preservation of cultures see Shivas and Beasley (2005), Management of Plant Pathogen Collections.

Lyophilisation by freeze drying

Lyophilisation, or freeze-drying, is the method of choice for long-term preservation of many fungi and is used routinely in most major culture collections. Its major drawback is the requirement and expense of specialised equipment. It is best suited to fungi which grow and sporulate well in culture on sterile plant tissue such as green rice stem-pieces or carnation leaf-pieces. There are also many fungi which cannot be freeze-dried successfully, such as Oomycetes, rusts and mildews.

Cultures are lyophilised by drying colonised stem or leaf pieces in small glass ampoules under high vacuum (10^{-1} to 10^{-2} Torr). The ampoules are prepared by inserting a small cotton wool plug and then autoclaving in a loosely covered beaker. Five stem or leaf pieces are taken from a culture (which is two weeks old and initiated from a single conidium), and aseptically transferred to the ampoule. The ampoule is replugged, labelled (with an internal label) then heated and drawn out to an hourglass shape using a gas torch. The ampoules are attached to the freeze dryer for 12–24 hours, then sealed under high vacuum and stored at room temperature or at 5 °C. Many species of *Fusarium* and other fungal genera have been successfully lyophilised using this technique and have retained viability for many years.

Cultures can be revived by aseptically plating the dried stem or leaf pieces onto a suitable medium. The ampoule is first surface sterilised before it is shattered to release the leaf pieces.

Other preservation techniques for living cultures

Cultures can also be stored as spore suspensions in glycerol in a –80 °C freezer for long-term storage. Many species have also been stored successfully in liquid nitrogen. However, these are very expensive techniques.

Preservation of fungal cultures for herbarium records

Holotype specimens grown on PDA must be lodged in an internationally recognised herbarium when a formal description of a new species is published.

Cultures are initiated from single germinated conidia and grown under standard conditions of temperature and light for 2 to 3 weeks. Cultures are then killed by exposing the plates to formalin in a closed container for 3 days. Preservation of the culture is achieved using agar and glycerine. Three grams of agar are dissolved in 147 mL water, which is then dispensed as 6 mL aliquots into test tubes before autoclaving. The lid of the culture dish is inverted, 1.5–1.75 mL glycerine is added and then the 6 mL aliquot of hot agar is poured over the glycerine. The culture is aseptically lifted from the Petri dish and floated on the mixture in the lid. Cultures

are then allowed to dry in a drawer for 3–5 days covered with a sheet of paper. When dried, the culture is rubbery and can be removed from the Petri dish for storage. This procedure was originally developed for use with *Fusarium* species at the Fusarium Research Centre, Pennsylvania State University. It is suitable for many fungi.

Preservation of fungi under mineral oil

Many fungi can be stored in culture under sterile mineral (paraffin) oil for 4–5 years at 15–20 °C. Cultures should be grown on PDA amended with 0.1% concentrated yeast extract (e.g. Vegemite®). The mineral oil should be prepared as follows:

1. Dispense 11 mL paraffin oil into 25 mL McCartney bottles without rubber seals.
2. Replace lids loosely and autoclave at 121 °C for 20 minutes.
3. Allow to cool completely in the autoclave.
4. Remove any water from the oil by heating at 120 °C in an oven for 8 hours and leaving in the oven overnight to slowly cool to room temperature. Discard any bottles that contain cloudy oil or repeat the oven-heating process.

Cultures should be grown on slopes of PDA with yeast extract in 25 mL McCartney bottles (without rubber seals) until all of the agar is covered with mycelium. To preserve the cultures, aseptically add 11 mL of sterile mineral oil to each culture in a sterile work chamber. Label carefully with the accession number and date.

Cultures can be re-grown as follows:

1. Aseptically remove a small piece of agar from the preserved culture.
2. Blot the piece on sterile filter paper or blotting paper to remove the oil.
3. Plate the piece of agar on appropriate medium.

Note: It is recommended that three cultures of each fungal isolate are preserved at any one time, and that mineral oil cultures are renewed every 4–5 years.

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Acronyms and abbreviations

ACIAR	Australian Centre for International Agricultural Research
ATSE	Academy of Technological Sciences and Engineering
CFU	colony-forming units
CLA	carnation leaf agar
DCNA	dichloronitroaniline
DCPA	dichloran chloramphenicol peptone agar
DNA	deoxyribonucleic acid
DON	deoxynivalenol
EDDHA	ethylmediamine-di-o-hydroxyphenylacetic acid
IDM	integrated disease management
KBM	King's B medium
PCA	potato carrot agar
PCNB	pentachloronitrobenzene
PDA	potato dextrose agar
PPA	peptone PCNB agar (Nash–Snyder medium)
PPSD	Plant Protection Sub-department
PSM	<i>Phytophthora</i> selective medium
RNA	ribonucleic acid
SNA	Spezieller Nährstoffarmer agar
SPA	sucrose peptone agar
UV	ultraviolet
WA	water agar

Glossary

Acervulus (pl. acervuli)

An asexual saucer-shaped fruiting body that produces conidia.

Antheridium (pl. antheridia)

The 'male' sexual organ found in some fungal species.

Antibiotic

A chemical compound, synthesised naturally or artificially, that inhibits or kills targeted organisms.

Ascospore

A sexually produced spore formed in an ascus, produced by ascomycetes.

Ascomycetes

A class of true fungi that produce ascospores sexually within asci.

Ascus (pl. asci)

A sac-like body in which ascospores are formed.

Asymptomatic

Possessing no symptoms.

Basidiomycetes

A class of fungi sexually producing basidiospores on a basidium.

Basidium (pl. basidia)

A clavate body on which basidiospores are produced.

Blight

A disease characterised by rapid death of plant tissue.

Chlamydospore

A thick-walled, asexually produced survival spore.

Colonisation

The process of colony establishment on a substrate.

Conidiophore

A specialised hypha on which conidia are produced.

Conidium (pl. conidia)

An asexually produced fungal spore.

Cultivar

A variety of a plant produced by selective breeding.

Damping-off

Rot at the soil level, causing young seedlings to collapse and die suddenly. Usually associated with excess moisture in the soil.

Deuteromycetes

A large and heterogeneous grouping consisting of true fungi of which no sexual reproductive stage is known.

Diagnostic

A characteristic that can be used to differentiate one organism from another.

Disease cycle

The cyclic sequence of events involved in the life of a pathogen, including infection, development, reproduction and survival stages.

Eukaryote

An organism in which the genetic material (DNA) is contained in a nucleus.

Fungicide

A chemical compound that is toxic to fungi.

Forma specialis (f. sp.) (pl. formae speciales)

A specific biotype of a pathogen that can only infect a specific genus or species of plants.

Gamete

A reproductive cell containing half the required genetic material for reproduction.

Heterothallic fungi

Fungi that require two individuals in order to sexually reproduce, each possessing either a 'male' or a 'female' gamete.

Homothallic fungus

An individual fungus capable of producing both 'male' and 'female' gametes for sexual reproduction.

Hypha (pl. hyphae)

A singular somatic filament produced by fungi.

Infection

The establishment of a parasitic organism within a host.

Infested

An individual plant or a specific area affected by a large number of parasitic organisms.

Inoculate

The process of artificially infecting a host organism with a pathogen.

Isolation

The process of obtaining a pathogen from a host for further study.

Koch's postulates

A set of conditions, formulated by Robert Koch, that are designed to test if a microorganism is the causative agent of a disease.

Macerate

To break up into small pieces with the aid of water.

Mosaic

Irregular angular patterning commonly found on leaves of plants infected by a viral pathogen.

Mottling

An irregular patterning of light and dark regions.

Mycelium

A mass of fungal hyphae.

Mycotoxins

Secondary metabolites produced by fungi on infected plant material that can cause illness in livestock and humans when ingested.

Necrotic

Dead and discoloured organic material produced in and around diseased regions of plants.

Nematode

An unsegmented round worm. Some nematodes are parasites of plants.

Non-specific symptoms

Symptoms that do not provide a diagnostic.

Oogonium (pl. oogonia)

The 'female' sexual organ found in some fungal-like species.

Oomycete

A classification of fungal-like organisms, some of which asexually produce motile spores for infection.

Oospore

A sexually produced spore from the phylum Oomycota.

Overseason

The ability of a pathogen to survive between infection stages on a host crop.

Pathogen

An organism possessing the ability to cause disease.

Pathogenicity

The ability to cause disease.

Perithecium (pl. perithecia)

A sexual fruiting body producing ascospores.

Phialides

A specialised cell on which conidia are produced.

Prokaryote

A micro-organism whose genetic material is not contained in a membrane-bound nucleus.

Propagule

A part of an organism that can be detached from a parent and give rise to a new organism.

Pycnidium (pl. pycnidia)

An asexual fruiting body producing conidia.

Rhizome

A horizontally growing underground stem capable of producing both shoots and roots.

Saprophyte

An organism that uses dead organic matter as a source of food.

Sclerotium (pl. sclerotia)

A compact mass of hyphae covered with a dark rind, capable of surviving for extended periods of time.

Septate

Hyphae possessing cross walls.

Sporodochia

An asexual spore-producing structure containing a mass of conidiophores on a hyphal mass.

Spore

The reproductive propagule of fungi. Spores may be either sexually or asexually produced.

Sporangium (pl. sporangia)

A sac-like structure containing asexually produced spores. In some cases, the sporangium itself can act as an infective propagule.

Vector

An organism that transmits a pathogen.

Virulence

The degree of pathogenicity of a disease-causing organism.

Zoospore

An asexually produced spore, possessing flagella. The flagella enable movement in free water.

Bookshelf

The bookshelf is the most important resource in a diagnostic laboratory. We suggest one of the most valuable books to have on the bookshelf is *Plant Pathology* (Agrios 2005). This book contains valuable information on each type of pathogen — fungi, bacteria, viruses, mollicutes and nematodes. A wide range of diseases are illustrated in this text and many excellent diagrams are provided.

The American Phytopathological Society publishes compendia of plant diseases of individual crops or groups of crops. These are valuable resources, and are highly recommended for any diagnostic plant pathology laboratory. Many international agencies such as ACIAR also produce manuals on a wide range of subjects, many produced at low or no cost.

A great deal of information is also available from official websites of government departments of agriculture and from universities. Make use of these sources of information and file them for future reference, either on the basis of the crop of interest or by taxonomic grouping.

The bookshelf should be continually updated with references on current diseases and methods in disease diagnosis.

The following is a list of literature recommended by the authors to be added to your bookshelf in the diagnostic laboratory.

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