3 The diagnostic process

The main activities involved in the diagnostic process are:

- collecting diseased plants in the field
- examining collected plants in the laboratory
- pathogenicity testing
- disease diagnosis.

These activities are shown in Figure 3.1.

3.1 Case studies

In this section, two case studies are presented to provide an illustrated overview of the diagnostic process:

- diagnosing the cause of pineapple heart rot—*Phytophthora nicotianae*
- surveying a complex disease—ginger wilt caused by bacterial and Fusarium wilts.

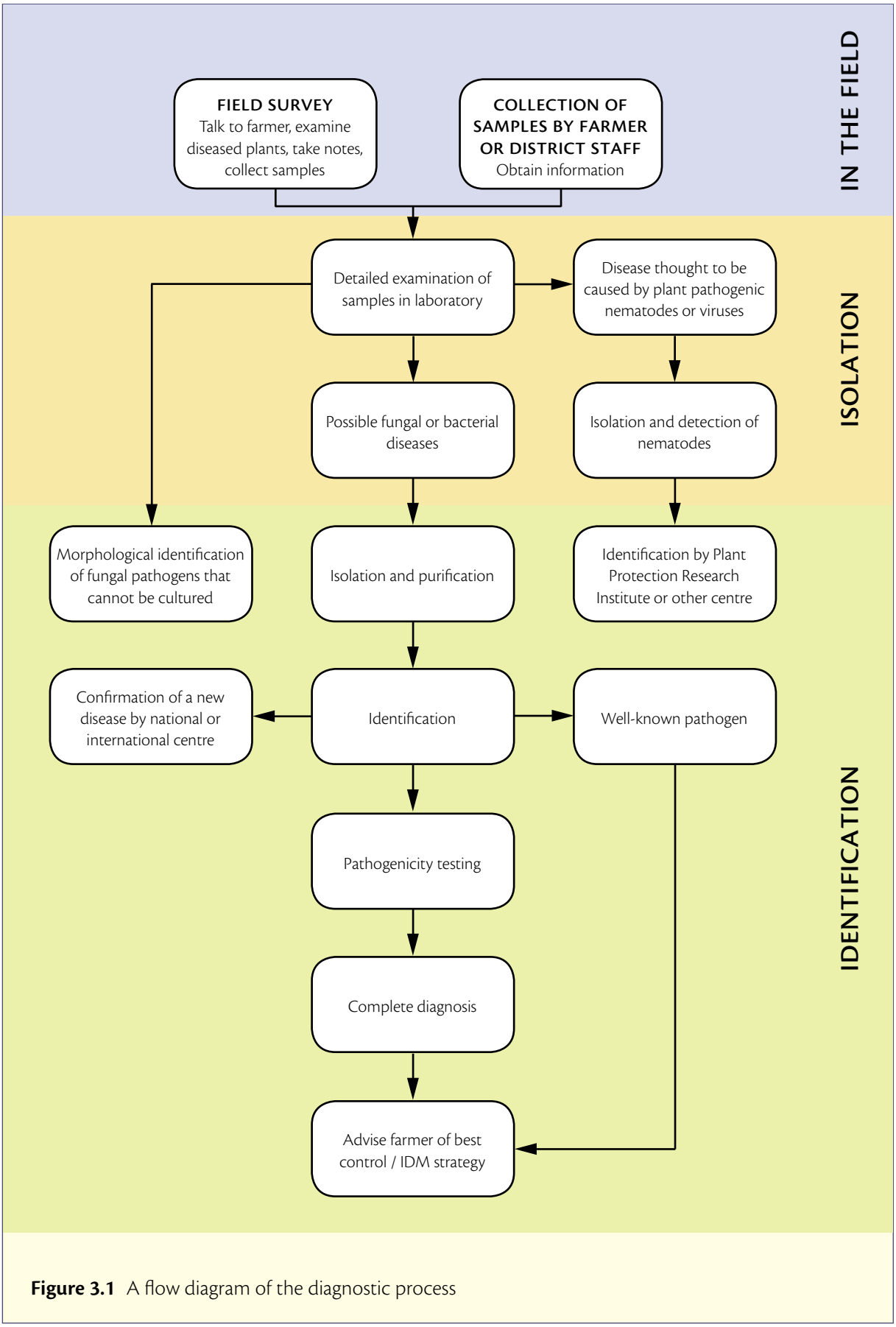


Figure 3.1 A flow diagram of the diagnostic process

DIAGNOSTIC CASE STUDY 1

Diagnosing the cause of pineapple heart rot—*Phytophthora nicotianae*

Figure 3.2 provides an example of the steps to follow during the diagnostic process.

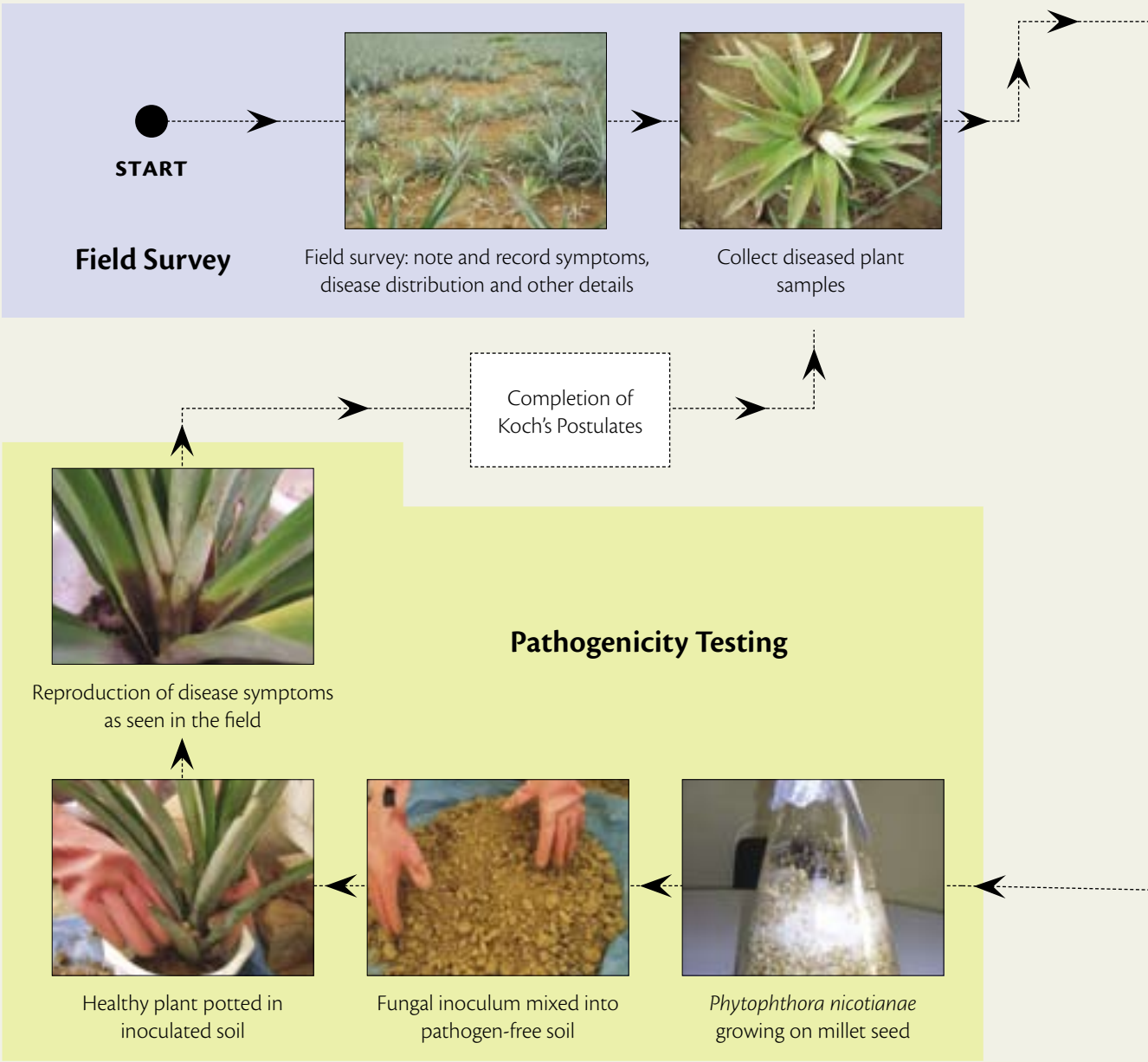
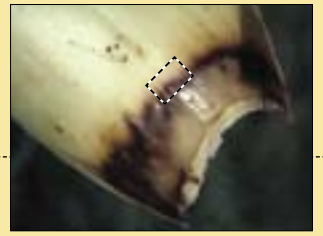


Figure 3.2 Steps involved in the isolation, purification, identification and pathogenicity testing of the pineapple heart rot pathogen, *Phytophthora nicotianae* (Images provided by Dang Luu Hoa)



Selection of material on margin of necrotic tissue

Wash and surface sterilise samples

Small segments cut and transferred aseptically

Plate segments on selective medium

Laboratory

Isolation and Purification

Incubation of plates

Colonies growing from segments

Subculture of fungal colony and hyphal tipping on water agar

Identification of pure culture from hyphal tip (*P. nicotianae*)

DIAGNOSTIC CASE STUDY 2

Surveying a complex disease—ginger wilt caused by bacterial and Fusarium wilts

Introduction

Ginger wilt was first recorded officially in Quang Nam in 2000. The disease has caused severe losses, with many farmers losing 100% of their crop. A preliminary study in 2006 indicated that bacterial wilt and Fusarium wilt were involved. A systematic survey of the disease complex was made in January 2007, as a part of the Australian Centre for International Agricultural Research project CP/2002/115, Diseases of crops in the central provinces of Vietnam: diagnosis, extension and control (2005–2008).

The objective was to isolate and identify potential pathogens associated with diseased ginger plants, and determine their relative importance. Ten diseased plants were collected from each of 10 crops from two districts, Phu Ninh and Tien Phuoc, which are the main areas of ginger production in Quang Nam. Crops were selected on an ad hoc basis for sampling before inspection.

In the field

Information was collected from the farmers at each crop site (Figure 3.3). The farmers maintained that there were two types of wilt: quick wilt and slow wilt. The leaves of plants with quick wilt appeared to have been 'boiled in water' and were translucent. In contrast, the leaves of plants with slow wilt appeared yellow (Figure 3.4). These comments suggested that two diseases were involved and the symptoms described were assumed to correspond to bacterial wilt (quick wilt) and Fusarium wilt (slow wilt).

Figure 3.3 Discussions with farmers on ginger wilt

Figure 3.4 A ginger wilt survey in Quang Nam in January 2007: (a) ginger with symptoms of quick wilt, (b) ginger plants with yellowing, a symptom of slow wilt, (c) adjacent crops, one crop with quick wilt, the other symptomless, (d) and (e) plants being removed carefully using a machete, keeping the root systems intact, (f) sample bag labelled with site number, farmer's name and date

DIAGNOSTIC CASE STUDY 2 (CONTINUED)

In the laboratory

Specimen preparation

Plant roots were washed carefully to remove soil. The plant was then examined and small samples from diseased areas on the plant were taken into the laboratory for microscopic examination and isolation of the pathogens (Figure 3.5).

Gently washing the soil from the roots

Examining the plant (leaves, shoot, rhizome, roots) and recording symptoms

Root knot nematode (*Meloidogyne* sp.) present in some root systems

Cut rhizome from root and stem

Figure 3.5 Preparation and examination of plants with ginger wilt for the laboratory

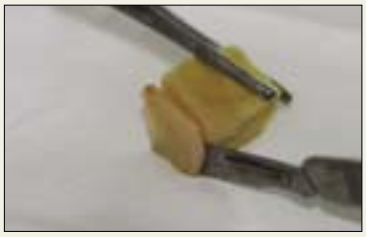
Isolation of potentially pathogenic organisms from diseased tissue

Ginger rhizomes were surface sterilised, peeled and surface sterilised again. A disc was removed from each rhizome, from which segments were cut and plated on peptone PCNB (pentachloronitrobenzene) agar and *Phytophthora* selective medium. Another segment was macerated and streaked on a plate of King's B medium to isolate bacteria (Figure 3.6).

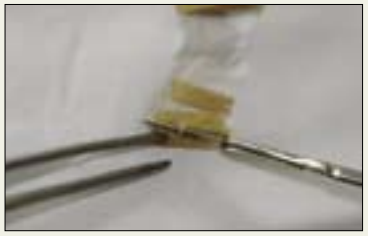
Rhizome was surface sterilised in 70% ethyl alcohol for 5 seconds and flamed

Rhizome was peeled (outer layer removed) and then surface sterilised again in 70% ethyl alcohol

Peeled rhizome was then flamed and a disc from the top of the rhizome was then aseptically removed



Five small segments were cut from the disc and two were plated on PPA and two on PSM



The remaining segment was macerated in sterile water on a glass slide and streaked onto a plate of King's B medium for isolation of bacteria

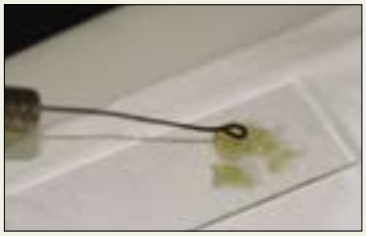


Figure 3.6 Isolation procedure for potential plant pathogenic organisms from ginger rhizome

DIAGNOSTIC CASE STUDY 2 (CONTINUED)

Recovery of potential pathogens

Fusarium spp. were isolated from rhizomes (Figure 3.7) from plants identified as having slow wilt—yellowing and root knot nematode symptoms—from some sites. The isolates were purified by single spore isolation and identified as *Fusarium oxysporum*.

The colonies of *F. oxysporum* were identical in culture on carnation leaf agar and potato dextrose agar, indicating that they could be pathogenic. (Cultures of saprophytic strains of *F. oxysporum* are usually quite variable in culture.)

Figure 3.7 Isolation of *Fusarium oxysporum* from some segments of ginger rhizomes on selective isolation medium (peptone pentachloronitrobenzene agar) for *Fusarium*

Phytophthora species were not isolated from the ginger rhizome.

A Pocket Diagnostic® Kit test for *Ralstonia solanacearum* was positive for rhizomes from three plants identified as having quick wilt, which indicates that this bacterium was present in the rhizome tissue. The kit test was negative for a rhizome from a plant identified as having slow wilt symptoms (yellowing)—*F. oxysporum* was isolated from this rhizome.

A variety of bacterial colonies were isolated on King's B medium and it was not possible to identify putative colonies of *R. solanacearum* with confidence. Because the plants that were sampled had obvious to severe symptoms and had been subjected to very wet soil prior to sampling, conditions would have favoured colonisation of diseased tissue by non-pathogenic bacteria.

Consequently, a bioassay procedure was used in an attempt to recover cultures of *R. solanacearum* (Figure 3.8). The inner tissue of additional rhizomes from plants previously sampled at six sites was cut into segments and shaken with 30 mL of sterile water. The water was then poured into small containers with freshly cut tomato and chilli stem cuttings—bait

seedlings—which were kept in a greenhouse at 25–30 °C. Within 4–8 days, some of the cuttings in the water extracts wilted and some of these showed signs of bacterial ooze. Control seedlings in sterile water remained healthy.

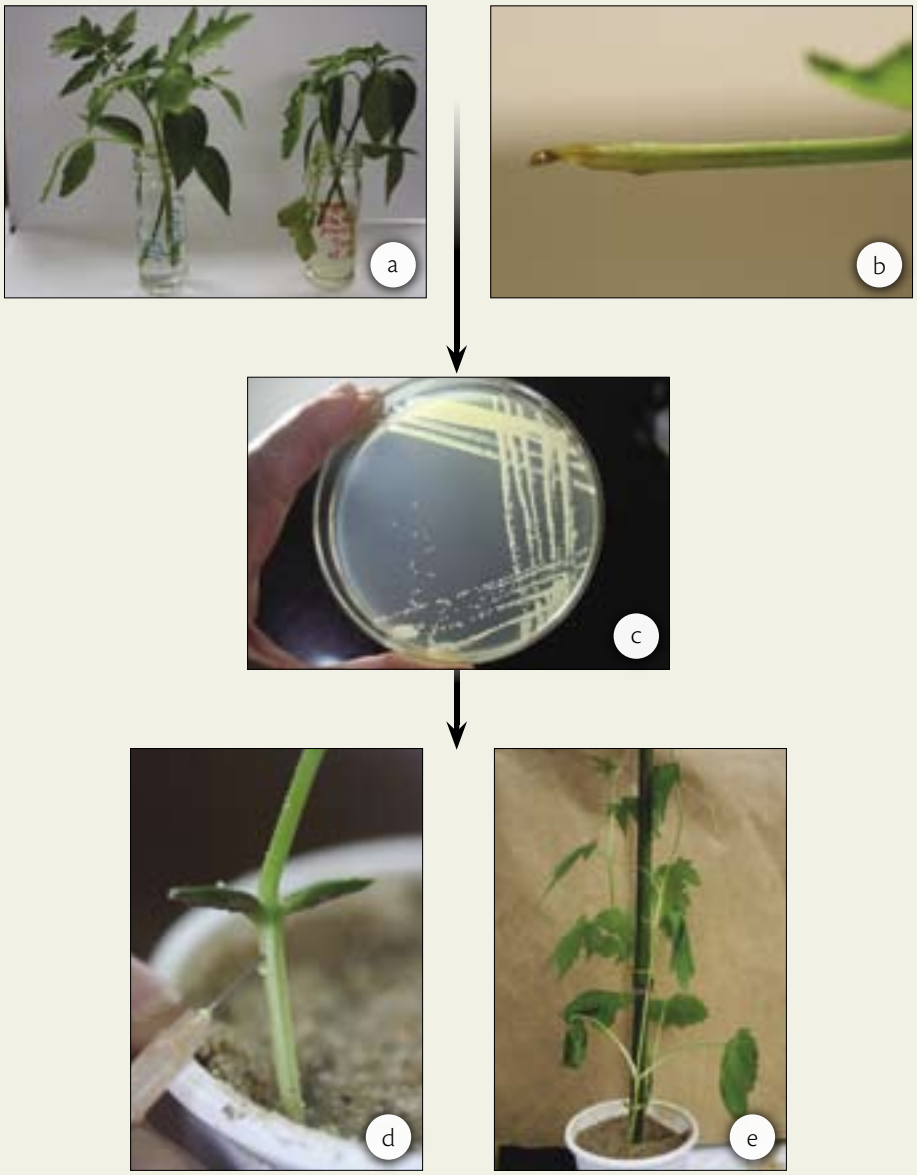


Figure 3.8 Bioassay procedure for isolating *Ralstonia solanacearum* from diseased ginger rhizome: (a) chilli and tomato cuttings in control (left) and wilted cuttings in water extract from rhizome segments (right), (b) wilted chilli cutting showing vascular browning, (c) isolation of *R. solanacearum* from chilli cutting, (d) and (e) pathogenicity test in bitter melon of bacterium isolated in the bioassay

DIAGNOSTIC CASE STUDY 2 (CONTINUED)

A stem section from each wilted bait seedling was macerated in sterile water and streaked on King's B medium. A single colony from this medium was then subcultured for purification and also inoculated into the stem of a 6-week-old bitter melon plant, to assess virulence. Some of the bitter melon plants showed severe wilting and the isolation process was repeated with these stems to obtain pure cultures of *R. solanacearum* for identification at an international reference centre.

Confirming identification with pathogenicity tests

Fusarium oxysporum f. sp. *zingiberi* has been reported widely in many countries as a cause of Fusarium wilt of ginger. However, it was essential that the isolates of *F. oxysporum* from ginger from Quang Nam were tested for pathogenicity in local ginger cultivars, to prove that they were pathogenic isolates and not saprophytic strains. Therefore, representative isolates were grown on millet seed/rice hull medium for use in pathogenicity tests.

Because *R. solanacearum* is also known to cause bacterial wilt of ginger, pure cultures of *R. solanacearum* were also tested for pathogenicity to local cultivars of ginger to complete Koch's postulates (criteria used to establish a causal relationship between pathogen and a disease).

For precise identification, pure cultures of *R. solanacearum* were also sent to an international reference laboratory. This species is variable, including a number of races, which differ in host range and require different crop rotations for control.

Samples of *Meloidogyne* galls were also forwarded to a reference laboratory for precise identification of species.

After proof of pathogenicity is obtained

Once it was established that wilt pathogens are responsible for the wilt disease on the ginger plants, staff developed a supply of pathogen-free ginger rhizomes for planting in small demonstration plots using the pathogen-free rhizomes and soil. The soil was deemed to be free of the pathogen where crops resistant to bacterial wilt (and root knot nematode) had been grown previously. Note that *R. solanacearum* has a wide host range.

Although *F. oxysporum* f. sp. *zingiberi* only causes disease on ginger, it may persist on roots of symptomless non-host plants. Good crop hygiene is important so that soil from diseased fields is not introduced into disease-free fields on shoes and digging implements.

4 Symptoms of disease

Diagnosis begins with careful observation of all parts of the diseased plant—foliage, flowers, fruit, stems and roots. It can be difficult to identify the pathogen responsible because many plant pathogens cannot be identified with the unaided eye. The visible effects of the pathogen on the plant—symptoms—can help in determining the type or types of pathogens present. Symptoms of disease may be caused by:

- damage to the plant tissues
- disruption of the normal physiological functions of the plant:
  - water and nutrient uptake
  - photosynthesis
  - growth.

Symptoms of disease should be recorded carefully in a field notebook and in photographs using a digital camera (if possible).

4.1 Common symptoms

Common non-specific symptoms may be caused by many different types of pathogens. Wilting, yellowing and stunting are common non-specific symptoms (see wilt pathogen sections). Wilting is commonly caused by vascular wilt pathogens, root rots and root galls, collar rots, stem rots and by dry soil. These and many other diseases also cause stunting and yellowing. Therefore, it is important to examine all parts of the plant—most importantly the roots.

Pathogens that damage roots or stem tissues—typically fungi and nematodes—disrupt the absorption of water and nutrients. This can cause wilting, yellowing and stunting, beginning with stunting of the plant and, as the disease progresses, wilting, yellowing and plant death. Bacterial wilts cause wilting and plant death and some virus diseases also cause wilting, stunting and plant death.

Leaf disease symptoms may be caused by fungal pathogens (leaf spot and blight), bacterial pathogens (leaf spot and blight) and plant pathogenic viruses (mosaics or mottling, leaf dwarfing and leaf rolling). Viruses may also cause non-specific symptoms (yellowing, wilting and stunting).

Plant parasitic nematodes mainly affect plant root systems, causing root lesions, root galls, and root proliferation in association with cysts. This leads to stunting, yellowing, wilting and sometimes plant death.

Some symptoms can help in determining the cause of a disease as they are specific to certain pathogens. For example, the distinctive root galls caused by *Meloidogyne* spp. (root knot nematodes) or club root caused by *Plasmodiophora brassicae*, enable an accurate diagnosis in the field.

Legumes such as peanuts and soybeans have root nodules—small swellings on the roots caused by infection with nitrogen-fixing bacteria such as *Rhizobium* spp. These are beneficial and important to the health of these plants. Healthy nodules are usually pink when cut with a knife.

Due to the range of possible symptoms that may be observed in the field, plant pathologists should examine diseased plants carefully and take clear notes to help in making an accurate diagnosis. In addition, plant pathologists should question farmers to get information about the history of the crop and the development of the disease.

A single plant may be affected by several different pathogens, causing a range of disease symptoms.

Diseases thought to be caused by plant viruses should be sent to a laboratory for examination by virologists experienced in identifying viruses. The accurate identification of plant parasitic nematodes, plant pathogenic bacteria and related pathogens also requires expert examination.

In contrast, many fungal pathogens can be identified successfully in the field or in a basic laboratory, provided adequate reference materials are available.

Seek help if you are not confident in determining the cause of a disease. Do not guess—the farmer's income will be affected by your diagnosis and advice.

Plant pathogens are identified as the likely cause of disease in only about half of plant samples sent to diagnostic laboratories. Plants may be affected by other factors such as pesticides or environmental stress.

4.2 Diseases of foliage, flowers or fruit

Many fungal pathogens cause diseases of the foliage (leaves, petioles and stems), flowers or fruit, and usually produce spores from spore-forming structures on the diseased tissue. The spores are carried by wind or splashed by rain onto other plants, which spreads the disease. Fungal pathogens can rapidly build up disease levels and cause epidemics under weather conditions suitable for the specific pathogen.

The presence of spore-forming structures such as pycnidia or perithecia, conidiophores or sporangiophores are the basis for morphological identification of fungal pathogens, and the diagnosis of plant fungal disease.

Use a hand lens to assist with the identification of fungal structures in the field. However, for accurate identification of spores and spore-forming structures, use a microscope in the laboratory.

Many fungal pathogens cause spots (lesions) on the leaves, flowers or fruit. Yellow spots are chlorotic lesions and brown spots are necrotic lesions. Brown spots are called 'necrotic' because the pathogen has caused death, or necrosis, of the tissue. Fungal pathogens that cause lesions on leaves, flowers and fruit can often be isolated and grown on culture media.

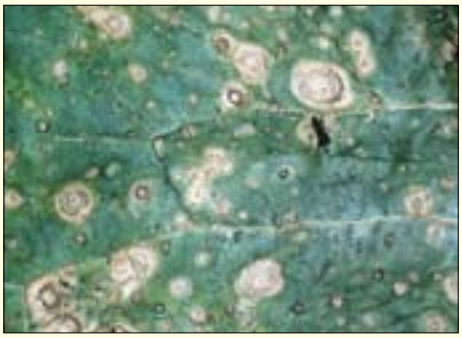
Saprophytic fungi also grow on diseased plant tissues as secondary colonisers. For example, saprophytic species of *Alternaria* and *Pestalotia* commonly grow on diseased leaf tissue and form spores on the tissue. These saprophytic species can lead to an incorrect diagnosis as they are usually visible under the microscope and grow rapidly on the isolation medium.

4.2.1 Spore production on diseased foliage

Fungi may form spores asexually from specialised hyphae called conidiophores. In the species *Stemphylium*, *Botrytis*, *Aspergillus*, *Penicillium*, *Bipolaris*, *Alternaria* and *Cercospora*, conidiophores develop on the diseased tissue from hyphae (Figure 4.1). Some species (e.g. *Septoria*, *Diaporthe*, *Didymella*, *Ascochyta* and *Phoma*) form conidia in specialised structures called pycnidia, others (e.g. *Colletotrichum*, *Cylindrosporium*) in structures called acervuli.

Conidia formed in conidiophores on surface of tissue

Stemphyllium
Botrytis
Aspergillus
Penicillium
Bipolaris
Alternaria
Cercospora



Conidia formed on an acervulus

Colletotrichum
Cylindrosporium

Conidia formed in pycnidia on tissue

Septoria
Diaporthe
Didymella
Ascochyta
Phoma

Figure 4.1 Formation of conidia on foliage by various fungal pathogens

Some fungal pathogens also produce spores from sexual reproductive structures on leaves, stalks, stems or fruit. For example, *Fusarium graminearum* (*Gibberella zeae*) forms ascospores in an ascus within perithecia on mature diseased stalks and cobs of maize. This fungus causes stalk and cob rot of maize.

Perithecia are similar in shape to pycnidia, but pycnidia form conidia and do not have asci.

4.2.2 Obligate foliar fungal and fungal-like pathogens

The downy mildews, powdery mildews and rusts are caused by obligate plant parasites. These pathogens are only able to infect, grow and produce spores in living host tissue—they cannot be isolated and grown in culture—and are usually only able to infect one or two host species or varieties (cultivars). For example, peanut rust can only infect peanuts.

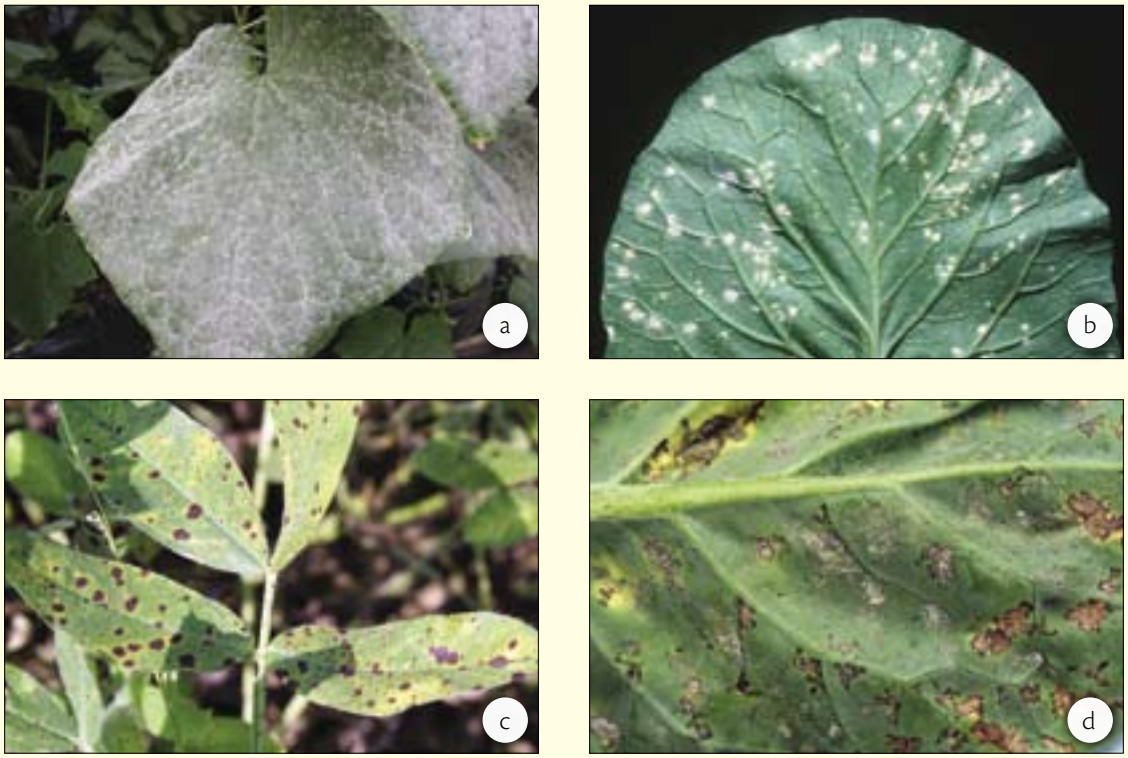


Figure 4.2 Fungal and fungal-like pathogens of the foliage: (a) powdery mildew on a cucurbit, (b) white blister on Brassica sp., (c) Cercospora leaf spot and rust on peanut, (d) downy mildew on cabbage

The symptoms of downy mildews, powdery mildews and rusts are usually obvious (Figure 4.2). They absorb nutrients from living plant cells, which commonly leads to yellowing of the tissue. Photosynthesis in the leaf tissue is reduced, leading to reduced plant growth.

4.2.3 Pathogens that produce sclerotia on infected tissue

In humid conditions, some fungal pathogens produce hyphae and/or sclerotia on infected plant surfaces. Hyphae of some *Rhizoctonia* species grow on infected stem bases and leaves. In Vietnam, some *Rhizoctonia* species produce irregularly shaped brown sclerotia on diseased tissue on maize leaves and cabbages (Figure 4.3a).

Sclerotium rolfsii causes stem base rot on many annual vegetable and field crops in hot wet weather in Vietnam. *S. rolfsii* forms obvious white hyphae (mycelium) on the infected stem base and small round brown sclerotia (Figure 4.3b) from these hyphae.

Sclerotinia sclerotiorum produces white mycelium and large black sclerotia on the stems and foliage of many broad-leafed crops, such as long and short beans, tomatoes, cabbages, potatoes and lettuce (Figure 4.3c).

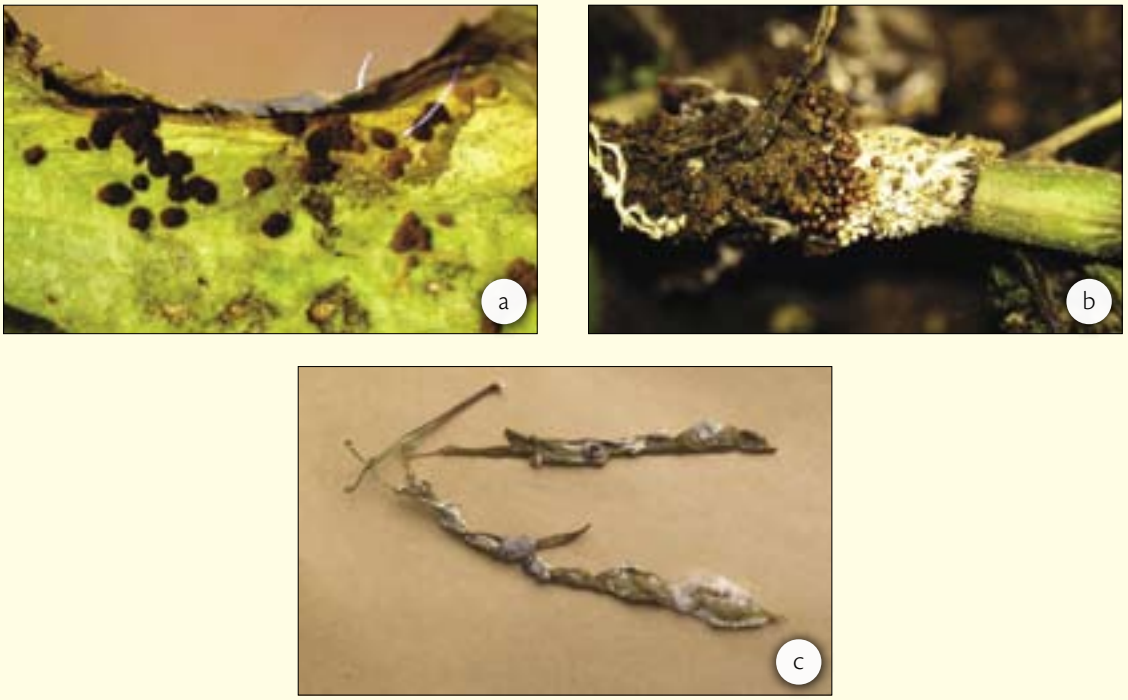


Figure 4.3 Sclerotial formation by (a) *Rhizoctonia solani*, (b) *Sclerotium rolfsii* and (c) *Sclerotinia sclerotiorum*

4.3 Diseases of roots, crown and stem

Fungal pathogens can cause serious diseases of the roots, crown (base of the stem) or the stem. Some fungal pathogens only affect seedlings, killing them before or after emergence; others only affect the feeder rootlets, and some species only affect the stem (e.g. *Sclerotinia sclerotiorum* and *Sclerotium rolfsii*).

Root symptoms may be non-specific. Rots of the feeder rootlets, main roots, crown or stem disrupt the uptake of water and nutrients, causing stunting and yellowing of the leaves, wilting and sometimes death of the plant.

Genera that commonly cause these diseases in Vietnam are *Phytophthora*, *Pythium*, *Fusarium*, *Sclerotinia*, *Sclerotium*, *Rhizoctonia* and *Phoma* (Figure 4.4). One root parasite, *Plasmodiophora brassicae,* which causes club root of crucifers, is an obligate parasite and cannot be grown on culture media. There are also Basidiomycota pathogens of the trunk and roots of perennial tree crops (Shivas and Beasley 2005), which are generally difficult to isolate into pure culture.

Root rot pathogens can be difficult to isolate as there can be many saprophytic fungi and bacteria in diseased root tissues, which will also grow on isolation media—commonly overgrowing pathogens.

4.4 References

Shivas R. and Beasley D. 2005. Management of plant pathogen collections. Australian Government Department of Agriculture, Fisheries and Forestry. At: <http://www.daff.gov.au/planthealth>.

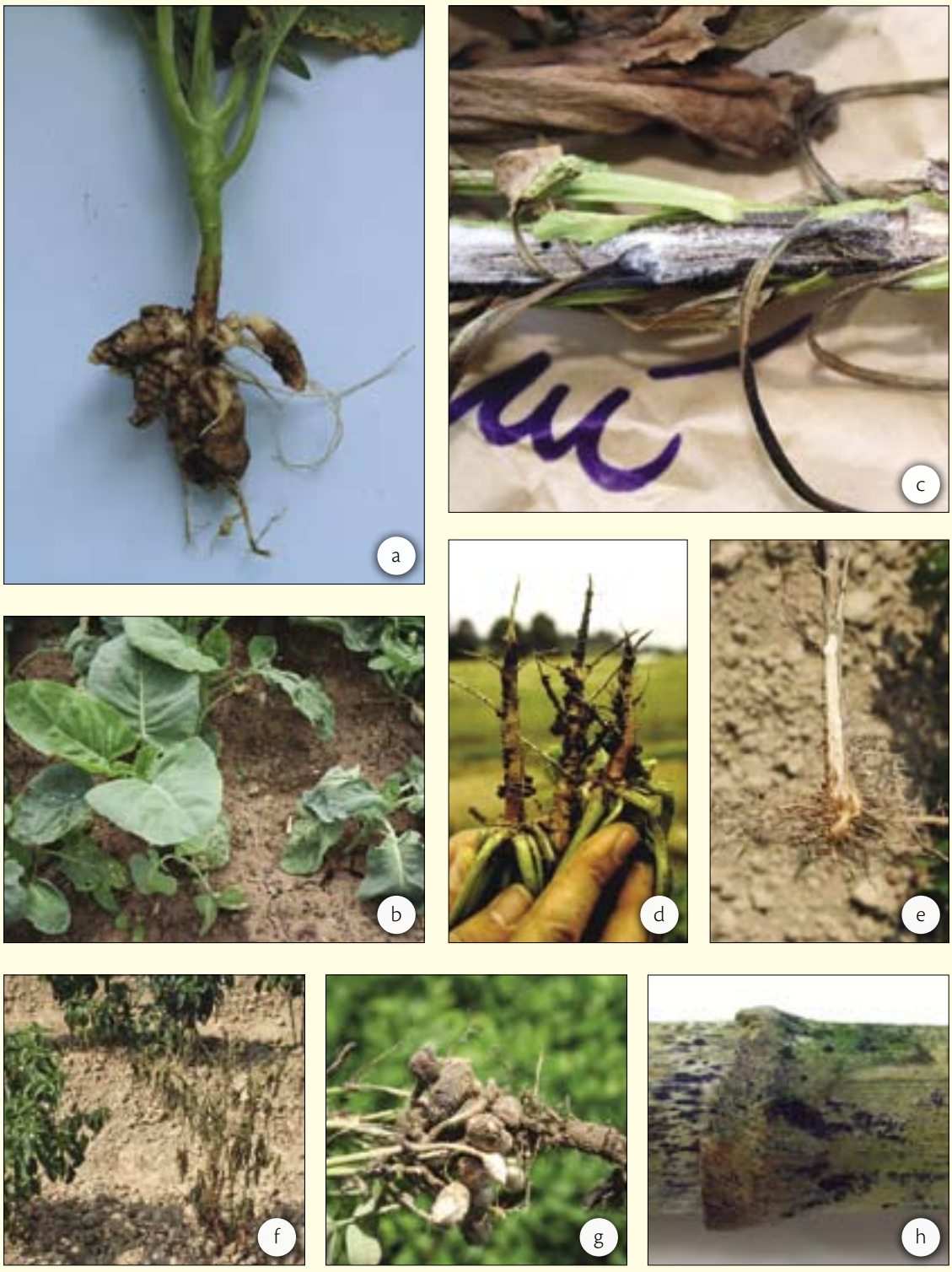


Figure 4.4 Diseases of the crown, roots and stem: (a) club root of crucifers, (b) wilting of crucifers (healthy [left] and diseased [right]) caused by club root (*Plasmodiophora brassicae*), (c) Fusarium wilt of asters (note the production of sporodochia on the stem), (d) spear point caused by *Rhizoctonia* sp., (e) Phytophthora root rot of chilli, (f) Phytophthora root rot of chilli causing severe wilt, (g) Pythium root and pod rot of peanuts, (h) perithecia of *Gibberella zeae* causing stalk rot of maize