

## 5 In the field

Farmers and district staff are usually the first to observe diseases on crops. Plant pathologists may then be asked to identify the disease and the pathogen.

If material is to be sent to a diagnostic laboratory, it is very important that all relevant information is obtained from the farmer or district staff and sent with them (Figure 5.1). District staff can provide information about the disease on a field notes diagnostic sheet (Section 5).

However, it is recommended that the plant pathologist visit the farmer to examine the diseased crop with the help of district staff. The plant pathologist can collect fresh samples for laboratory study and obtain information such as crop variety, management practices, previous crops and climatic data. This information is particularly important in the diagnosis of the disease. A list of field equipment used by plant pathologists in the field is provided in Section 5.1. Samples should be kept cool and transported to the diagnostic laboratory as quickly as possible for detailed examination.



**Figure 5.1** Talking with farmers in the field

## Disease Surveying: Field Notes

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Collector's name: ..... Collection date: .....

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### Farmer's details:

Name: ..... Phone: .....

Address: .....

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### Diseased sample:

Type of sample collected: .....

Crop species: ..... Variety/rootstock: .....

Source of seeds/plants: ..... Plant maturity: .....

Symptoms (compare with healthy specimens): .....

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Signs (evidence of the pathogen): .....

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### Field observations:

Percentage affected: ..... Distribution/patterning: .....

Paddock history: .....

Other plant species present: .....

Rainfall, irrigation events: ..... Soil type: .....

Association with terrain (slope of land): .....

Where was the problem first observed: .....

Chemical use (herbicides, insecticides, fungicides): .....

.....

Additional information (insect damage, other diseases): .....

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## Phiếu điều tra bệnh trên đồng ruộng

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Tên mẫu: ..... Ngày lấy mẫu: .....

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### Thông tin về chủ ruộng:

Tên: ..... Điện thoại: .....

Địa chỉ: .....

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### Thông tin về mẫu bệnh:

Phân loại mẫu: ví dụ mẫu thân, lá, rễ hay đất: .....

Tên cây trồng: ..... Tên giống: .....

Nguồn gốc hạt giống: ..... Tuổi cây: .....

Mô tả triệu chứng bệnh: .....

.....

.....

Những dấu hiệu điển hình chứng tỏ cây bị bệnh: .....

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### Thông tin quan sát được trên ruộng:

% số cây bị bệnh: ..... sự phân bố của bệnh: .....

Tên các cây trồng trên ruộng trước đó: .....

có luân canh với cây trồng khác không?: .....

lượng mưa và hệ thống tưới tiêu: ..... Loại đất: .....

đất bằng phẳng hay đồi dốc: .....

vị trí phát sinh bệnh đầu tiên: .....

những loại thuốc trừ cỏ, trừ sâu và trừ bệnh đã sử dụng: .....

.....

Sơ qua về tình hình các loại sâu bệnh khác đang tồn tại trên ruộng: .....

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## 5.1 Field equipment for diagnostic studies

It is important to prepare equipment carefully before a field survey. The following equipment is essential for sampling plant material and recording relevant information. Many of the items in this basic diagnostic kit can be obtained from local markets in Vietnam. Box 5.1 is a checklist of field equipment (Figure 5.2).

A notebook is essential for recording disease symptoms and taking notes on the crop and the history of the site. A hand lens or magnifying glass is used to examine fungal structures, such as sclerotia and pycnidia. A digital camera, if available, can be used to record images of symptoms of disease in the field.

Envelopes and several different sizes of paper and plastic bags should be included in the kit for storing plant samples. Critical information should be written on the bags using a permanent marker pen. This should include the name and address of the farmer, the location of crop and the sample number.

Different cutting implements are required for collecting samples:

- a large machete is used to cut large stems (e.g. banana) or woody stems (e.g. cotton), and to dig out entire plant specimens and collect small soil samples
- a small machete is used to cut small hard stems (e.g. chilli)
- two or three small sharp knives are used to cut soft stems (e.g. melon, tomato) to check for stem browning and bacterial ooze, or to collect samples for laboratory use
- secateurs are used to collect large numbers of samples.

### Box 5.1 Field equipment checklist

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|--|--|
| ■ Notebook and pen                       | ■ Knives and secateurs                                     |
| ■ Digital camera                         | ■ Small glass bottle (vial)                                |
| ■ Hand lens                              | ■ Bottle of clean water                                    |
| ■ Paper bags, plastic bags and envelopes | ■ Squeeze bottle of 70% ethanol                            |
| ■ Permanent marker pen                   | ■ Ice box and bottles of ice (keep a store in the freezer) |
| ■ Small and large machetes               | ■ Drinking water and a hat                                 |

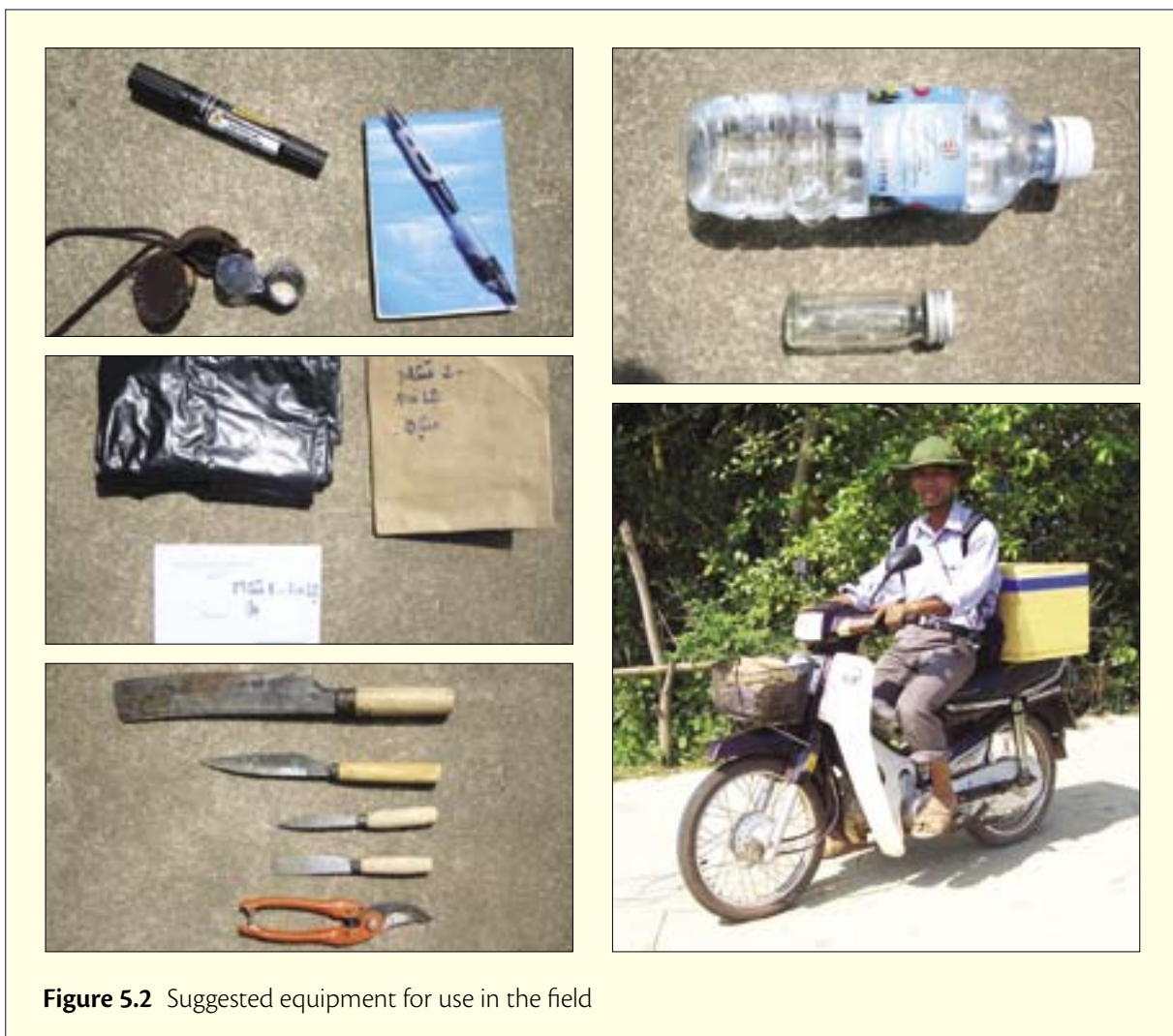
This equipment can be transported easily on a motorbike. Digging implements can usually be borrowed from a farmer—they are awkward and dangerous to carry on a motorbike.

A small glass bottle (or vial) is essential for checking for bacterial ooze from the cut stem of a plant suspected of bacterial wilt. Clean water must be used to wash cutting implements and for use in the bacterial ooze test. Cutting implements should be surface sterilised with 70% ethanol before moving to another part of the field or sampling uninfected material.

A polystyrene ice box is essential to keep samples cool during transport between the field and the laboratory. Bottles of ice are ideal for keeping the ice box cool and should be stored in the freezer for this purpose.

It is also very important to take drinking water and a hat on field trips, to protect against the sun and to avoid dehydration.

Do not carry mud between crops on shoes or other clothing or equipment. Mud may contain pathogens and transfer them from diseased crops to healthy crops.



**Figure 5.2** Suggested equipment for use in the field

## 5.2 Conducting a field survey

### Step 1

Prepare the field equipment (Section 5.1) and arrange transport.

### Step 2

Discuss the disease(s) with farmers and district staff.

### Step 3

Walk slowly through the crop and note the types of symptoms present on the plants, the distribution patterns of the disease and any soil or other factors associated with the disease (see disease symptoms in Section 4).



Check carefully for signs of insect damage, the presence of virus vectors, weeds and pesticide damage as you walk through the crop. Talk to farmers about their observations.

### Step 4

Carefully remove diseased plants from the soil and examine all parts of the plant for symptoms. Compare the diseased plant with an apparently healthy plant.

Wilting, stunting and leaf yellowing usually indicate a disease of the roots or stem (see Box 5.2). Check the roots carefully for root rot or root lesions, root galls and the proliferation of rootlets from one section of the roots.

Check for fungal structures at the stem base, such as sclerotia of *Sclerotium rolfsii* or *Sclerotinia sclerotiorum*.

Leaf mosaic or mottle, leaf yellowing, leaf curling, leaf roll, stunting or dwarfing of the plant can indicate the presence of a disease caused by a plant virus. Some viruses also cause wilt-like symptoms.

Check leaf spots for the presence of fungal structures, such as pycnidia or acervuli, with a hand lens. Powdery mildew, downy mildew and rust also reproduce on the leaf surface and form distinct spore masses. Downy mildew growth is usually on the underside of the leaf. In contrast, powdery mildews are usually more obvious on the upper side of the leaf.

Leaf spots with a watery, greasy or oily appearance often indicate the presence of a bacterial pathogen (see Box 5.3).

Cut the stem to check for stem browning, which can indicate a fungal or bacterial wilt. Check the cut stem for bacterial ooze by placing it in water (see Box 5.3).

More than one disease may affect different parts of the plant at the same time.



## Step 5

Record disease symptoms with diagrams. Look carefully for the presence of fungal structures using the hand lens and take photographs.

### Box 5.2 Detecting nematodes

Nematodes can cause non-specific symptoms (stunting, yellowing and wilting) in a range of crop species. The symptoms occur because nematodes reduce the ability of plants to take up water and nutrients through the roots.

Nematode damage can be identified by examining the plant roots closely (see nematode section).

Nematode levels in the soil can be determined using techniques that rely on nematode movement for separation of nematodes from the soil (e.g. Whitehead trays or Baermann funnels), or passive techniques (e.g. sieving).

Parasitic nematodes can be identified by the presence of a piercing stylet. This structure can be observed under a compound microscope or a good dissecting microscope.

There are a number of different genera of nematodes which can cause diseases of crop plants. Common pathogens include *Meloidogyne* spp. and *Pratylenchus* spp.





## Step 6

Collect samples for examination and the isolation of potential pathogens in the laboratory. Most plant and soil samples, including samples with roots and soil, should be collected and stored in paper bags. Samples stored in plastic bags tend to 'sweat', encouraging saprophytic bacterial growth. Such growth can interfere with the isolation of the pathogen.

Small leaf samples are best carried in a small plastic box with some paper tissue to 'cushion' the specimen.

Label the samples carefully and store them in an ice box for transport.

## Step 7

Analyse the information collected from farmers, the notes on disease symptoms and the patterns of disease to determine the most likely causes of the disease. Use this analysis to guide laboratory work. Examine samples in the laboratory within a few hours of collection, if possible.

## Step 8

Do not enter the laboratory wearing field clothes or shoes. Shower and change into clean clothes before entering the laboratory.

### Box 5.3 Detecting bacterial pathogens

Bacterial pathogens can only infect (enter) plants via natural openings, such as those on leaf margins and root tips, or via wound sites, which may be created by insect damage or lateral root emergence.

Once inside the plant, some bacterial pathogens can quickly spread through the vascular system.

The bacterial ooze test provides a quick diagnostic test to determine if a plant has been infected by a bacterial wilt pathogen. This method quickly differentiates between wilt diseases caused by bacteria and those caused by fungi. A short stem section of the diseased plant is cut and placed in a small glass vial with clean water. If ooze emerges from the cut surface and the water solution becomes cloudy after a few minutes, the plant is probably infected by a bacterial pathogen. To be more confident about the outcome of the test, a control for this method can be set up using an asymptomatic stem (a stem from a healthy plant).





## 6 In the laboratory

In this section, guidelines are provided to assist inexperienced staff to develop skills essential for a diagnostic plant pathologist, including:

- microscope use
- isolation
- subculturing
- purification
- identification
- pathogenicity testing.

With experience, diagnostic staff can modify the procedures to obtain the most efficient and effective results. Techniques for preserving living fungal cultures, recipes for growth media and the principles and methods used in sterilisation are provided in Appendix 3.

### 6.1 Laboratory examination of the samples

The following procedures are for examining plants with wilting, stunting and foliar disease symptoms in the laboratory. The first step is to examine the whole plant carefully and to compare these observations with observations made in the field.

### 6.1.1 Wilting and stunting

If the plant is wilted and stunted, the cause is most likely a root or stem rot or vascular wilt disease.

#### 1. Check for vascular browning.

- (a) If present, suspect bacterial or fungal wilt. Do bacterial ooze test.
  - If ooze is present, set up streak plates.
  - If no ooze is present, suspect *Fusarium* or *Verticillium* wilt. Plate out stem to recover fungus.
- (b) If absent, suspect a fungal or fungal-like stem or root rot or nematodes. (Note: also check for virus symptoms as some viral pathogens cause wilting and stunting.)

#### 2. Check for sclerotia and fungal mycelium.

- (a) If present, attempt to isolate *Sclerotinia sclerotiorum*, *S. rolfsii* or *Rhizoctonia* spp. as indicated by the type of sclerotia observed.
- (b) If absent, suspect root rot, nematodes or club root of crucifers.

#### 3. Examine for evidence of nematodes (root knots or root lesions).

- (a) If present, extract nematodes to confirm the pathogen and forward to a nematology laboratory for species identification.
- (b) If absent, and there is root rot (browning), attempt to isolate a fungal or fungal-like pathogen from the roots.

### 6.1.2 Leaf diseases

If the leaves show signs of disease, examine them under a dissecting microscope.

#### 1. Check for mosaic, mottling, leaf rolling or dwarfing of leaves.

- (a) If present, suspect a viral disease. Forward the material to a plant virus diagnostic laboratory. (Note: yellowing and spots may also be caused by viruses, for example papaya ring spot virus and tomato spotted wilt virus.)
- (b) If absent, suspect a bacterial or fungal pathogen.

#### 2. Check for oily leaf spots or blights, or bacterial ooze.

- (a) If present, suspect a bacterial pathogen. Isolate by streaking a bacterial suspension made from plant sap on King's B medium.
- (b) If absent, suspect a fungal pathogen.

### 3. Examine under a dissecting microscope for the presence of fungal structures.

With practice, downy mildews, white blister (*Albugo candida*), powdery mildews and rusts can be identified using dissecting and compound microscopes. As these are obligate plant parasites, they cannot be grown on culture media.

If signs of other fungal pathogens are present (hyphae, spores or reproductive bodies), microscope slides should be made for viewing under a compound microscope. Common leaf spot genera that may be identified include *Alternaria*, *Cercospora*, *Stemphylium*, *Septoria* and *Phomopsis*. Sporulation may also be induced by incubating diseased leaves in a moist chamber in the light. Leaves should be examined every day for signs of a fungal pathogen. (Note: saprophytic fungi and bacteria will grow quickly in a moist chamber, which may lead to an incorrect diagnosis.)

It is also recommended that tissue be plated from the margin of leaf spots from freshly collected material onto a low nutrient medium for isolation of the pathogen (see Section 6.3).

## 6.2 Microscopy

Dissecting and compound microscopes are essential items in a diagnostic laboratory and diagnostic plant pathologists should be familiar with their set up, maintenance and use.

### 6.2.1 Using a dissecting microscope

A dissecting microscope is used to examine diseased material for the presence of small fungal structures, such as pycnidia, acervuli, sporodochia and perithecia, under low magnification (up to approximately  $\times 100$ ). Using the dissecting microscope, such structures can be easily transferred to a slide preparation for examination under a compound microscope, at higher magnification (up to  $\times 400$ ).

A dissecting microscope is also used for fine work such as the transfer of germinated single spores or hyphal tips during purification of fungal cultures, and the examination of nematodes, which can also be transferred to a slide for examination under a compound microscope. In this way, it can be determined whether nematodes are plant parasitic or non-parasitic. Developing colonies are best examined under a dissecting microscope (Figure 6.1).



**Figure 6.1** Examination of colonies under a dissecting microscope

Each dissecting microscope should be fitted with an adjustable mirror that tilts to provide direct and oblique lighting for low-contrast specimens. A built-in light source for illuminating the upper surface of material is ideal, but a separate light source can also be used.

### **6.2.2 Using a compound microscope**

The instructions provided with a compound microscope should be followed carefully to ensure that it is used correctly and to avoid damage.

It is very important that objective lenses are not scratched or touched by agar, fungus or stained preparations. Coverslips (cover glass) are very thin and, if broken, can cut fingers.

#### **Adjusting the compound microscope**

1. Place a slide containing a specimen on the stage of the microscope.
2. Turn the lamp on and adjust the transformer to approximately 50% brightness.
3. Bring the specimen into focus with the  $\times 10$  objective.
4. Close the field iris diaphragm so that it becomes small.
5. Adjust the condenser height to bring the field iris diaphragm into focus.
6. Turn the two condenser centring knobs until the image of the field iris diaphragm is centred—this is very important!

7. Open the field iris diaphragm until it just disappears from view (i.e. until it is slightly larger than the field of view).
8. Adjust the aperture iris diaphragm to clearly show the specimen. The setting number (numerical aperture) on the aperture iris diaphragm should be approximately 75% of the numerical aperture on the objective being used.
9. Examine the specimen (Figures 6.2 and 6.3).

### 6.2.3 Preparing slides

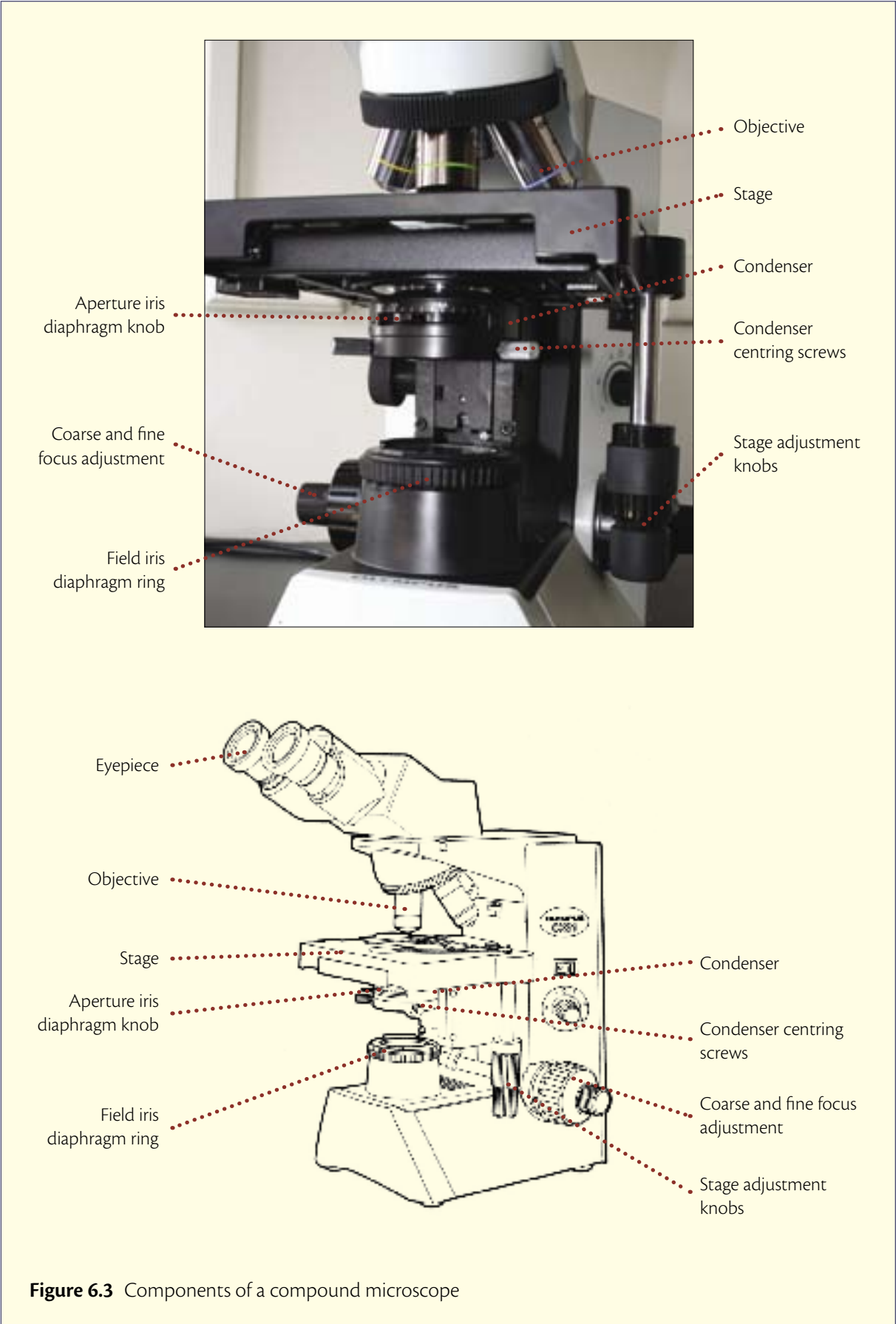
Specimens such as fungal spores or spore forming structures such as pycnidia, perithecia or cleistothecia can be mounted on slides in water.

#### Mounting specimens in water

1. Place a small drop of filtered water on a slide.
2. Place material into the water drop, under a dissecting microscope.
4. Place a coverslip with one side touching the slide near one edge of the water drop.
5. Gently lower the other side of the coverslip onto the water drop—this method excludes air bubbles from the preparation.
6. Use a strip of blotting or filter paper to blot excess water at the edge of the coverslip.



**Figure 6.2** Examination of fungal spores under a compound microscope



**Figure 6.3** Components of a compound microscope

Spores from a diseased plant or culture can be scraped off with a transfer needle and transferred to the water drop.

Larger spore-forming structures should be examined under the dissecting microscope and then placed in the water drop and squashed (flattened) by pressing gently on the coverslip using a flat surface. On re-examination, pycnidia can be identified as containing only spores (conidia), while perithecia and cleistothecia will contain both asci and ascospores.

Structures with soft tissue, such as apothecia of *Sclerotinia sclerotiorum*, should be cut into thin slices (sections) with a wet razor blade or scalpel and transferred to the water drop.

### 6.3 Isolating fungal pathogens

The following isolation techniques for root, stem and leaf rot fungi do not need to be followed exactly, but can be used as a guide and modified to give the best recovery according to different pathogens and crop situations. It is important to learn and gain experience by experimenting with different methods.

It is recommended that staff participate in laboratory training workshops if they have no previous training in laboratory techniques. It is important to learn how to recognise pathogenic species and distinguish them from common saprophytes.

Practice isolation and subculturing procedures with a range of fungal pathogens.



Isolation techniques may be improved by:

- changing the duration of surface sterilisation of plant material
- removing outer layers of plant material
- making modifications to media (e.g. pH, nutrient and agar concentrations)
- adding antibiotics to the media.

Ethyl alcohol (70%) is a standard surface sterilant for laboratory equipment and diseased material. Sodium hypochlorite may also be used as a surface sterilant, but it becomes ineffective with age and exposure to light.

Always damp dry (blot) the plant tissue on sterile paper before plating.





Select newly diseased tissue for isolation. Do not use older diseased tissue as it will be colonised extensively by saprophytic fungi and bacteria.

The surface of plant tissue harbours many saprophytic fungi and bacteria, which must be killed before the disease-causing pathogen can be isolated. Many of these saprophytes will also grow quickly on isolation medium, so that the pathogen cannot be isolated.

Do not use potato dextrose agar (PDA) or other carbohydrate-rich media for isolation from diseased plant tissues, especially if isolating from roots. Saprophytic fungi and bacteria grow quickly on carbohydrate-rich media and suppress the growth of slower growing fungal pathogens.

The successful isolation of fungi from diseased plants depends on several factors:

- type of diseased tissue (leaves, stems, roots)
- method of surface sterilisation
- plating procedure
- isolation medium
- incubation conditions of isolation plates.



Past experience is an invaluable tool in selecting appropriate isolation procedures. Experience will often provide an indication of which types of fungal pathogens are likely to cause particular symptoms. When in doubt, draw on information from picture databases, accession books and published material.

### 6.3.1 Isolation from leaves and stems

Isolation from stems is often improved by removing the bark or outer stem tissues before surface sterilisation.

#### Basic isolation from leaves or stems

1. Wipe the work area with 70% ethyl alcohol.
2. Dip instruments (forceps and knife or scalpel) in 70% ethyl alcohol and flame dry. (Methylated spirits can be substituted for ethyl alcohol.)
3. Rinse leaf or stem tissue in water to remove soil and other debris.

4. Surface sterilise leaf or stem tissue by wiping the leaf surface with soft paper (paper tissue) dipped in 70% ethyl alcohol or by briefly dipping thick leaves in 70% ethyl alcohol for 5 seconds, rinsing in sterile water and damp-drying on sterile paper tissue.
5. Aseptically cut small pieces (approximately  $2 \times 2$  mm) from the margin of the healthy and diseased tissue, and transfer them to a low-nutrient medium (e.g. water agar [WA]) or a selective isolation medium, placing the pieces near the side of the plate.
6. Incubate the plates at approximately 25°C, ideally under lights.
7. Check plates each day, and when fungal colonies develop from the pieces of plant tissue, transfer material from the margins to a medium such as PDA or WA that contains sterile pieces of plant tissue, for example, pieces of green rice stem, carnation leaf or bean pod. (Sterile pieces of plant tissue encourage sporulation, which aids in identification of the pathogen.)
8. Make a final identification using pure cultures grown from a single germinated spore or a hyphal tip. (Techniques for doing this are described in Sections 6.5.1 and 6.5.2.)

The medium used for isolation depends on the fungus suspected to be the cause of the disease. Water agar or one-quarter strength PDA, containing antibiotics if necessary, are the most useful general purpose isolation media. Selective isolation media may be used, such as peptone pentachloronitrobenzene agar (PPA) for *Fusarium* spp. and *Phytophthora* selective medium (PSM) for *Phytophthora* spp.

Saprophytic species of fungi, such as *Alternaria*, *Pestalotia* and *Cladosporium*, commonly colonise dying leaf tissue. The presence of these fungi can make it difficult to isolate pathogenic species of *Alternaria* or other foliar fungal pathogens, such as *Stemphylium* and *Bipolaris*.

#### **Alternative method for isolating from leaf spots**

1. Place the leaf or leaf piece on moist paper in a Petri dish in a humid chamber.
2. Incubate at approximately 25 °C under lights to promote sporulation.
3. Examine after 1–2 days under the dissecting microscope to locate spores or spore-forming structures such as pycnidia, acervuli or sporodochia.
4. Pour isolation plates containing WA with a drop of lactic acid (which reduces the pH and suppresses bacterial growth) or with added antibiotics (as used in PPA).
5. Using a sterile transfer needle, transfer the spores to the plates.

### 6.3.2 Isolation from small, thin roots

Small, thin feeder rootlets and lateral roots absorb nutrients for plant growth and are important to plant health. Pathogens such as *Rhizoctonia*, *Pythium*, *Phytophthora* and *Phoma* commonly cause diseases of these rootlets.

Many saprophytic fungi (e.g. some *Fusarium* spp. and *Trichoderma* spp.) and bacteria colonise the outer cells of the root cortex. Therefore, the isolation of pathogens from rootlets can be difficult.

Do not use severe surface sterilisation of small rootlets as the sterilant may kill all the fungi in the rootlet, including the pathogen.

#### Isolation from small, thin roots

1. Select diseased rootlets with both healthy (symptomless) and diseased parts, and wash them in three changes of sterile water in a small bottle. Add a small drop of detergent to the first wash.
2. Wipe the work area with 70% ethyl alcohol.
3. Dip instruments (forceps and knife or scalpel) in 70% ethyl alcohol and flame dry. (Methylated spirits can be substituted for ethyl alcohol.)
4. Dip the rootlets briefly in 70% ethyl alcohol, rinse quickly in sterile water and then damp-dry on sterile paper tissue. Alternatively, surface sterilise the rootlets in 1% sodium hypochlorite in 10% ethyl alcohol for 10–15 seconds only, immediately rinse in sterile water and allow to air-dry on sterile paper tissue in a sterile work chamber.
5. Aseptically cut root pieces 1–2 mm in size at the margin of healthy and diseased tissue and transfer onto WA or a selective medium.
6. Press the pieces gently into the surface of the agar to ensure good contact between the entire root segment and the antibiotics in the agar.
7. Incubate at approximately 25 °C and check each day under the dissecting microscope for fungal growth from the root pieces.
8. Subculture each colony onto PDA or WA containing sterile pieces of plant tissue, such as green rice stem pieces.
9. Purify by hyphal tipping (see Section 6.5.1) or by the single germinated spore technique (see Section 6.5.2) before final identification.

Note that it is usual to isolate some saprophytic fungi along with pathogenic fungi from diseased root tissue. With experience, some pathogenic fungi can be recognised on PDA by simple examination. The pathogenicity of isolates must be proven before a decision can be made on the cause of the disease.

### 6.3.3 Isolation from woody roots and stems

Often fungal root pathogens must be isolated from the main root or stem base of woody plant material. Isolation is generally more successful if stem tissue is plated. Commonly, there are fewer saprophytes in the stem base than in the woody root.

The choice of preparation depends on the amount of lignification (woodiness) of the tissue. Surface sterilisation of softer stems can be as simple as wiping or spraying the stem with 70% ethanol before plating onto media.

#### Isolation from woody roots and stems

1. Cut off and discard the lateral roots (Figure 6.4).
2. Wash the sample in water with a little detergent to remove soil and other debris.
3. Cut away the outside of the stem or root, an area which is often the source of saprophytes.
4. Remove the lower section of the stem at the soil line. Selection of tissue for isolation will depend on disease severity. Do not attempt to isolate from old diseased tissue. Ideally, plate segments from the margin of healthy and diseased tissue.
5. Spray the sample with 70% alcohol.
6. Flame off excess alcohol, or if stem is soft, allow the alcohol to evaporate.
7. Cut thin segments of stem tissue and plate onto selective or low-nutrient media.

### 6.3.4 Soil baiting

Soil baiting is an indirect method of isolating *Phytophthora* and *Pythium* species from soil or roots.

#### Isolation from soil using apples or other fruits as bait

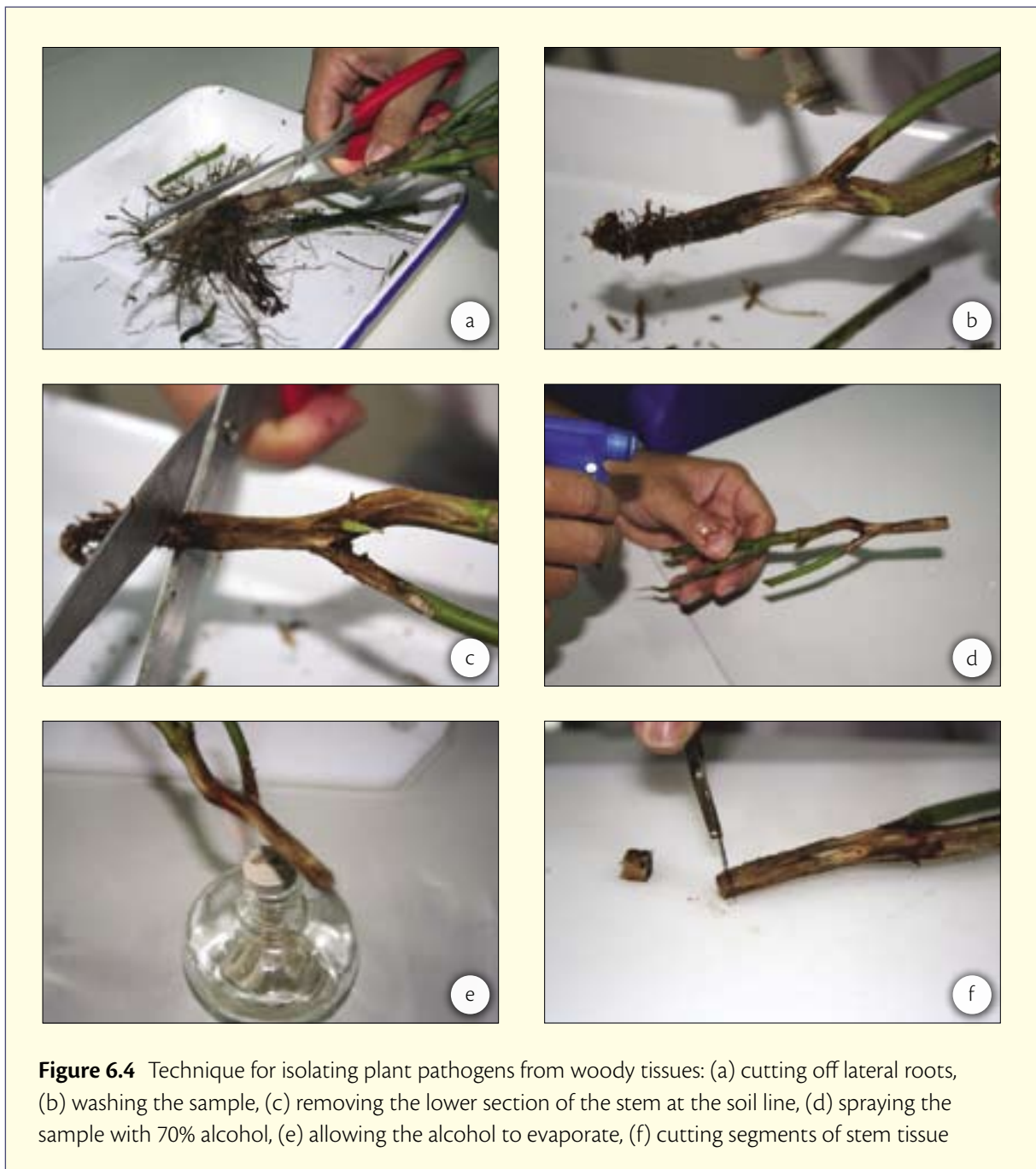
1. Swab the apple with alcohol.
2. Cut a hole approximately 10 mm in diameter through to the core on one side using a sterile cork borer.
3. Pack the hole with soil and cover it with sticky tape to retain the soil.
4. Incubate the apple at room temperature in the light.
5. Isolate fungi after 1–3 days from the margins of the fast-spreading, brown lesions.

This method is not completely selective as fast-growing zygomycetes may also cause similar lesions.

### Isolation from flooded soil using leaves or petals as bait

*Phytophthora* and *Pythium* species can be isolated from soil by floating clean leaves or rose petals over flooded soil. If these species are present in the soil sample, zoospores are produced that move up to and infect the leaf or petal. This method is selective as it favours the isolation of species that produce zoospores.

1. Place up to 100 g of soil in a plastic cup.
2. Cover soil with sterile or filtered water to a depth of 5–10 cm.



3. Float pieces of susceptible plant material on top of the water.
4. Incubate the cup for 2–4 days.
5. Isolate fungi after 2–3 days from the margins of lesions that have developed on the bait, using a selective medium (e.g. PSM), after rinsing in sterile water and sterilising the surface.

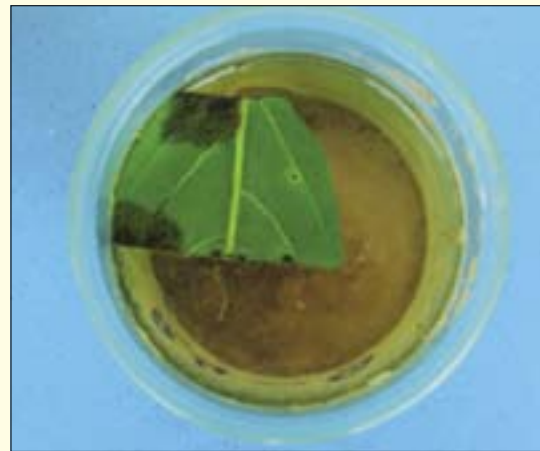
The bait material can also be the crop species thought to be affected by *Phytophthora* or *Pythium*. Potential baits include chilli leaves, rose petals, citrus leaves, and seedlings of chilli, lupins and soybean. If the bait will not float by itself, it can be suspended from a lid over the container, or from a piece of polystyrene foam or other suitable float.

#### Isolation from rootlets using host plant as bait

1. Wash diseased rootlets (Figure 6.5).
2. Place rootlets in a plastic cup and fill the cup with sterile or filtered water.
3. Float a leaf from the host plant on the surface of the water.
4. Incubate the cup for 2–4 days.
5. Isolate fungi after 2–3 days from the margins of lesions that have developed on the bait, using a selective medium (e.g. PSM) after rinsing in sterile water and sterilising the surface.

#### 6.3.5 Soil dilution plate method

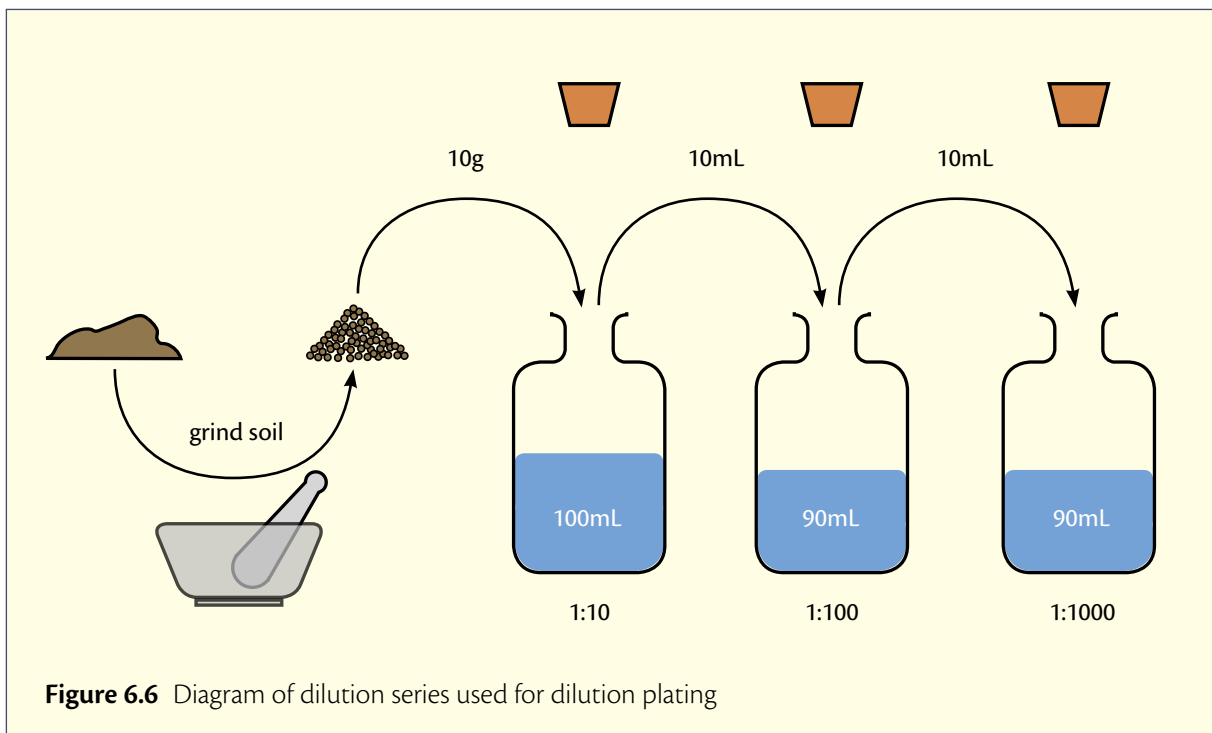
The soil dilution plate method is used for the isolation of *Fusarium* species from dry soil using PPA. It can be adapted for the isolation of other fungal species using appropriate selective media.



**Figure 6.5** Baiting soil for *Phytophthora* using flower petals and leaves

### Isolation by the soil dilution plate method from dry soil

1. Air-dry the soil sample, grind it lightly with a mortar and pestle (a marble kitchen mortar and pestle is appropriate) and mix thoroughly.
2. Transfer a 10 g subsample to 100 mL of sterile 0.01% WA in a bottle, to give a dilution of 1:10.
3. Transfer 10 mL to a second bottle containing 90 mL of 0.01% WA to give a dilution of 1:100 and mix well to ensure an even spread of the soil in the solution. Repeat this step to give a 1:1000 dilution, which is usually satisfactory for the isolation of *Fusarium* from vegetable or field crop soils (Figure 6.6).
4. Disperse (spread) 1 mL of soil suspension across the medium in a 90 mm diameter Petri dish:
  - prepare the plates and let them dry for a few days to eliminate water from the surface of the plate
  - carefully pipette 1 mL of soil suspension onto the edge of the medium to one side of the plate
  - hold the plate on a slight slope away from the suspension and gently shake at right angles to the slope, spreading the suspension in a uniform wetting front across the plate.
5. Incubate the isolation plates under lights for 5–7 days until colonies develop.
6. Subculture the colonies and purify them using the single spore technique on PDA, carnation leaf agar (CLA), or WA containing a sterile plant tissue fragment.





Although a 1:1000 dilution is usually satisfactory, a dilution should be used that gives 10–30 *Fusarium* colonies per plate (Figure 6.7). As the level of *Fusarium* in soil depends on cropping history and soil type, a different dilution may be required to achieve the desired result.



**Figure 6.7** Dilution plate containing *Fusarium* spp. on peptone PCNB agar (ideally the number of colonies should be between 10 and 30)

If the isolation procedure is designed to provide quantitative data, use 3–5 replicate plates per dilution and replicate (use several) soil subsamples. There may be considerable variation between replicate plates and between soil samples.

This technique does not determine the number of spores in the soil, but rather the number of ‘propagules’ of a species in the soil. Propagules might include conidia, chlamyospores and hyphal fragments in infested plant residues. The number of these colony-forming units (CFU) per gram of soil can then be calculated for each species using the following formula:

$$\text{Dilution} \times \text{mean no. colonies of fungal species on isolation plates} = \text{CFU/g soil}$$

## 6.4 Subculturing from isolation plates

Subculturing is the stage between isolation from plant material and the creation of pure cultures. This stage helps to determine which organism has been isolated.

### The subculturing process

1. Examine the plates under the dissecting microscope each day and assess the growth of fungal hyphae from the segments of plant tissue.
2. Determine if there is more than one fungal species growing.
3. Subculture when there is approximately 5 mm of hyphal growth from the plant tissue.
4. Cut out a small block of agar ( $2 \times 2$  mm) from the margin of each colony and transfer it to PDA or a natural substrate medium (e.g. CLA or green rice stem agar).

There are some fungal diseases where the pathogen is readily isolated and where saprophytes rarely cause problems. For example *Sclerotinia sclerotiorum* and *Sclerotium rolfsii* are readily isolated from the margins of healthy and diseased tissue in green stems.

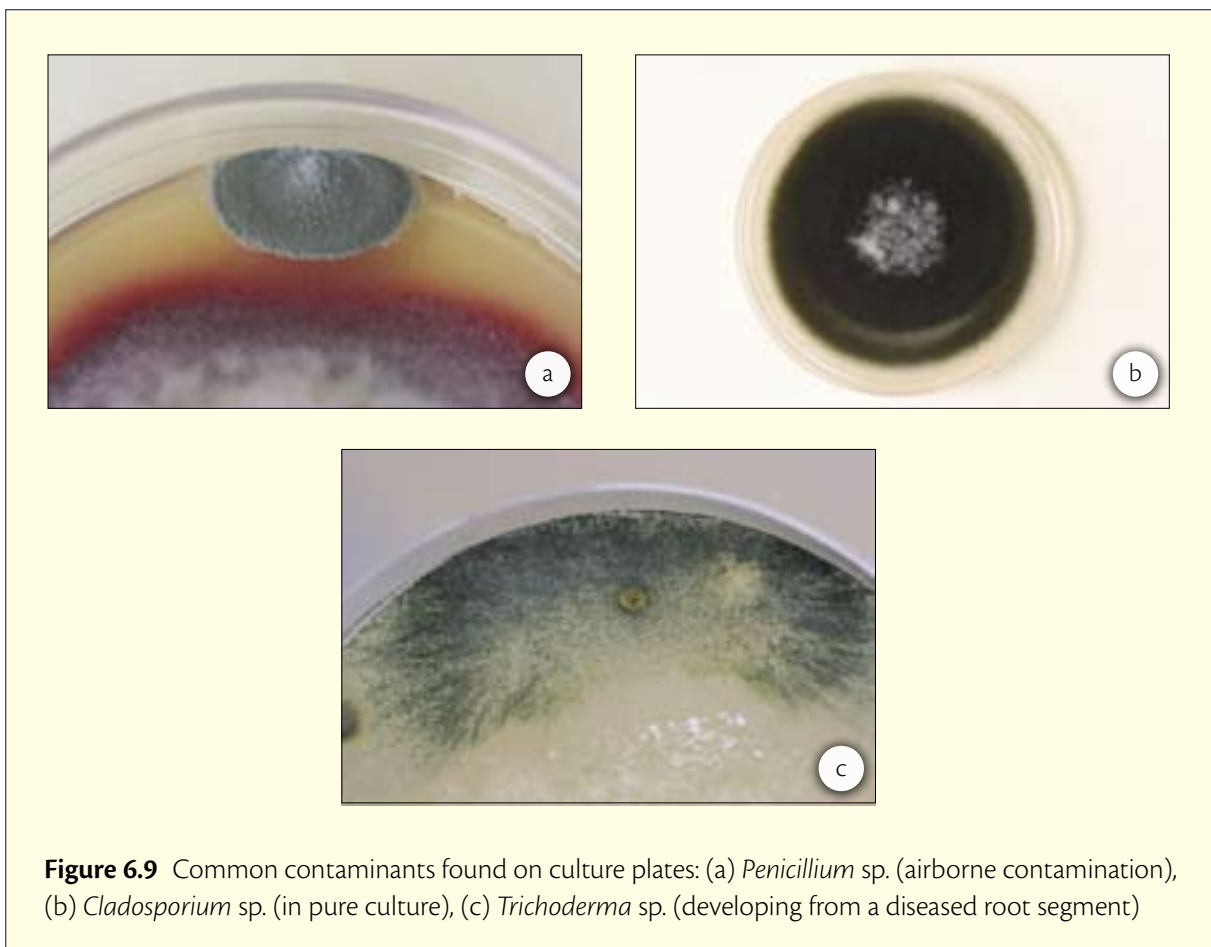
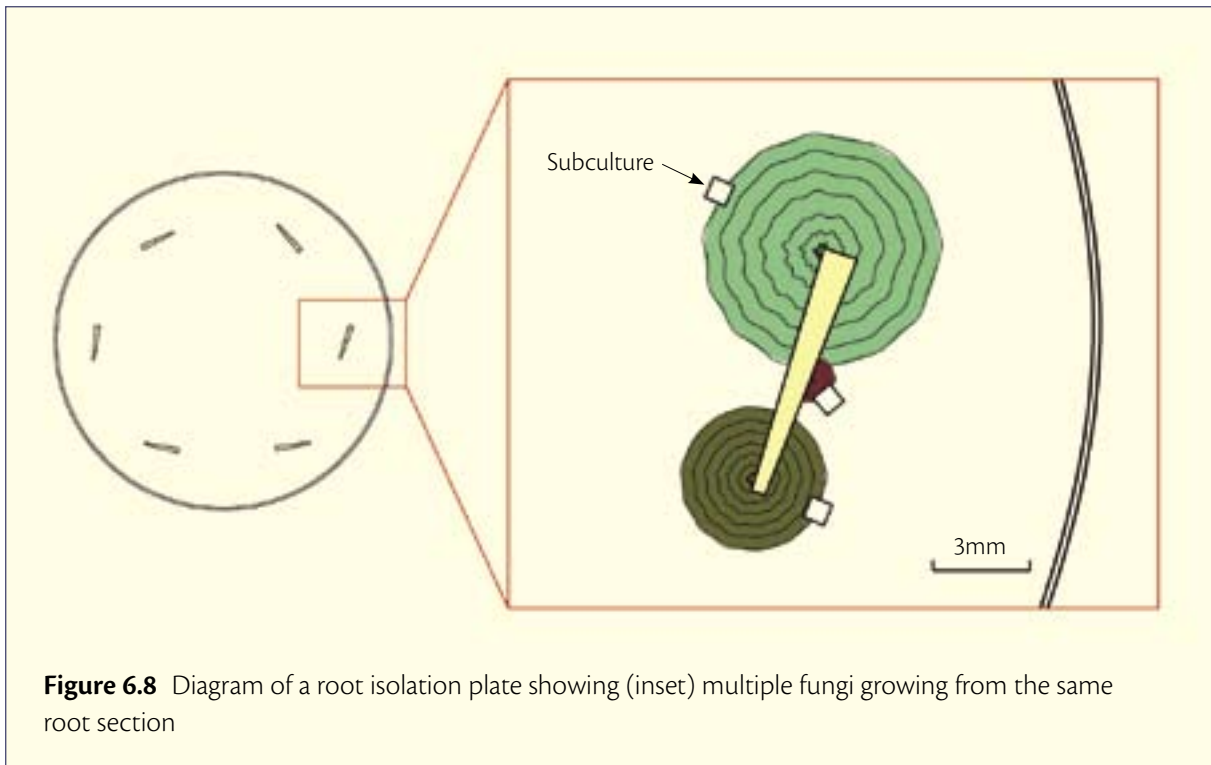
Isolation from other plant parts can be more difficult. When isolating from diseased roots, generally two or more fungi grow from the root, even on selective isolation media (Figure 6.8). The same problem can occur when attempting to isolate a pathogen from a fungal leaf spot, because saprophytic fungi quickly colonise leaf tissue weakened or killed by the pathogenic organism. It is important to subculture when these colonies are small, as it is easier to subculture before the fast-growing species grow over the slower-growing species.

Therefore, examine isolation plates each day for fungal growth. Check the tissue segment for sporulation, which may give an indication of the identity of the pathogen. However, be aware that the sporulation may be from a saprophyte.



Practice makes perfect! Practice isolating a wide range of fungal pathogens to gain experience and to learn to recognise how common fungal pathogens develop on isolation plates.

Saprophytic fungi are common in diseased tissue and often colonise isolation plates (Figure 6.9). Some, such as *Trichoderma*, interfere with the isolation process. Airborne fungal saprophytes, such as *Penicillium* and *Cladosporium* can also contaminate plates.



Once subcultures develop into colonies, these can be grouped by colony type and microscopic features for preliminary identification. Well-known saprophytes can be identified and discarded and potential pathogens can be purified by hyphal tip or single spore transfer to appropriate media for subsequent identification.

## 6.5 Purification of cultures

The final stage in identifying fungal pathogens is the creation of pure cultures. Only a single spore or hyphal tip is transferred to ensure a pure culture is produced.

### 6.5.1 Single sporing

Single sporing involves the transfer of a single germinated conidium to obtain a pure culture (Figure 6.10). This method is suitable for species of fungal genera that produce spores in culture, for example, *Fusarium*, *Colletotrichum*, *Alternaria*, *Stemphylium*, *Bipolaris*, *Verticillium* and *Phoma*.

#### Single sporing

1. Sterilise transfer needle.
- 2 & 3. Create a spore suspension by removing a small amount of surface mycelium with conidia or a small scraping of sporodochia from *Fusarium* spp. and place in 10 mL of sterile water in a test tube.
4. Shake the suspension to disperse the spores and check the spore concentration by holding the tube against the light or by examining a drop of the suspension under a dissecting microscope. Avoid high concentrations of spores. With experience, the concentration can be assessed visually in the test tube.
5. Dilute with sterile water if needed.
6. Pour the spore suspension onto a Petri dish containing a thin layer of water agar.
7. Pour out excess water. This leaves some spores on the agar.
8. Store the plate on its side for 18 hours until the spores germinate.
9. Examine the Petri dish under a dissecting microscope with a light source underneath. (Adjust the mirror on the light source carefully to obtain a good contrast between the agar and the conidia and germ tubes.)
10. Remove a single germinating spore using a flat transfer needle (Figure 6.11) and transfer it to a new medium (Appendix 1 describes how to make a flat transfer needle).

Sterilize transfer  
needle



Pour spore  
suspension  
onto thin water  
agar plate



Scrape spores  
from colony  
edge



Pour off excess  
water



Add spores to  
sterile water



Store plates on  
their side for 18  
hours



Check spore  
concentration



Aseptically cut  
out a single  
germinated  
spore



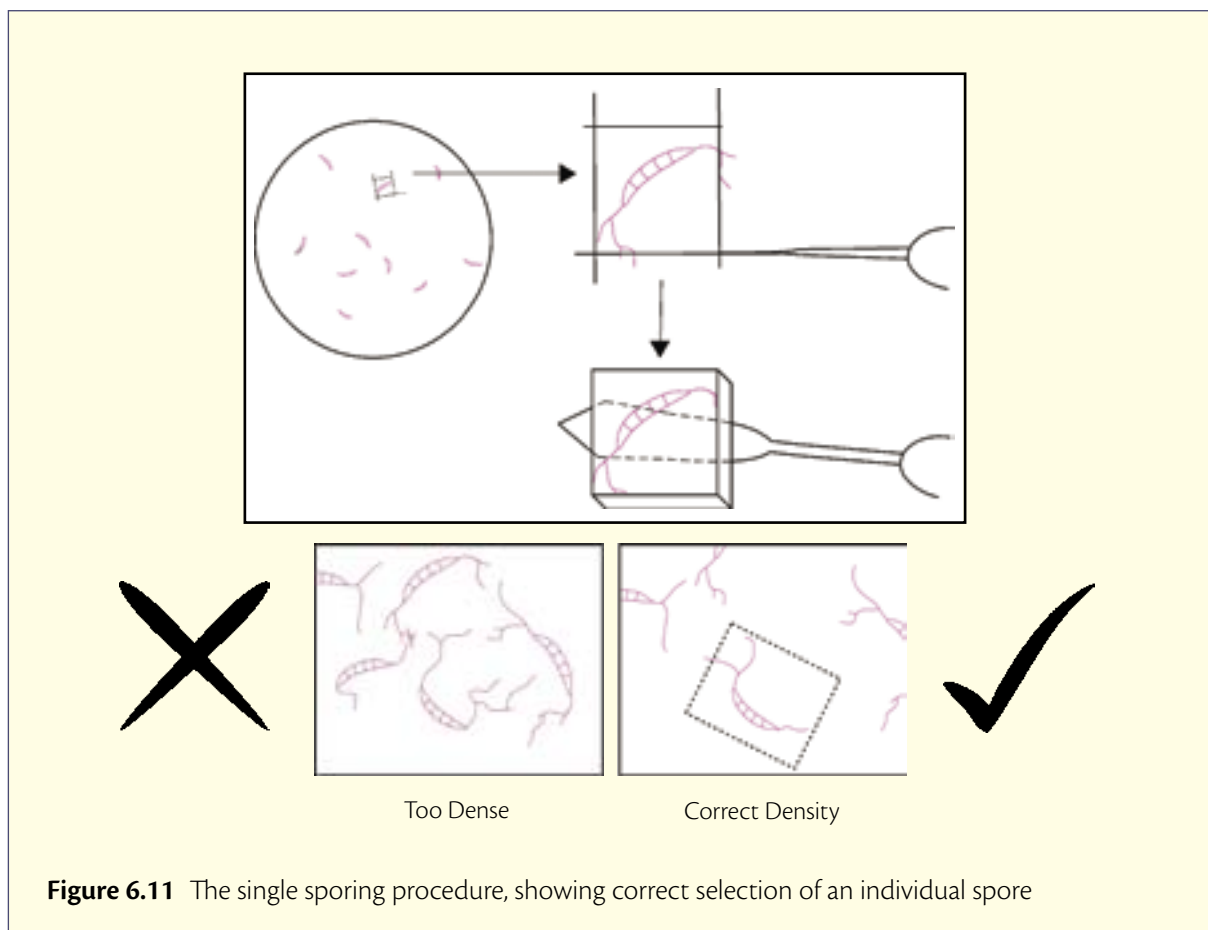
Dilute if  
necessary



Transfer to  
a new agar  
medium



**Figure 6.10** Steps in the single sporing process



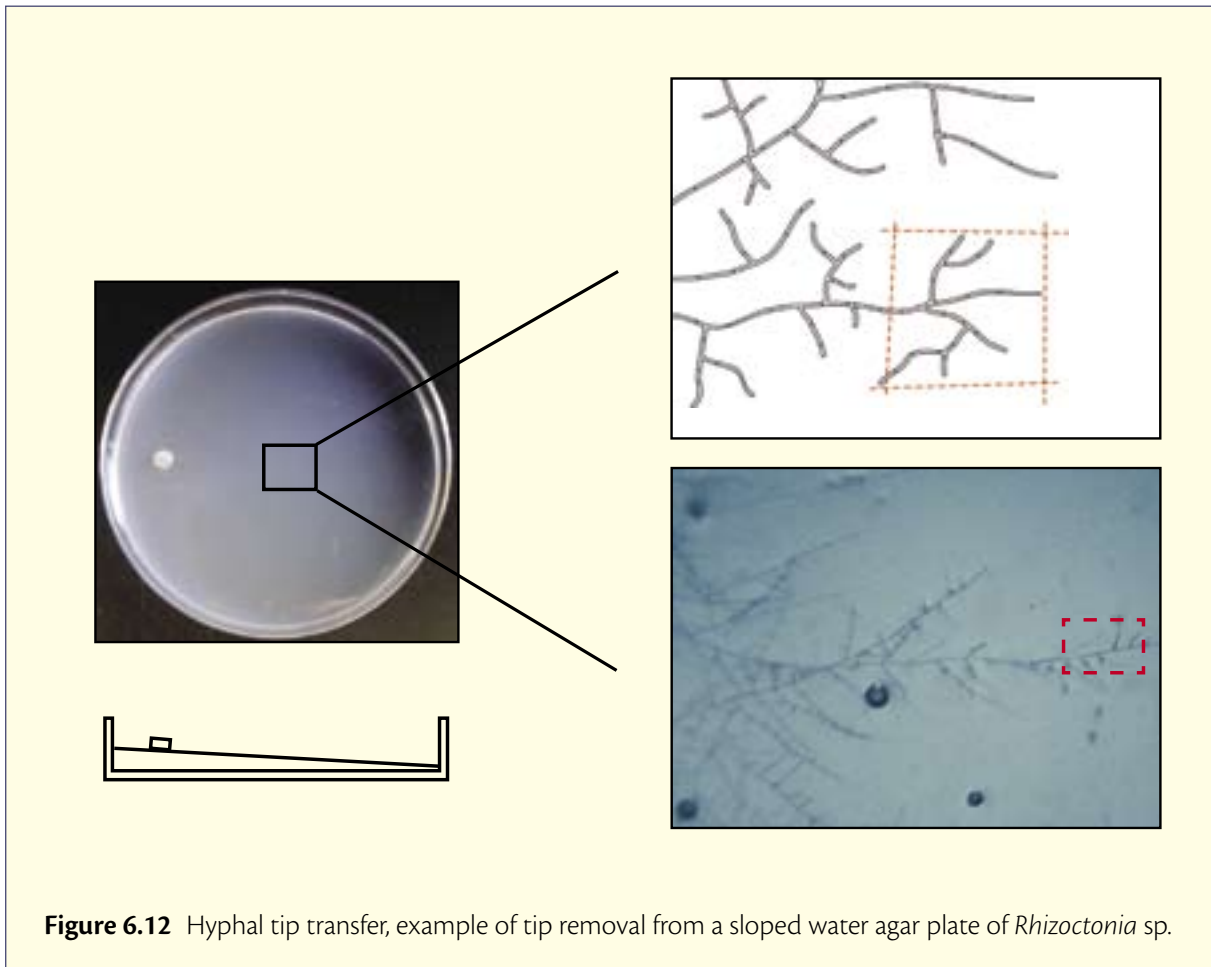
### 6.5.2 Hyphal tip transfer

Hyphal tipping involves the transfer of a single hyphal tip to obtain a pure culture. This method is suitable for species of fungal genera such as *Phytophthora*, *Pythium*, *Rhizoctonia*, *Sclerotium* and *Sclerotinia*.

#### Hyphal tip transfer

1. Pour a plate of water agar so that it is shallow on one side of the plate (Figure 6.12).
2. Inoculate on the side of the plate where the agar is deeper with a small agar block taken from a subculture isolation plate.
3. Place the plate under a dissecting microscope and focus on the hyphae at the growing margin of the colony. (The hyphae will grow sparsely across the shallow region of the agar.)
4. Adjust the light source (mirror) to obtain a good contrast between the medium and the hyphae.
5. Aseptically transfer a small block of agar containing a single hyphal tip to a plate of suitable agar medium, using a flat transfer needle.

Transfer only a single hyphal tip to ensure a pure culture.



**Figure 6.12** Hyphal tip transfer, example of tip removal from a sloped water agar plate of *Rhizoctonia* sp.

## 6.6 Recognising pure cultures

There can be considerable variation in colony morphology and pigmentation within a species. Diagnosis becomes easier as staff learn to recognise colonies of common fungal species on growth media (Figure 6.13). Cultures may then be sorted easily by eye into potential pathogenic species and probable saprophytic species. This can reduce the number of pure cultures that need to be prepared, saving resources and time.





*Pythium aphanidermatum*



*Pythium irregulare*



*Phytophthora nicotianae*



*Fusarium oxysporum*



*Fusarium solani*



*Aspergillus niger*



*Rhizoctonia* sp.



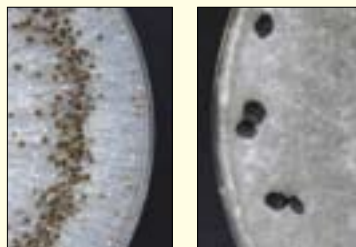
*Rhizoctonia* sp.



*Rhizoctonia* sp.



*Sclerotium rolfsii*



Close-up *S. rolfsii* (left) and *S. sclerotiorum* (right)



*Sclerotinia sclerotiorum*



*Pestalotia* sp.



*Phoma terrestris*



*Colletotrichum* sp.

**Figure 6.13** Colonies of common fungal pathogens on potato dextrose agar

Final identification of pure cultures should be based on:

- micromorphological features (e.g. asexual and sexual fruiting bodies)
- spore morphology or morphology of sclerotia
- mode of formation of spores (e.g. nature of the conidiogenous cell and the presence or absence of chains of conidia).

Some important plant pathogens do not produce spores and can only be distinguished by the presence and morphology of sclerotia (e.g. *Sclerotinia* spp., *Sclerotium* spp. and some species of *Rhizoctonia*). If asexual or sexual fruiting bodies, spores or sclerotia are not present, identification to species level can be difficult.

## 6.7 Identification of fungal pathogens

There are over 10,000 species of fungal and fungal-like plant pathogens. These pathogens cause a wide range of diseases, including leaf spots and blights, stem rots, cankers, root rots, wilt diseases and dieback, seedling diseases, fruit, head and grain rots, galls, rusts, smuts and mildews.

The accurate identification of a pathogen is the critical first step in the development of control measures and integrated disease management. If the name of the pathogen is known, information on its biology, epidemiology and potential control measures can be obtained from relevant publications, or via the internet.

Accurate identification is essential in selecting an appropriate fungicide for chemical control. For example, some fungicides that control downy mildews are not effective against powdery mildews.



The identification of fungal pathogens is based initially on morphological features, such as spores and spore-forming structures. Microscopic examination (see Section 6.2) is generally required to enable identification of the pathogen and diagnosis of the disease. Keys and guides to morphological identification will assist with this process, and there are also excellent illustrations of many common fungal pathogens in Agrios (2005). Other books and manuals should be kept as resources in diagnostic laboratories, and fortunately many scientific journal publications on taxonomy and identification can be accessed via the internet.

Obligate fungal parasites only grow on living host tissue (e.g. downy mildews, powdery mildews and rusts) and cannot be isolated into pure culture on agar media. Therefore, morphological identification depends on careful examination of the spores and spore-forming structures on the diseased tissue.

Many other fungal pathogens can be isolated and grown as pure cultures on artificial media under standard conditions. Spore forming structures and spores of these pathogens can be examined in culture for identification purposes. For example, most fungal pathogens that cause leaf diseases produce spore forming structures—perithecia, pycnidia, acervuli, sporangiophores or conidiophores—and these are readily examined microscopically.

Vegetative compatibility is another technique that can be used to identify strains. Sexual compatibility studies and molecular techniques can provide an understanding of genetic variation, and may be used to distinguish species that are morphologically similar. However, these techniques require more experience and resources than are available in small diagnostic laboratories.

## **6.8 References**

Agrios G.N. 2005. Plant pathology, 5th edition. Elsevier Academic Press: San Diego, California.