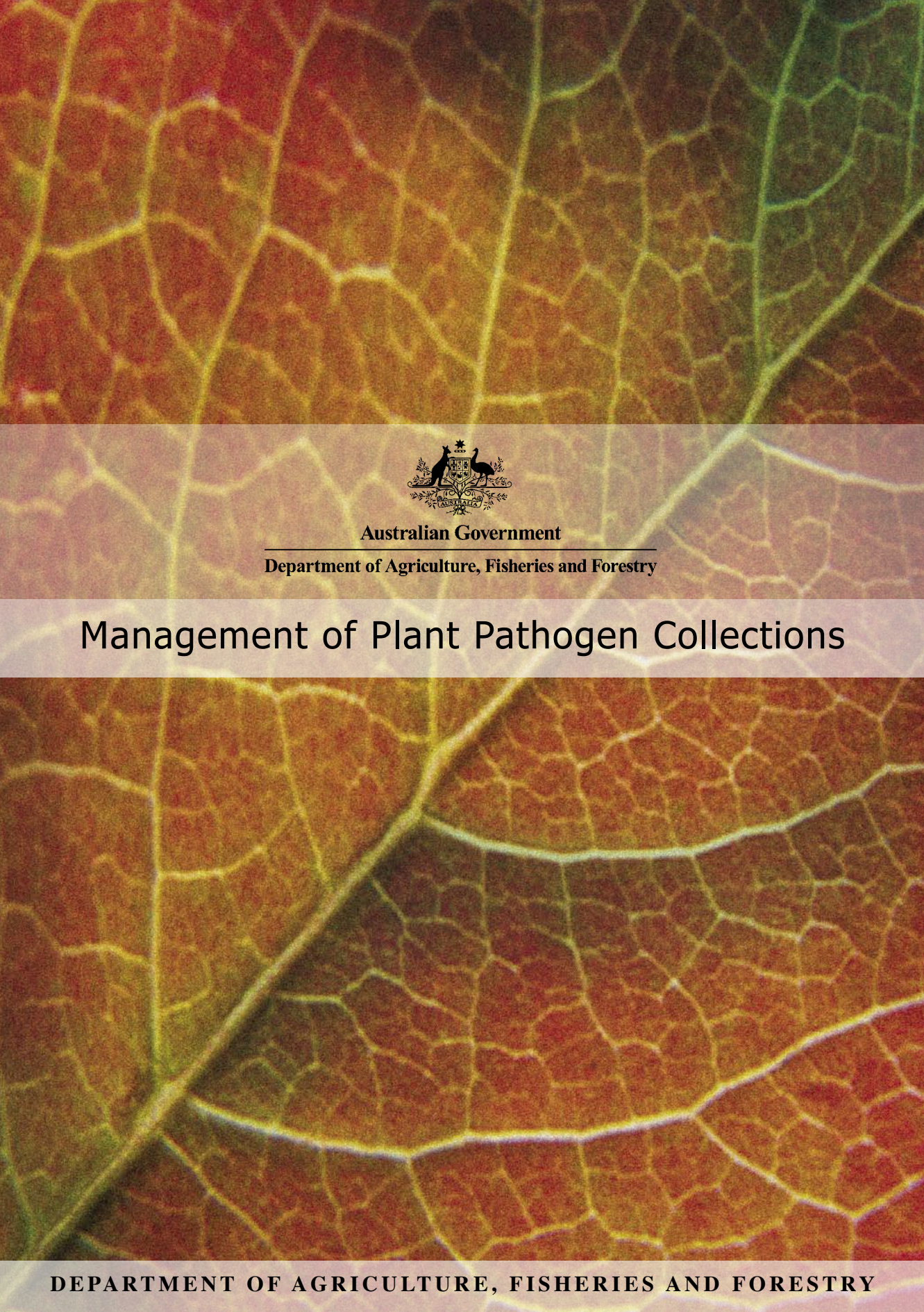

Australian Government

Department of Agriculture, Fisheries and Forestry

Management of Plant Pathogen Collections

DEPARTMENT OF AGRICULTURE, FISHERIES AND FORESTRY

Australian Government

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All enquiries should be directed to

Office of the Chief Plant Protection Officer
Australian Government Department of Agriculture, Fisheries and Forestry
GPO BOX 858
Canberra ACT 2601
Australia
Phone: 61 2 6272 5118

The attached CD includes copies of the *Management of Plant Pathogen Collections in Thai, Bahasa and Vietnamese.*

Lists of records that cannot be verified are mere waste paper.

R.W.G. Dennis, in *British Ascomycetes* (1968),
J. Cramer, Lehre, Germany.

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Primary authors

Dr Roger Shivas and Dr Dean Beasley, Plant Pathology Herbarium, Queensland Department of Primary Industries and Fisheries, Queensland, Australia

Contributing authors

Dr John Thomas and Dr Andrew Geering, Virology, Queensland Department of Primary Industries and Fisheries, Queensland, Australia

Dr Ian Riley, Nematologist, The University of Adelaide, South Australia, Australia

Abbreviations

ASEAN	Association of Southeast Asian Nations
ASEANET	Southeast Asian LOOP of BioNET INTERNATIONAL
AusAID	Australian Agency for International Development
APPD	Australian Plant Pest Database
BRIP	Queensland Department of Primary Industries Plant Pathology Herbarium
DAFF	Department of Agriculture Fisheries and Forestry
DAR	New South Wales Plant Pathology Herbarium
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
GA	glycerol agar
GBIF	Global Biodiversity Information Facility
GPS	global positioning system
IJSB	International Journal of Systematic Bacteriology
IJSEM	International Journal of Systematic and Evolutionary Microbiology
IPPC	International Plant Protection Convention
ISPM	International Standard for Phytosanitary Measures
KOH	potassium hydroxide
LOOP	Locally Organised and Operated Partnership
PCR	polymerase chain reaction
PDA	potato dextrose agar
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SEM	scanning electron microscope
SPS Agreement	WTO Agreement on the Application of Sanitary and Phytosanitary Measures
TWA	tap water agar
WFCC	World Federation for Culture Collections
WTO	World Trade Organization

Foreword

This handbook on the management of plant pathogen collections was commissioned by the Australian Government Department of Agriculture, Fisheries and Forestry (DAFF). Its purpose was to provide a reference for countries in the Southeast Asian region seeking to build specimen-based pest lists for plant diseases, to facilitate international trade in agricultural commodities.

Establishment of the World Trade Organization (WTO) in 1995 was heralded as providing a new era in trade liberalisation. While trade in agricultural commodities has expanded as a result of the obligations imposed by the WTO Agreement on the Application of Sanitary and Phytosanitary Measures (the SPS Agreement), trade expansion has eluded many developing countries. This outcome is attributed to deficiencies in arthropod pest collections and plant disease herbaria, which limit the ability of many countries to describe the health status of their agricultural and forest industries. As a consequence, some national plant protection organisations cannot undertake robust and credible pest-risk analyses. While this situation continues, the developing countries of the region will be at a significant disadvantage in negotiating access to new markets for their agricultural commodities.

Under Article 9 of the SPS Agreement, developed countries agreed to provide technical assistance to developing country members to build capacity in relation to sanitary and phytosanitary measures. Australia has responded to its obligations in a number of ways, including regional and bilateral assistance programs funded through the Australian Agency for International Development (AusAID). The assistance activities cover a wide range of issues, including the building of specimen-based pest lists, which is the focus of a program managed by the Office of the Chief Plant Protection Officer within DAFF, targeting the needs of countries in Southeast Asia.

The program emphasises building the capacity of scientists in five key areas as follows:

- surveillance for plant pests
- diagnostics
- specimen preservation
- curation and collection management
- data management.

This handbook provides a summary of methods and techniques used in all disciplines of plant pathology, and includes information on the major groups of economically important pathogens, methods for collecting specimens in the field, growth media, isolation and preservation of specimens, as well as information and tips on how to manage a herbarium and a culture collection. To expand its impact, the handbook has been translated into Vietnamese, Thai and Bahasa.

Lois Ransom
Chief Plant Protection Officer
Australian Government Department of Agriculture, Fisheries and Forestry

Contents

Tables

Figures

Boxes

Introduction 1

The importance of biological collections, including plant disease herbaria and living cultures of plant pathogens, has been the subject of a number of papers and reviews published in the scientific literature. In a paper titled 'Mutual responsibilities of taxonomic mycology and plant pathology', Walker (1975) cites no less than 18 references in which the authors have written on the role of taxonomy in applied biology and the importance of taxonomic work on fungi in fields such as mycology and plant pathology. The impact of these papers on the level of support provided for plant disease herbaria and taxonomy is unknown. To judge from the situation in Australia, where most collections are the responsibility of State government agencies, it seems that there has been very little impact.

Just why governments should be so reluctant to support the biological collections that underpin taxonomic studies is debatable, but it may have something to do with the failure of taxonomists to give attention to the needs of potential users: '…many taxonomists have often been unaware of the needs and work of applied biologists, including plant pathologists, and some have tended to cut themselves off from the wider fields of biological practice and research' (Walker 1975). Despite this, with the establishment of the World Trade Organization (WTO) in 1995 and its rules applying to trade in agricultural commodities, plant health has become a major trade policy issue. Governments everywhere are under pressure from their constituents to use the provisions of the WTO Agreement on the Application of Sanitary and Phytosanitary Measures[1] (the SPS Agreement) to maximum competitive advantage — that is, to prise open markets previously closed on questionable quarantine grounds and to exclude commodities that pose a risk to domestic industries. The SPS Agreement sets conditions, based on scientific principles and risk assessment, to protect agricultural industries from exotic pests,[2] yet at the same time facilitate trade in agricultural commodities. The SPS Agreement allows members to manage trade in agricultural commodities on health and safety grounds, but restrictions must be transparent and technically justified.

1.1 International obligations

The International Plant Protection Convention (IPPC) and the SPS Agreement impose obligations on a prospective exporting country to provide the prospective importing country with a list of pests likely to be associated with the commodity to be exported.

[1] Sanitary and phytosanitary measures are domestic standards or regulations covering such matters as the presence of microbial contaminants, toxins, heavy metals and pesticide residues in food, and pests, weeds and pathogens.

[2] The term 'pest' is used to include arthropod pests, plant pathogens and nematodes.

- The IPPC established obligations to provide official technical and biological information necessary for pest-risk analysis in recognition of the essential nature of specific information on the pest status of a product that is held by exporting Members.[3,4]
- Article 6.3 of the SPS Agreement states that '…exporting Members claiming that areas within their territories are pest- or disease-free areas or areas of low pest prevalence shall provide the necessary evidence thereof in order to objectively demonstrate to the importing Member that such areas are, and are likely to remain, pest- or disease-free areas or areas of low pest prevalence, respectively. For this purpose, reasonable access shall be given, upon request, to the importing Member for inspection, testing and other relevant procedures'.
- Annex B, paragraph 3(b) of the SPS Agreement states that '…each Member shall ensure that one enquiry point exists which is responsible for the provision of answers to all reasonable questions from interested Members as well as for the provision of relevant documents regarding: (b) any control and inspection procedures, production and quarantine treatment, pesticide tolerance and food additive approval procedures, which are operated within its territory'.

In order to meet these obligations, and to be able to conduct pest-risk analyses and establish phytosanitary regulations to prevent the entry, establishment or spread of a pest, countries need to maintain reliable pest records.

According to International Standard for Phytosanitary Measures (ISPM) 8,[5] '…the provision of reliable pest records and the determination of pest status are vital components of a number of activities covered under the IPPC and by the principles noted in the ISPM 1: "Principles of plant quarantine" as related to international trade, and the international standards for phytosanitary measures that have been developed from them'.

ISPM 8 states that:

All countries may use pest status information for:

- pest risk analysis purposes
- planning national, regional or international pest management programs
- establishing national pest lists
- establishing and maintaining pest free areas.

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[3] International Plant Protection Convention, 1997: Article VII: International cooperation: '1. The contracting parties shall cooperate with one another to the fullest practicable extent in achieving the aims of this Convention, and shall in particular:…c) cooperate, to the extent practicable, in providing technical and biological information necessary for pest risk analysis'.

[4] International Standard for Phytosanitary Measures (ISPM) 11, Pest Risk Analysis for Quarantine Pests, 1.2 Information: 'The provision of official information regarding pest status is an obligation under the [International Plant Protection Convention] IPPC (Art. VIII.1c) facilitated by official contact points (Art. VIII.2)'.

[5] ISPM 8, Determination of Pest Status of an Area, International Plant Protection Convention (IPPC), Food and Agriculture Organization of the United Nations, Nov 1998.

In order for countries to benefit from the spirit of trade liberalisation embodied in the agreement establishing the WTO, they must be able to comply with obligations imposed by the IPPC and the WTO under the SPS Agreement. The capacity of the infrastructure underpinning plant health is critical if quarantine services are to assess the risk of moving pests with traded commodities.

1.2 The status of biological collections in ASEAN countries

In 2001–02 the Australian Agency for International Development (AusAID) supported an initiative to review and assess the arthropod pest collections and plant disease herbaria in Association of Southeast Asian Nations (ASEAN) countries. The initiative stemmed from a decision made at the second ASEANET[6] Locally Organised and Operated Partnership (LOOP) Coordinating Committee meeting held in 2000, when delegates endorsed a recommendation to document the biological collections throughout member countries.

The authors of the reports on the state of plant pest collections in ASEAN countries were to observe that, to a greater or lesser extent, none of the countries of the region could provide an adequate description of the health status of its agricultural industries. The problem was attributed, in large part, to the small number of specimens of plant diseases held in herbaria throughout the region. The arthropod pest collections were much better populated than the plant disease herbaria, and entomologists were generally more conversant with specimen preservation and collection management than were their counterparts in plant pathology.

1.3 The importance of specimen-based records

Information on the presence or absence of a pest or pathogen is available from many sources with varying levels of reliability. However, in the context of international trade, records based on voucher specimens that are held in properly curated collections provide the most reliable evidence of a country's plant health status. Voucher specimens, together with accompanying data documenting such matters as the location where the specimen was collected, date of collection, collector, host and the identity of the pathogen constitute pest records. Well-populated collections contain multiple entries of the same species from different hosts and from across different geographic and production areas. These specimens can be re-examined to check their identity or to obtain more precise data on the circumstances under which the specimens were obtained and on their distribution. On the other hand, published reports that are not supported by voucher specimens cannot be validated and are a potential impediment to agricultural trade. It can be extremely difficult, time-consuming and expensive to disprove erroneous reports to the satisfaction of a prospective trading partner. Specimens and other material contained in biological collections provide a powerful tool to assist bids for market access and to justify measures to exclude potentially harmful exotic species.

[6] ASEANET is the Southeast Asian LOOP of BioNET INTERNATIONAL, a body that works collaboratively to develop self-reliance in taxonomy and biosystematics.

1.4 Building and populating plant disease herbaria in ASEAN countries

The establishment of plant disease collections in ASEAN countries will be greatly assisted if practising plant pathologists are made aware of the need to lodge specimens in designated laboratories. A priority for Australian Government assistance programs is to run small workshops targeting practising plant health scientists and their supervisors to explain the importance of biological collections in underpinning trade, and the role that plant health professionals can play in populating the collections. These workshops are supplemented by training programs that target practising plant pathologists, to ensure that they know how to preserve specimens and transport them to designated herbaria. This handbook contains information that will assist all practising plant pathologists to fulfil their responsibility in populating national plant disease herbaria and collections of plant pathogens, as well as providing a useful ready reference for the curators of the herbaria and collections.

Plant pathogen collections 2

2.1 Herbaria

Herbaria are collections of biological specimens where dead, dried, pressed or preserved plants and fungi are permanently stored along with information about the specimens. Many botanical herbaria contain at least some mycological and plant pathological specimens. There are, by comparison, fewer dedicated mycological or plant pathological herbaria. Plant disease herbaria are in fact dual collections containing specimens of the host and the pathogen — fungi, bacteria, viruses, viroids, nematodes, bacteria, phytoplasmas and rickettsia-like organisms.

Mycological herbaria and the information they contain are used by taxonomists, mycologists, plant pathologists, plant health scientists, quarantine personnel, bioprospectors and policy makers from a range of disciplines, including biosecurity and biodiversity conservation. All officially recognised herbaria have abbreviations, for example, Herbarium Bogoriense and CABI Bioscience, UK Centre. Every specimen in a collection will have a unique number, which is preceded by the herbarium abbreviation. If the accession number of a particular specimen is known, then it can be located with the aid of *Index Herbariorum*, which is a published directory of more than 3000 public herbaria across the world and more than 9000 staff associated with them. The *Index Herbariorum* is also available online, and information can be searched by institution, abbreviation, staff member and research speciality.[7] *Index Herbariorum* is a joint project of the International Association for Plant Taxonomy and the New York Botanical Garden.

2.2 Culture collections

Culture collections maintain living isolates of fungi and bacteria that are kept in a stable state until required for future use. Culture preservation techniques range from continuous growth to methods that reduce, or ideally suspend, metabolism.

The World Federation for Culture Collections (WFCC)[8] plays a major international role in the collection, authentication, maintenance and distribution of cultures of microorganisms and cultured cells. Its purpose is to promote and support the establishment of culture collections and related services, to provide liaison and to set up an information network between the collections and their users, and to ensure the

[7] http://sciweb.nybg.org/science2/IndexHerbariorum.asp (Accessed 31 May 2005)
[8] http://www.wfcc.info (Accessed 31 May 2005)

long-term survival of culture collections. The WFCC has developed an international database on culture resources worldwide. This database is maintained at the National Institute of Genetics in Japan and has records of almost 500 culture collections from 62 countries. The records contain data on the organisation, management, services and scientific interests of the collections. The database forms an important information resource for all microbiological activity and also acts as a focus for data-sharing activities among WFCC members.

2.3 Pest lists

Pest lists are a compilation of recorded pests and diseases in a country or region that are known to affect a particular host plant. Information relating to the health (disease) status of agricultural crops, forest trees, native and introduced species has many uses, but these lists are particularly important when countries are seeking to export commodities to foreign markets.

The most reliable pest lists are those supported by voucher specimens; less reliable are those pest lists supported not by specimens, but only literature or institutional reports. Reliability also depends on the skill of the collector and determiner, the method of identification and the level of recording or publication. Reports made by a taxonomic specialist published in an internationally accepted refereed journal will be more reliable than a report that lacks this level of validation (Box 2.1).

Box 2.1 Information sources for generating pest lists in order of preference

Information sources in order of preference

1. Pest collections in agricultural departments, research facilities, universities and other institutions.
2. Primary literature: scientific journals, research papers, books, quarantine reports, correspondence from plant health and quarantine authorities.
3. Secondary literature; CABI crop protection compendium.
4. Grey literature: conference proceedings, pamphlets, pest risk analyses.
5. Other information: consultation with local and overseas experts, newspaper reports, electronic sources (the internet).

The format of the pest list depends on the users' needs. What information do they want? What do they want to do with it? Box 2.2 provides an example of a pest list detailing the pathogens found on the host in a particular location and the common names of the diseases they cause.

Box 2.2 Example of a pest list for pawpaw

CARICACEAE
Carica papaya L. (Pawpaw)
Alternaria tenuis Nees — Leathery fruit spot
Ascochyta caricae Pat. — Black spot
Botryosphaeria rhodina (Berk. & M.A. Curt.) Arx — Fruit rot
Colletotrichum acutatum (J.H. Simmonds) — Ripe fruit spot
Corynespora cassiicola (Berk. & M.A. Curt.) C.T. Wei — Leaf spot
Glomerella cingulata (Stoneman) Spauld. & H. Schrenk — Ripe fruit spot
Macrophomina phaseolina (Tassi.) Goid. — Stem girdling
Phytophthora cinnamomi Rands — Root rot
Sclerotium rolfsii Sacc. — Damping-off
Thanatephorus cucumeris (A.B. Frank) Donk — Damping off
Verticillium dahliae Kleb. — Wilt

Pest lists are built as a result of:

- surveillance
- specimen collection
- specimen preservation in well-maintained collections
- well-planned surveys that ensure the relevant commodities and production areas are targeted
- collaboration with other agencies
- ensuring specimen collection records meet International Standard for Phytosanitary Measures (ISPM) 8 standards.

Historically, the compilation of pest lists was difficult, reflecting the problem of accessing information contained in herbaria that are dispersed and frequently belong to different institutions including:

- national/state ministries of agriculture and forestry
- museums of natural history
- crop-specific research institutes
- research scientists within academic institutions.

Given the importance of the information contained in plant disease herbaria and pathogen collections, records should be entered into a database and made available to users electronically. Specialised information technology systems that facilitate the convenient and rapid location of specimens and disease records held in collections are available. The software needed for this task is relatively cheap; however, the process of adding records to a database is more costly. Data capture is a persistent

and widespread problem during the early stages of database development and, consequently, an ongoing commitment is required from the relevant institutions to allocate resources for data entry. Another major consideration in the development of any database is the quality and quantity of the underlying information. In many institutions, a significant amount of work is required in the taxonomic area, both in validating existing records and clearing a backlog of unidentified specimens. It is also important that countries establish minimum data standards for their pest records, to ensure that they can meet the international standard set out under the International Plant Protection Convention (IPPC) (ISPM 8). ISPM 8 lists the basic information needed to establish a pest record (Box 2.3).

Box 2.3 Information needed for a pest record, modified from ISPM 8

What constitutes a valid pest record?

1. Pest scientific name (genus, species, infraspecies).
2. Life stage or state.
3. Taxonomic group.
4. Identification method (including determiner's name).
5. Collection date (including collector's name).
6. Collection location details.
   a. place (town and state, district or province)
   b. country
   c. global positioning system (GPS) coordinates (latitude & longitude)
7. Host scientific name (genus, species, infraspecies).
8. Host damage.
9. Prevalence.
10. Bibliographical references.

In most countries, there is no central repository of plant health information, because reference collections holding disease records are usually scattered among numerous institutions. This impedes the ready flow of information and, because different levels of priority may be accorded to the individual collections, the quality of the data can also be highly variable. Recent developments in information technology mean that it is now possible to overcome this problem. Distributed database technology enables diverse, geographically isolated databases to be linked so that the available data can be accessed from a single point. In a sense, the technology creates a single, virtual national (or regional) database that can be maintained and regularly updated at the local level. The disease records can then be accessed via a website, with appropriate consideration given to ownership, access control and authorisation (certain data may be password-protected). User queries can generate lists of species, and provide information on their distribution and host range.

A distributed database system that has the capacity to collect and integrate plant health data from a number of institutions is possible provided that:

- institutions responsible for pathogen collections have their disease records held in an electronic database that has an internet connection
- resources are available to develop and adapt the specific ‘gateway’ or ‘broker’ software needed to link the diverse underlying database sources
- the institutions are willing to share their data and have an ongoing commitment to maintain their collection as part of the network (this will include meeting agreed minimum data standards).

How to establish collections 3

The specimens found in herbaria and culture collections are very different. Herbaria house dead specimens and culture collections maintain living isolates of microorganisms, so plant pathologists need to work with both types of material. Often, a plant pathology herbarium will maintain a culture collection. Sometimes different groups within an organisation may be responsible for each collection. It is not uncommon for different agencies or government departments to have responsibilities for maintaining herbaria and culture collections. Ideally, there should be a close linkage between herbaria and culture collections that maintain plant pathogens in any particular region or country.

The basic equipment needed to house a herbarium and a culture collection is shown in Figure 3.1. Both herbaria and culture collections may share some administrative and external equipment; for example, a specimen database of collection records.

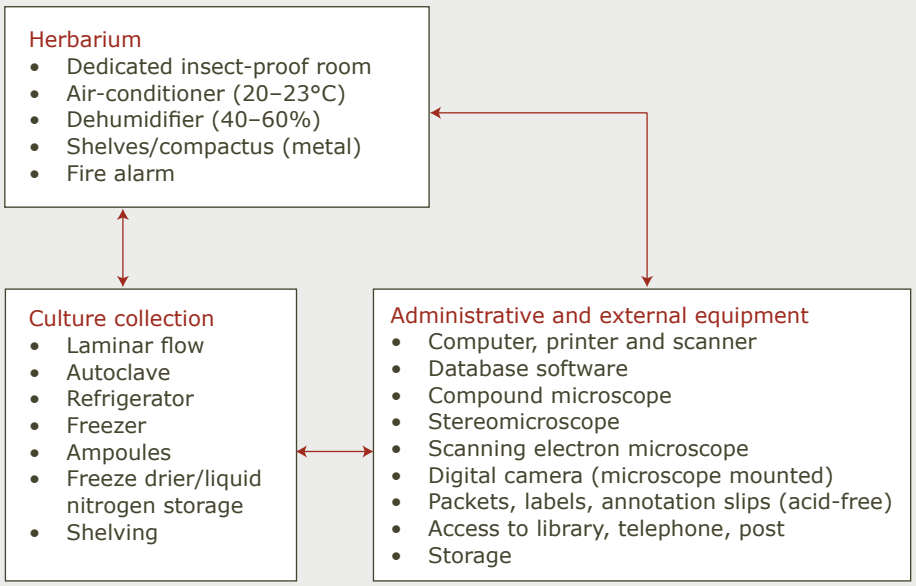


Figure 3.1 Equipment required for housing a plant disease herbarium and culture collection

3.1 Populating a collection

The primary source of specimens in a plant pathology collection is from professional plant health scientists working in diagnostic laboratories, which are usually located in government departments, research centres or universities. Specimens collected from the field, particularly during surveys, are usually sent to a diagnostic laboratory where a decision is made to keep the specimen or not. Any specimen that represents a new record of a pathogen, host or location, should be deposited in a recognised herbarium. Some herbaria have diagnostic staff and most have specialists who can determine the identity of particular pathogen groups.

Specimens often come from staff and students at universities and research centres, particularly as all published works should include at least the name of the herbarium where the specimens have been deposited. These specimens provide a reliable way to verify or correct the identity of pathogens recorded in surveys and research studies (ecological, epidemiological, phylogenetic, morphological and molecular).

Further specimens may arise when duplicates and exsiccata (a set of dried specimens with labels) are exchanged amongst herbaria. Gifts, loans and purchases are other means of adding to a collection. Permanent loans, where an entire collection is lent indefinitely from one institution to another, are a useful way to preserve valuable collections that are either inaccessible or unable to be adequately housed and maintained.

3.2 Collection of specimens from the field

Most specimens in a plant pathogen collection will have originated from the field, whether from an agricultural or natural environment. Diseased plant specimens are recognised by particular symptoms and signs. A symptom is the visible change in the appearance of a plant or its parts arising from a disease (Table 3.1). Symptoms may result from disruption to the capacity of the plant to photosynthesise efficiently, reproduce, absorb water or translocate nutrients.

A sign of disease is the visible presence of the pathogen, such as a fruiting body or discharge associated with the disease. Some common signs of disease are:

- ascomata, acervuli, conidiophores, pycnidia: minute, fungal, fruiting structures that produce conidia
- basidiocarps: the fruiting body of a polypore or agaric
- mycelium: the mass of hyphae (fungal threads) of the fungus
- ooze: the sticky fluid exuded from a wound or opening
- rhizomorphs: string-like strands of fungal hyphae (often dark).

Table 3.1	Some common symptoms and descriptions of plant disease
Symptom	Description
anthracnose	black, necrotic lesions caused by *Colletotrichum*
black mildew	dense, black colonies of parasitic fungi (Meliolales), usually on the surfaces of leaves of tropical plants
blight	widespread rapid death of plant tissue
canker	sunken necrotic lesion, often on a woody stem, branch or root
damping-off	collapse and rot of seedlings near soil level before emergence or soon after emergence, caused by fungi such as *Pythium* and *Rhizoctonia*
dieback	partial defoliation, twig, branch death and even complete death of plants
downy mildew	whitish bloom on leaves and stems caused by the presence of sporangiosphores and sporangia of members of the Peronosporales
enation	small abnormal outgrowth of host tissue, often flat extensions from veins, especially on leaves and flowers
fasciation	shoot proliferation appearing as thin, flattened bundles of curved or curled shoots
gall	an abnormal swelling or tumour
gummosis	leaking of gum from host tissues
lesion	defined, localised area of diseased tissue (a wound)
mosaic	patchy variation of light and dark green colour in leaves, symptomatic of many viral diseases
phyllody	flowers transformed into leaf-like structures
powdery mildew	white, powdery bloom on the plant surface consisting of fungal mycelium, conidiophores and conidia of powdery mildew fungi (Erysiphales)
pustule	blister from which a fungus erupts
root knot	a swelling or gall on a root caused by certain nematodes (*Meloidogyne*)
rot	softening and disintegration of plant tissues by enzymes produced by pathogens (may be hard, soft, dry, wet, black or white)
rust	pustules formed by members of the rust fungi (Uredinales)
scab	crust-like, superficial, roughened diseased area
scald	tissue that appears as if burnt with hot water
shothole	leaf disease where necrotic tissue falls out leaving circular holes
smut	black spore masses on leaves, stems and flowers caused by the smut fungi (Ustilaginomycetes)
sooty mould	saprobic dense mats of dark superficial fungi (often Capnodiales) living on exudates from insects (often aphids and scale) on the surface of leaves and stems
virescence	presence of green colouration in plant parts that are not normally green, especially the flowers
wilt	loss of turgidity and drooping of plant parts
witches' broom	proliferation of buds and shoots arising at or close to the same point

3.3 Handling specimens

The choice of specimens, whether for diagnosing plant disease or as a taxonomic resource, requires care. The best plant samples to collect are those in the early to middle stages of disease, when the pathogen is still active. Severely diseased plant samples are often unusable because the pathogen may no longer be viable and saprobic organisms may colonise necrotic tissues, which makes isolation of the pathogen difficult. Choice of material is also important. A basic knowledge of symptoms and how these are caused is needed to ensure that the part of the plant collected is infected by the pathogen. In some cases, the symptoms may appear in one part of the plant, but the pathogen is found elsewhere; for example, wilt disease symptoms appear in the leaves, although the pathogen occurs in the vascular system of the roots and stems. A list of useful equipment for a collection trip is included in Figure 3.2.

Equipment

Secateurs	Plant press	Newspaper
Hand lens	Trowel	Paper bags
Scissors	Ink markers	Envelopes
Maps	Hand saw	Machete
Global positioning system (GPS)	Literature	Labels
Plastic bags	Pencils	Ice box

Figure 3.2 Commonly used equipment for collecting plant disease specimens

Collectors should remember some important principles when collecting and handling specimens of diseased plants.

- Determine the identity of the host plant. If the identity of the host plant is uncertain, then healthy plant material, particularly flowers and fruits, should be collected. One should be sure that healthy material selected for host identification is actually the host plant. This is particularly important when collecting smuts and other diseases, which destroy the inflorescence on grasses that often grow in mixed populations.
- Use paper bags for specimens. Never wrap fresh herbaceous plant material in plastic; this causes the sample to sweat, encouraging saprobic organisms to rapidly colonise and decompose plant tissues. Plastic bags should only be used for short-term storage of damp specimens.
- Pack plant samples to avoid damage by crushing and to avoid condensation. Surface moisture will encourage the growth of saprobes and render the sample useless.
- Write labels in pencil (ink is not suitable as it will run if it becomes damp).
- Obtain the necessary permits allowing collection and movement of the specimens. In some areas, the collection of biological specimens may be restricted; for example, in national parks and on private land. The movement of specimens between countries may require import and export permits as well as quarantine permits.

3.3.1 Leaves, stems and fruit

Collect leaves when surfaces are dry, or if this is not possible, blot the leaves dry with newspaper prior to placing them between layers of fresh newspaper or other absorbent paper (do not use tissue paper, because this can disintegrate when wet and become difficult to remove from the sample). Press and dry leaf material, making sure to spread out the leaves out so they do not overlap. If the leaves are particularly fleshy, change the newspaper daily until the leaves dry out.

The sections of stems collected should have both healthy and diseased areas of tissue. Carefully wrap the samples individually in newspaper, because they can be easily damaged if they are packaged together.

When collecting fleshy specimens such as fruit, choose samples in the early to intermediate stages of symptom development. Secondary rots and saprobes often overcome fruit in the late stages of symptom development, which makes diagnosis of the causal pathogen difficult. Wrap fruit separately in dry newspaper. Do not wrap the sample in plastic.

Rusts and smuts

When collecting rusts, search both sides of leaves for brown-black teliospores as well as the orange-yellow urediniospores. Smuts often destroy the inflorescence, or parts of the inflorescence, of grasses and sedges. Careful identification of the host plant is essential for the accurate identification of smuts, but this can prove difficult if the inflorescence has been destroyed. Fold the diseased samples in newspaper so the rust and smut spores are contained.

Bacterial diseases

Samples with suspected bacterial diseases can be more difficult to collect and send to a distant laboratory, because tissue disintegration is often rapid. Place the sample in a paper bag and wrap in moistened newspaper to prevent the sample from drying out. If possible, keep the sample cool and out of the sun. Pressed and dried specimens of leaf spots and blights should be retained for use as herbarium material and also as a reference resource. Many bacterial pathogens survive for periods of months, or even years, in dried material stored at room temperature.

Viral diseases

Plant material that is suspected of being infected with a virus can be collected and temporarily preserved using small desiccators (Figure 3.3). The desiccators are made by filling a small plastic container with anhydrous calcium chloride ($CaCl_2$) crystals or silica gel up to one-third of its volume. The specimen should be separated from the desiccant by cotton wool. This technique is best carried out at temperatures of 0 to 4°C, but will also work quite well at ambient temperatures.

Use scissors or a safety scalpel blade to cut up leaf tissue. If the leaves are dusty or covered in sooty mould or scale insects, swab them with water or alcohol to clean them. Leaf sections should be collected from near the centre of the lamina. Cut the leaf into 3–5 mm squares and place 5–10 squares in each dessicator. Sterilise the scissors or safety blades in alcohol or a 10% sodium hypochlorite (NaOCl) solution between samples to prevent cross-contamination.

Alternatively, specimens with suspected virus infection can be placed in plastic bags with some moistened paper and kept in an ice-box for transport to specialist diagnostic laboratories. These conditions allow the plant material to remain turgid.

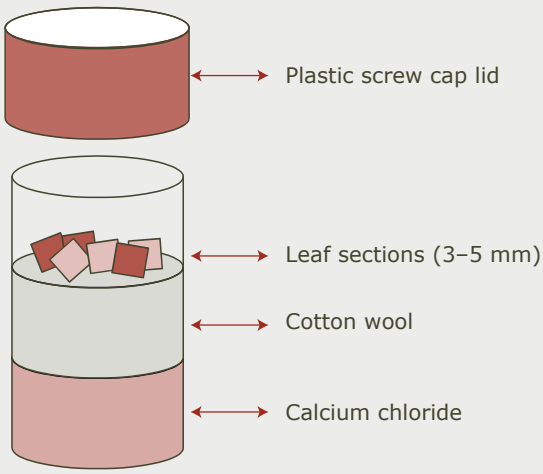


Figure 3.3 Desiccator for leaf samples suspected of being infected by virus

3.3.2 Roots and soil

Often, diseased root tissue or pathogen structures associated with roots are delicate. Do not pull plants from the soil, because this may shear off diseased tissue or pathogens, making diagnosis more difficult.

Shake off excess soil or, if possible, wash the roots gently unless the sample is to be tested for nematodes. Soil contains many microorganisms that readily colonise dead or dying tissue. These saprobes can interfere with the recovery of pathogens from diseased tissue. When removing soil from roots, do not scrub the roots, because this can lead to the loss of root tissue that may be important in disease diagnosis. Wrap the roots in newspaper for transport back to the laboratory.

Enough soil samples need to be collected to provide a representative picture of the disease situation. The major limiting factors in collecting numerous samples are the weight and volume of soil involved. The time and space required for processing must also be considered.

Soil-borne pathogens are not usually evenly dispersed in the soil; but tend to form groups or aggregations, either where conditions are most favourable or around infection points. The best strategy is to collect a number of random samples to take into account the uneven distribution of the pathogen. It follows that the more soil samples collected, the more accurate the overall disease assessment.

The number of samples collected will vary depending on the prevailing conditions, but generally a number of individual samples should be taken and then bulked together to form the sample proper. The material in the bulked sample can then be thoroughly mixed and a smaller subsample taken. If sampling for nematodes, care must be taken in handling the soil so as not to physically damage the nematodes through abrasion.

Sampling of soil that is very wet or very dry should be avoided. The soil for the sample should be taken at least 5–10 cm below the surface as it is here, in the root zone, that the plant pathogens will tend to be most abundant. If a crop shows patches of poor growth, separate soil samples should be taken from the badly affected and healthy areas so that a comparison can be made. Individual soil samples should weigh approximately 250–300 g.

If at all possible, roots should be either included in the sample or taken separately. For herbaceous plants, approximately 25–100 g of root tissue should be sufficient (the lower weight is suitable for vegetables, while the higher weight is more applicable to plants with large roots, such as banana). For woody plants, it may be necessary to excavate to a depth of up to 30 cm near the base of the tree or until roots are found showing the margin between healthy and diseased tissue.

Soil samples should be placed in strong plastic bags and labelled using a pencil-written paper or plastic label placed inside the bag. Samples should be kept cool. Do not leave soil samples in the sun or in a vehicle parked in the sun. Samples should be treated with care and processed or despatched for analysis as soon as possible. If immediate despatch or processing is impossible, samples can be stored in a refrigerator at 4–8°C for several days without deterioration.

3.3.3 Larger fungi

The fleshy fungi, particularly the larger members of the Agaricales, are the easiest to find, but quite often are the most difficult to transport back to the laboratory. Several healthy specimens at different stages of development should be collected. Do not harvest macrofungi by pulling them up and try not to break the stem. Dig them out so as not to damage their bases. They should be wrapped individually in newspaper and placed carefully in a container so they are not squashed.

It is important that specimens of larger fungi are dried quickly. Depending on the situation, this can be done in a number of ways:

- in a fan-ventilated oven (45°C, overnight)
- with an electric fruit dryer
- using a source of heat; for example, camp fire, gas heater, oil lamp, in the sun.

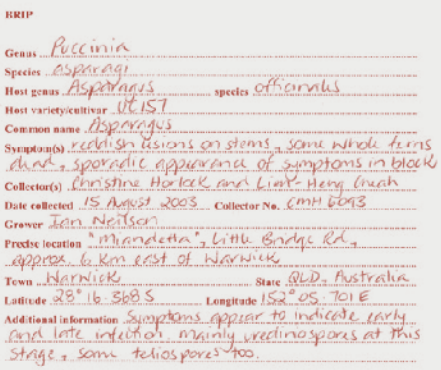
Spore prints, particularly their colour, are an important aid for identifying many larger fungi that have gills. A spore print is made by cutting off the pileus (cap) and placing it (gills downwards) onto a piece of white card for 15 min to a few hours. A container should be placed over the material to keep air currents from disturbing the print. It may be hard to see the spore print of white-spored fungi, so black card should be used for these. The colour of spores is easily seen from spore prints, and prints also provide a clear impression of the gill pattern.

3.4 Labelling specimens

Specimens that are not properly labelled are virtually useless, no matter how good the actual sample. The collection details that should accompany specimens include:

- name of host plant and part of plant affected
- precise location, town, state, province, district, country (longitude and latitude and altitude, if known). A global positioning system (GPS) is the best means of obtaining precise coordinates. A GPS tracks satellites that orbit the earth. By using three satellites, a GPS can calculate exact longitude and latitude. By using four satellites, a GPS can determine altitude. GPS-determined coordinates enable accurate distribution maps to be developed for plant pathogens.
- collection date
- collectors' names (collection number if given)
- disease symptoms and severity (eg number of plants affected).

All specimens submitted to a herbarium should be properly labelled (Figure 3.4). The name of the person submitting the specimen and their contact details are essential. The reason for submitting the sample should also be clearly stated; for example, for diagnosis or deposition.


BRIP

Genus Puccinia

Species asparagi

Host genus Asparagus species officinalis

Host variety/cultivar UC157

Common name Asparagus

Symptom(s) reddish lesions on stems, some whole ferns dead, sporadic appearance of symptoms in block

Collector(s) Christine Horlock and Liah Heng Chuah

Date collected 15 August 2003 Collector No. CMH 6093

Grower Ian Neilson

Precise location "Mandetta", Little Bridge Rd, approx. 6 km east of Warwick

Town Warwick State QLD, Australia

Latitude 28° 16' 36" S Longitude 152° 05' 10" E

Additional information Symptoms appear to indicate early and late infection, mainly uredinospores at this stage, some teliospores too.

Figure 3.4 Example of a field collection slip used at Queensland Department of Primary Industries and Fisheries Plant Pathology Herbarium (BRIP)

3.5 Surveys and sampling

Surveys are used to determine the type and severity of plant diseases affecting host plants. Surveys of plant diseases are needed to:

- establish pest distribution and the pest status of an area; this may be for area freedom requirements that facilitate exports and market access by determining the presence or absence of particular diseases of quarantine significance
- prioritise disease problems and guide pest management to allow the allocation of crop protection resources
- detect exotic and emerging diseases
- assess crop losses and determine levels of crop health
- understand pest dynamics
- identify host preferences
- evaluate the efficacy of control measures
- establish pest prevalence and link to crop damage
- identify natural enemies.

A survey can be qualitative or quantitative. Qualitative surveys determine whether a specific plant disease is present or absent. Quantitative surveys determine how much of a particular plant disease is present, particularly in relation to the amount of damage caused to host plants.

The survey must include sites that are representative of the region and cover an area of sufficient size to obtain the required level of accuracy. The objectives of the survey will determine the equipment, techniques and skills required. The amount of information to be gathered and the degree of accuracy will determine the scale of the survey.

Sampling procedures should be simple, representative and reliable. A rough estimate of sample size can be obtained from a pilot survey in which disease intensities are estimated for a number of different sample sizes. Comparing these results will enable the selection of the minimum sample size required to obtain a reliable estimate of disease intensity.

It is important that any sampling pattern takes the distribution of the disease into account. Diseased plants may have a random, regular or clustered distribution. Random sampling often yields good results because many plant diseases have a clustered distribution. Sometimes more systematic sampling patterns may be needed.

For specific surveys, more detailed information must be obtained about the best survey method. Other considerations are the timing of the survey and the appropriateness of the plant part sampled as an indicator of disease. For further information on conducting surveys in the Asia–Pacific region, refer to Dr Teresa McMaugh[9] in the Office of the Chief Plant Protection Officer, Australian Government Department of Agriculture, Fisheries and Forestry (DAFF).

[9] Dr McMaugh is coordinating the production of a publication, *Guidelines for Plant Pest Surveillance in Asia and the Pacific*, funded by the Australian Centre for International Agricultural Research and the Rural Industries Research and Development Corporation.

How to examine specimens 4

Examination starts with a study of the macroscopic features visible to the naked eye and under a stereomicroscope. The next step is to prepare a microscope slide and examine any fungal structures under a compound microscope. It is important during this stage, especially if the pathogen is unfamiliar to the collector, to take measurements, draw pictures and, if possible, take photographs. Ideally these notes, drawings and photos should be kept with the herbarium specimen.

4.1 Staining fungi and bacteria

Most fungi and bacteria can be observed microscopically in a droplet of water under a cover slip. Lactic acid may be used by itself as a mounting medium for fungi. Often it is necessary to stain fungi that have hyaline structures. Two useful stains for fungi with hyaline structures are **cotton blue** and **lacto-fuchsin**. Cotton blue stains the chitin present in the cell walls of fungi and lacto-fuchsin also binds well to fungal cell walls. Cotton blue stains blue and lacto-fuchsin stains red.

Cotton blue (or trypan blue)	
Cotton blue (or trypan blue)	0.1 g
Lactic acid	25 mL
Glycerol	50 mL
Distilled water	25 mL

Lacto-fuchsin	
Acid-fuchsin	0.1 g
Lactic acid	100 mL

The observation of bacteria in infected plant tissue can be facilitated using 0.1% aqueous **Toluidine blue O**. Cut a small section from the boundary between diseased and healthy tissue, place it on a clean microscope slide and add a drop of stain. Place a cover slip over the preparation and examine at a magnification of ×400. If present, it may be possible to see motile bacteria around the cut tissue edges. Bacteria are stained a deep blue; plant tissues a paler, greenish-blue.

Toluidine blue O	
Toluidine blue O	0.05 g
Distilled water	50 mL

One of the major divisions in bacteria is that between those that can retain a complex of **crystal violet** and **iodine** against elution with ethanol (gram-positive) and those that cannot (gram-negative). Observation of unstained gram-negative bacteria is enhanced by **safranin counterstain**. The gram stain is useful as a primary determinative character for many plant bacteria. The chemicals and procedure for the gram stain follow:

Crystal violet solution	
Crystal violet	2 g
95% ethanol	20 mL
Ammonium oxalate	0.8 g
Distilled water	80 mL

Dissolve 2 g crystal violet in 20 mL 95% ethanol. Dissolve 0.8 g ammonium oxalate in 80 mL distilled water. Mix the two solutions.

Lugol's iodine solution	
Iodine	1 g
Potassium iodide (KI)	2 g
Distilled water	300 mL

Grind together the iodine and potassium iodide, and then dissolve in the water and stir in a closed container for several hours to complete dissolution.

Safranin counterstain	
Safranin O	2.5 g
95% ethanol	20 g

Prepare a stock solution of safranin counterstain by dissolving 2.5 g safranin O in 100 mL 95% ethanol. Dilute this 1:10 in distilled water for use.

Make a turbid suspension of bacterial cells from an actively growing culture (usually 24–48 hours-old) in sterile water. Smear a loopful of this suspension on a clean microscope slide. The smear should be approximately 1 cm^2 and just visible. Air dry the slide then fix by passing the slide, bacterium up, several times through the flame of a Bunsen burner. Do not overheat the slide. Fixing ensures the bacteria will adhere to the slide surface during the subsequent staining procedure. Flood the slide with crystal violet for 1 minute. Pour off excess fluid and wash in a gentle stream of tap water until no more stain is obviously being removed from the smear. Flood the slide with Lugol's iodine for 1 minute. Wash in a gentle stream of tap water and blot dry. Wash in a gentle stream of 95% ethanol for a few seconds (not more than 30 seconds) to remove any stain and blot dry. Counterstain by flooding with safranin for 20 seconds. Wash again with

water and blot dry. Examine the slide under ×1000 magnification with oil immersion. Gram-positive bacteria stain blue-violet, while gram-negative stain red-pink.

The gram reaction can be confirmed by a potassium hydroxide (KOH) solubility test. Take a loopful of bacteria from a young actively growing agar culture and mix in a drop of 3% aqueous KOH on a clean glass slide until an even suspension is obtained. Lift the loop a few centimetres from the slide. If a string of slime is lifted with the loop (about 5–20 mm in length), the bacterium is gram-negative. If a watery suspension is produced and no string of slime observed after repeated strokes of the loop, the culture is gram-positive. The destruction of the cell wall of gram-negative organisms and subsequent liberation of deoxyribonucleic acid (DNA), which is viscid in water, produces the string of slime. The gram-positive wall is more resistant to KOH and remains intact, thus no DNA is released. This test is useful in cases of doubtful stain results.

4.2 Light microscopy

A stereomicroscope is useful for examining specimens initially. A high-power compound microscope providing magnifications up to ×1000 is essential for examining the morphology of bacteria and microfungi.

To examine fungi, a minute portion of the sporing tissue is removed under a stereomicroscope with the aid of a mounted needle or pointed scalpel, and placed in a small drop of stain, usually lacto-fuchsin, or a mounting fluid such as lactic acid. If the plant material is bulky, light pressure on the cover slip will help to disperse the structures into a thin optical plane. Air bubbles can be removed from the mount by gently passing the microscope slide through a flame (making sure not to deposit carbon on the lower surface of the glass slide). If the slide is heated too vigorously the cover slip will pop off.

The adhesive tape technique is particularly useful for showing the orientation of dry spores (as well as spore chains and the shape of conidiophores). A small piece of tape is held with forceps against a fungal colony (or leaf surface). It is then placed sticky side up on a small drop of stain or mounting fluid on a clean microscope slide. Finally, a cover slip is placed over the preparation and it can be examined under a microscope.

Some fungi have fragile chains of spores that fall apart at the slightest movement of air. Slide cultures may overcome this problem. Slide cultures are made using a Petri dish as a moist chamber and placing a bent glass tube inside the Petri dish, resting on moistened filter-paper. A sterile block of agar (approximately 1 cm^2) is placed on a flame-sterilised microscope slide resting on the bent glass tube. The fungus is inoculated at the four edges of the sterile agar block and a sterile cover slip is placed over it. After a few days, the slide can be mounted on a microscope, and the undisturbed mould structures viewed as they are growing. Later, the agar block can be removed and two conventional slide mounts made from the slide and the cover slip. If they are allowed to dry before conventional mounting, the fungal structures are less

likely to disintegrate.

For some pathogens, the spatial relationship of fruiting bodies to host tissues, or the position of conidiophores within a fruiting body, are important taxonomic characters. Thin tissue sections allow these structures to be seen under a microscope. Tissue sections can be cut by hand with a scalpel under a stereomicroscope or by more sophisticated means with a microtome. A freezing microtome is especially useful for cutting thin to semi-thin sections of fresh, frozen tissue. Freezing microtomes are usually equipped with a stage upon which tissue can be frozen, either thermoelectrically or with a low-temperature re-circulating coolant.

Bacteria are often difficult to observe in infected plant tissue. The use of Toluidine Blue O may facilitate bacterial detection. Gram staining is useful to differentiate between two biochemically different bacterial groups, gram-positive and gram-negative (gram-positive cells stain dark purple, while gram-negative cells stain red). More information on this technique can be found in Chapter 7.

Maintaining the optical quality of a microscope necessitates regular maintenance. Each microscope should be serviced every 6–12 months. In humid tropical environments, fungi can grow on the lenses and optical surfaces, affecting the optical qualities of the microscope, and damaging these surfaces permanently if left unchecked. In the tropics, all microscopes should be kept in an airconditioned and dehumidified room.

Keeping the microscope in an airconditioned room will not reduce humidity enough to prevent fungal growth. An alternative to using a commercial dehumidifier is to store the microscope in a dehumidifying cabinet or ‘hot box’ when not in use. A ‘hot box’ is a reasonably airtight, wooden or plastic box, big enough to house the microscope and a 25-W light bulb. The light bulb acts as a dehumidifier by providing enough heat to vaporise the humidity in the box, keeping it, and the contents, dry. In the tropics, camera equipment should also be stored in a ‘hot box’.

4.3 Photography

If possible, take photographs (colour slides or digital images) of the plant disease specimen while collecting in the field. Taking photos in the field will allow you to build up a pictorial reference collection of plant disease symptoms as they occur in nature. Take a number of photographs and select the best ones when they are developed or downloaded (in the case of digital images). It is also advisable to keep a logbook (ie record specimen collection details for each image) to avoid confusion back at the laboratory.

Photographing plant disease specimens in the laboratory allows greater control over environmental conditions, although additional lighting may be required. Unfortunately, artificial light can also create unwanted shadows. Light grey is the best background for photographs; black and white backgrounds can result in the under or over exposure of the subject.

Digital images of plant disease symptoms are extremely useful, because these can

be sent to colleagues quickly, and are not prone to degradation as are negatives and transparencies, which can become covered in fungal growth, particularly in the tropics. Digital images may be sourced directly from a digital camera, a scan of a colour print or transparency, or even a direct scan of the specimen.

4.3.1 File naming and management

Digital cameras automatically give image files unique names (eg IMG_001.jpg, DSCF0001.jpg). These names are often meaningless when browsing files on the computer. Access to image management software (see below) overcomes this problem as does renaming the image files. Descriptive filenames should be used (eg anthracnose on mango.jpg).

If your collection of digital images is large, use numbers as well to help catalogue the images. Do not forget to use leading zeros; for example, 001 rather than 1, because computer software tends to sort 100 before 99. If the image shows the symptoms of an actual herbarium specimen, it may be easiest to use the accession number of the herbarium specimen as the file name for the image.

It is best not to keep too many files in one folder. If there are numerous files, it can take considerable time to find a specific image. Create folders for new projects and subdivide these according to pathogen, host, country or date. One of the best ways to organise images is by using one of the many software packages available for image management. These programs allow you to organise your images, add descriptions, create contact prints and slide shows, and batch-process them so they can be uploaded to the worldwide web. Image management software can be used to:

- acquire images from cameras
- view images
- catalogue images
- edit images
- post thumbnails of the images on the worldwide web.

Some of the image management software currently available are Ablaze Image Manager, ThumbsPlus, Cumulus, Epson's File Factor, Piccolo, PhotoWallet, Polybytes Polyview, Thumber, PIE, ACDSee (PC and Mac), PicaView32, Image Fox, Graphic Workshop, Professional, GraphicConverter, Power Browser, IrfanView32, Photopage, Quisknailer, Compupic, Image Viewer, Photo Recall, Photo Explorer, EXIFRead, VuePrint, ImageAXS, iView Multimedia, Compupic Pro, Sony PictureGear, FlipAlbum, PictureWorks MediaCenter, JASC Media Center, Pictureshow, Qpict Media Organizer and DigiPics.

How to isolate fungi, bacteria and nematodes in the laboratory 5

After returning from a collecting trip, specimens should be sorted into groups. Priority should be given to those specimens that will deteriorate quickly (eg larger fleshy macrofungi), or those from which pathogens need to be isolated. Other samples that will not be adversely affected by leaving them for a time (eg rusts and smuts) can be dried in a plant press, then frozen at –20°C for 7 days, and examined later.

Plant viruses can be temporarily preserved using desiccators described in Section 3.3.1 and shown in Figure 3.3. Plant viruses are not considered further in this chapter, because they are not routinely isolated and purified (virus particles separated from plant cellular components) in diagnostic procedures.

Bacterial and fungal pathogens often have to be isolated and cultured from diseased plant specimens before these can be identified. Pathogens capable of saprobic growth (facultative parasites or necrotrophs) can generally be grown in culture, although some of these are fastidious in their requirements. Isolation of fungi from plant material is usually achieved by placing small portions of tissue onto a suitable agar medium (eg water agar) in sterile Petri dishes. Spores removed directly from fruiting bodies with a fine sterile needle can also be placed on the surface of the agar.

Many saprobic fungi and bacteria grow on or contaminate plant tissue as secondary colonisers of disease lesions. It is therefore important to take simple precautions and use sterile techniques to avoid isolating these contaminants. Surface sterilisation of excised tissue is often necessary to remove saprobic microorganisms, which commonly grow on plant surfaces.

Wipe the working surface with a tissue or cotton wool dipped in 70% ethanol and allow to dry. A smooth, hard non-porous surface such as glass is best for dissecting pieces of plant material. Sterilise forceps and scalpels by dipping them in 95% ethanol and carefully passing them through a flame. The instrument should not be held in the flame, because this will damage it. Alcohol is highly flammable and great care should be taken with flaming instruments near containers of alcohol. Sterilise inoculating loops by heating to redness in a hot flame. The needle must be allowed to cool before use.

Surface sterilisation of diseased plant material removes saprobes and permits the bacterial or fungal pathogen to grow unimpeded when the material is plated onto agar. Ethanol (70%) used as a surface swab or a dip will sterilise most surfaces. It does not need post-treatment washing, because it can be flamed off or left to evaporate.

Sodium hypochlorite is also widely used and effective as a liquid disinfectant. Commercial bleach contains 10–14% available chlorine. It is commonly used at 10% dilution (giving 1–1.4% available chlorine) with immersion times of 1–5 minutes. It

should be stored in the refrigerator, because it loses potency with age. A fresh dilution should be prepared every 2–3 weeks. The disadvantages of hypochlorite solutions are that they have strong odours, leave residues and damage clothing if spilt.

The exact procedure chosen for the isolation of bacterial and fungal pathogens depends on the nature of the host plant material as well as the pathogen itself.

5.1 Isolation of fungi

The following is a basic procedure for the isolation of plant pathogenic fungi from plant tissue.

1. Wash the tissue sample under running tap water to remove surface soil, dust and other contaminants.
2. If the sample is badly overgrown with saprobes, swab with 70% ethanol. This is recommended for surface sterilising diseased woody tissue.
3. Cut out tissue pieces from the leading edge of lesions.
4. Place tissue pieces in 1% sodium hypochlorite in 10% ethanol (this may need to be adjusted depending on the nature of the tissue; eg some leaf tissues are very porous and may absorb enough surface sterilant to kill the pathogen). The immersion time for sterilisation is usually 1–5 minutes.
5. Remove the tissue pieces from the sterilising solution and wash by transferring these briefly to sterile distilled water. The pieces should then be dried on sterile filter paper (under filtered air in a laminar flow, if available) before cutting out small tissue pieces (approx. 2 × 2 mm) and plating them onto tap water agar or potato dextrose agar. Drying is important, because it inhibits the growth of contaminating bacteria. When plating pieces of tissue onto agar, Petri dish lids should be carefully lifted and replaced to avoid entry of airborne contaminants.
6. Isolation plates should be incubated in the inverted position to prevent condensation of water vapour on the agar surface. Most plant pathogenic fungi grow well at 25°C.
7. Pure cultures can be obtained from the primary isolation plates by colonies initiated from single spores or from hyphal tips. Single-spore cultures can be made by preparing a suspension of spores in distilled sterile water and spreading it over tap water agar (or another suitable medium). These plates are then incubated in the dark at room temperature for approximately 24 h. The plates are then examined under a stereomicroscope and single germinated spores are removed on a small amount of agar with a transfer needle to a suitable medium.

These procedures should be modified as experience or literature dictates.

From leaves

Choose leaves with young lesions, because the fungus will be at its most active. Carefully cut out small pieces of tissue from the edges of the lesion using sterile

scissors or a sterile scalpel. Surface sterilisation is usually needed for leaf material (1–3 min in 10% sodium hypochlorite, followed by a rinse in sterile water). Using sterile forceps, place leaf pieces onto the agar surface.

From stems

Where there are deep lesions or internal lesions of the vascular tissue, samples may be taken from internal tissue to avoid the need for surface sterilisation. The sample is split longitudinally from the healthy to diseased area, using a flamed knife or appropriate tool for woody material.

With a sterile scalpel, carefully remove tissues from the leading edge of the lesion on the newly exposed internal surface. Using sterile forceps, transfer 3–5 mm-long slivers of tissue to a Petri dish containing tap water agar (or another suitable agar medium). On thin stems where the lesion is mainly confined to the outer tissues, or in other situations where it is not possible to take internal tissue samples, small pieces of diseased tissue should be excised from the lesion margin using a sterile scalpel, surface sterilised (1–3 min in 10% sodium hypochlorite), washed in sterile water and then placed onto the agar surface.

From roots

Wash small and fine roots to remove excess soil and place roots in a flat-based bowl or a glass Petri dish containing 2–3 cm water so that the diseased root portions are easily visible. Tease out the roots using a scalpel or a pair of forceps. Using a sterile scalpel or scissors, cut out leading edges from lesions on roots (pieces should be approximately 5 mm in length). A light surface sterilisation can be undertaken, but with such delicate material, a prolonged washing procedure may be best. This can be achieved by placing root pieces in a fine sieve under a gentle stream of clean running tap water for 30–90 min. Using a sterile scalpel or a pair of forceps, transfer the pieces to a Petri dish containing tap water agar (or other suitable agar medium), placing tissue pieces into the agar surface.

5.1.1 Moist chambers

Sometimes disease symptoms are apparent, but the causal pathogen may be difficult to isolate. If this is the case, the plant material can be incubated in a moist chamber to induce the formation of fruiting bodies and sporulation. A problem is that growth of saprobes occurring on the plant surface is also encouraged. A brief surface swab with industrial alcohol or 10% sodium hypochlorite may be helpful, but may damage existing surface structures of the pathogen. Alternatively, specimens can be washed in sterile water and dried before incubation.

Moist chambers should be incubated at temperatures below 25°C and are best kept in the light (eg on the laboratory bench), but out of direct sunlight and at a fairly constant temperature to avoid condensation. They need to be checked daily and examined under a low-power stereoscopic microscope for sporulation. Sporing

structures can then be removed with a fine sterile needle for closer microscopic examination or culturing on agar.

Glass or plastic Petri dishes are useful containers for small or flat specimens, such as leaves or twigs. Plastic boxes are best for larger material, and very large specimens can be incubated in large plastic bags or autoclave disposal bags.

Items used to prepare moist chambers should be sterile. Clean, non-sterile containers should be swabbed with 70% alcohol and allowed to dry. Plastic bags should be unused. Add sterile filter papers (autoclaved) to the Petri dishes or boxes and moisten them with sterile distilled water. Sterilised absorbent laboratory tissues are suitable for plastic bags and large plastic boxes. Specimens are best kept above the damp paper on small pieces of plastic grid or similar supports; sterilise these by dipping them in 70% alcohol and carefully flaming them.

5.1.2 Dilution techniques

Fungi may be isolated from soil and from plant surfaces (eg leaves, flowers) by washing and then using a serial dilution to obtain single colonies on a suitable agar medium, for example tap water agar. Nutrient-rich media should be avoided, because these promote excessive vegetative growth at the expense of sporulation. An antibiotic such as streptomycin sulphate should also be added to the agar medium to suppress bacterial growth.

A serial dilution in sterile water can be prepared by washing a small amount of finely ground or sieved soil or plant material, thoroughly mixed in 10 mL of sterile water in a 20 mL McCartney bottle. Use a sterile pipette to transfer 1 mL of this suspension to a tube containing 9 mL of sterile water. Use a fresh sterile pipette to stir this mixture, and transfer 1 mL of the suspension to a second tube containing 9 mL of sterile water.

This process continues until the required number of dilutions has been prepared. Transfer a small amount of the final dilution to a sterile Petri dish containing agar and spread using a sterile glass rod. Incubate the Petri dish until fungal colonies begin to appear. These colonies must be subcultured quickly onto a suitable agar medium before they begin to overgrow one another.

Sometimes there may be only a small amount of sporulating, fungal material available; for example, an ascomycete or pycnidial fungus. In this case a useful method is to suspend a fruiting body in a drop of sterile water on a flamed microscope slide and wait for the spores to be released. Monitor the spore density under a compound microscope and, when adequate, streak a tap water agar plate with the suspension. Transfer single, germinated spores to a suitable growth medium after approximately 24 h.

5.1.3 Growth and sporulation

For most fungal pathogens, spore production is the main method of reproduction and dispersal in their natural habitat. The conditions under which a fungal culture is

incubated will determine how well it sporulates. Most fungi will grow well in the laboratory at room temperature. Sporulation of fungi is essential for their identification and is also required for the production of inoculum for pathogenicity tests.

Unnatural environments, such as warm dark incubators and nutrient-rich agar media, are not ideal conditions for sporulation of most plant pathogenic fungi. Sporulation is often improved by the addition of sterilised host leaf material (eg wheat straw, maize leaves, carnation leaves) to 'weak' media, such as tap water agar.

Many fungi can be induced to sporulate under near ultraviolet light ('black light'). Although black light may affect pigmentation, the gross morphology of the colony and even the spore morphology, these effects are not sufficient to interfere with identification. A black light box can be constructed to induce sporulation in those fungi that require near ultraviolet light (Figure 5.1).

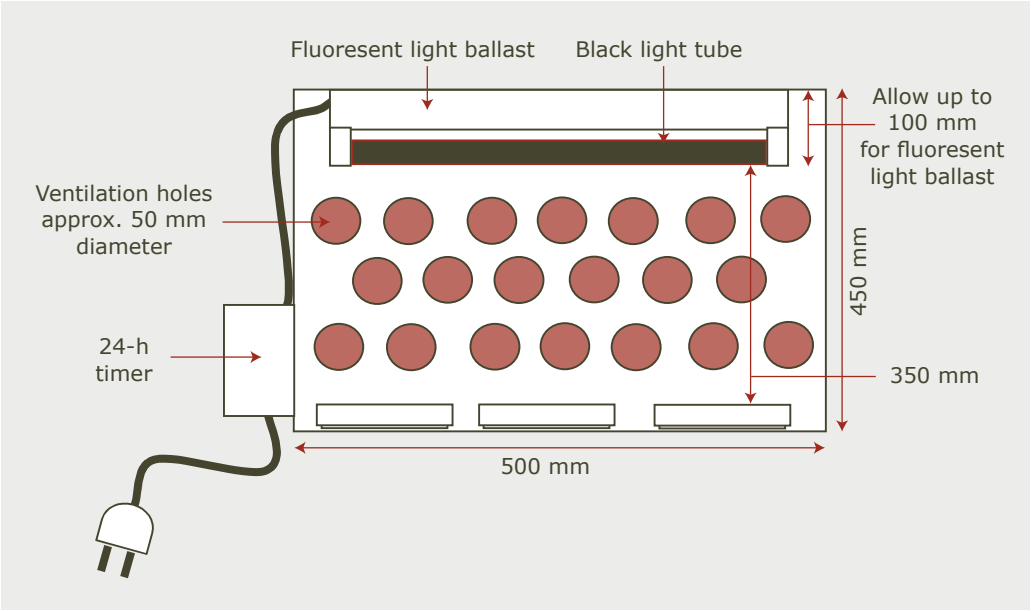


Figure 5.1 Cross-section showing how to construct a 'black light' cabinet

The following points are a guide for using black light to induce fungal sporulation.

- Near ultraviolet light (black light) fluorescent tubes are commercially obtainable in 20 W, 40 W and 80 W, and various lengths. Cool white daylight fluorescent tubes emit a significant amount of near ultraviolet radiation and can be used if black light tubes are unavailable. A combination of tubes (eg one black light tube between two cool white daylight tubes) may also be used. Incandescent lamps should not be used.
- An alternating cycle of 12 h ultraviolet and 12 h darkness can be established with a time switch.

- Some fungi require a period of darkness for sporulation to be initiated; therefore, the box should be light proof.
- Exposure to black light should start 1–4 days after inoculation of the culture, and continue until the end of growth. It is pointless to expose a culture to black light if the fungus has not had time to grow.
- For some fungi, the effect of near ultraviolet light is lost at high temperature.
- Near ultraviolet radiation is effectively transmitted through plastic Petri dishes and by many other colourless plastics.

5.2 Isolation of bacteria

If bacteria are the primary pathogens, bacterial cells can be found in large numbers when material is teased out from the advancing edge of a lesion or discoloured vascular tissue. A basic procedure for the detection and isolation of pathogenic bacteria from plant tissue follows.

1. Wash the tissue sample under running tap water to remove surface soil, dust and other contaminants. If the original plant material has not been handled excessively, surface sterilisation is unnecessary. Sterilisation is undesirable, especially with near-dry or dry material. This is due to the rapid tissue penetration of the sterilising agent, which effectively kills the bacteria. When required, a quick dip into 10% sodium hypochlorite is sufficient to sterilise most material. The sample should be rinsed in sterile water after treatment. When dealing with thicker plant parts, such as buds, fruits, cankers and galls, these can be immersed in 70% ethanol and flamed, prior to isolation.
2. Make clean cuts through small pieces of tissue selected from the boundary between diseased and healthy tissue and mount in a drop of sterile water on a flamed microscope slide. Thicker plant tissues should be teased apart after mounting, prior to microscopic examination. Cover with a gently flamed cover slip and examine immediately under phase contrast microscopy at ×400 magnification. It may be necessary to lower the condenser or close the substage diaphragm to see the bacterial cells. A cloud of bacteria will stream from the cut edge if the lesion is bacterial. Care must be taken not to confuse particulate material, such as latex, plastids or starch granules, with bacteria. If streaming of bacteria from the cut edges of the tissue is seen, the cover slip may be removed and a loopful of the suspension streaked onto suitable agar media. Sometimes it is necessary to leave the plant tissue in water for several hours to allow a sufficient quantity of bacterial cells to ooze from the tissue, before streaking suspensions on isolation media.
3. Streaking should be done with a platinum loop made from 24 standard weight guage platinum wire, by rubbing a loopful of the suspension onto the surface of an agar plate. There are several ways this can be done. One is to make a zigzag pattern on a quadrant of the plate starting at the circumference. Further quadrants should be progressively streaked each overlapping the last at the circumference (Figure 5.2). The loop should be flamed and then cooled by touching the agar surface between

streaks. A second method is to streak a loopful of the suspension over the whole surface of a plate, moving the loop slowly down from top to bottom, while simultaneously moving it rapidly across the plate from side to side. The object of these methods is to produce well-separated individual colonies.

4. The medium used must be suitable for growth of the suspect pathogen. Plates of isolation media should be dried after pouring, either by inverting the lid and propping the base on the edge of the inverted lid for 24 h in a clean incubator, or by removing the lids in a laminar flow cabinet for 30 min. Care must be taken to avoid air contamination of the agar.
5. Isolation plates should be incubated in the inverted position to prevent condensation of water vapour on the agar surface. Most plant pathogenic bacteria grow well at 25–28°C.

These procedures should be modifies as experience or literature dictates.

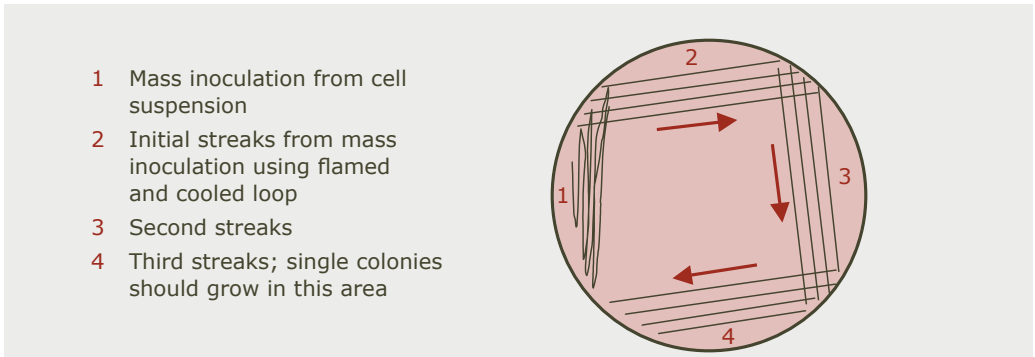


Figure 5.2 Bacterial isolation streak plate

After streaking, the plates are incubated at room temperature and examined daily for 4–5 days, or up to 10–14 days if a slower-growing pathogen is suspected. If the diagnosis based on the symptoms is borne in mind, the expected pathogen can often be recognised and subcultured, even if various other bacteria are present on the plates. With a good specimen of diseased plant and well-chosen pieces of tissue, the pathogen is often the only organism recovered. If more than one type of colony grows, the relative abundance of each should be noted. The most frequently isolated organism, especially if recovered from different sites, is worthy of examination unless readily recognised as a saprophyte. Subculturing must only be performed with well-separated colonies, preferably onto slopes of the same medium.

Another method of bacterial isolation is similar to that used for fungi. Pieces of infected tissue are placed directly onto the agar medium and bacterial growth occurs around these. If saprobes are present, microbial growth is liable to consist largely of the saprobes, and the slower growing pathogens are lost. For this reason, the method should be used only when the dilution method is deemed unsuitable.

5.3 Isolation of nematodes

Nematodes (unsegmented roundworms) are a diverse group of mostly microscopic animals. They can be found in virtually all situations in which free water occurs, even if only intermittently. Nematodes derive their nutrition from a range of sources, with many species being highly specialised parasites. Most plants and animals are hosts for one or more parasitic nematode species. Consequently, many parasitic nematodes are important economic pests. In contrast, species that feed on bacteria and fungi, regarded as free-living, have significant ecological functions. For example, free-living nematodes play an essential role in nutrient cycling in soil ecosystems.

Plant parasitic nematodes generally fall into two important groups: those that remain motile and feed on or within roots (migratory nematodes); and those that become sedentary, inducing the plant to produce specific feeding and protective structures (sedentary nematodes). Migratory species often have a wide host range and, except for a few genera, cause economic impact only when population densities are high. Sedentary species are highly specialised parasites that manipulate host plants at the molecular level, with significant impact on yield. However, they often affect a narrower range of hosts than migratory species.

To identify plant parasitic nematodes, it is necessary to extract and preserve specimens from soil or plant samples.

5.3.1 Soil samples

For diagnostic and collection purposes, field soil is collected from the active root zone of plants suspected of being infested. If investigating a patchy disorder of a crop, it is advisable to collect near the margins of patches where nematode population densities are likely to be highest, and to also collect from unaffected areas for comparative purposes. The depth of sampling and the number of subsamples can be important, but are beyond the scope of this section.

It is best to sample moist soil because dormant nematodes in dry soil are readily damaged. Samples are placed in plastic bags and must be kept cool during transport and processed as soon as possible. Refrigerated storage before processing is acceptable for some, but not all species. Friable samples can be sifted (10 mm sieve) to remove debris and thoroughly mixed, but this is not always feasible for heavy soils.

Methods for extracting nematodes are either active, relying on nematode motility, or passive, separating by size and density. The simplest method is extraction of active nematodes using a Whitehead tray or Baermann funnel. Although simple, extraction takes 2–4 days and may give poor recovery of slow-moving or large nematodes. Passive methods involve sieving, decanting and flotation in a variety of combinations with a consequent range of bias and effectiveness. Wet sieving and differential centrifugation is described below. Cysts of heteroderid species can also be recovered from soil by wet sieving.

Whitehead tray extraction

A sample of soil is spread on tissue or finely woven cloth on a coarse mesh, supported above the base of a tray to which water is added to just saturate the soil (Figure 5.3). Over a period of 1–4 days, active nematodes will move downwards and through the tissue into the water. The volume of soil used will depend on the size of the tray; large trays (450 × 300 mm) can be used to spread 200 g of soil in a thin layer. A thin layer of soil improves extraction efficiency and reduces extraction time.

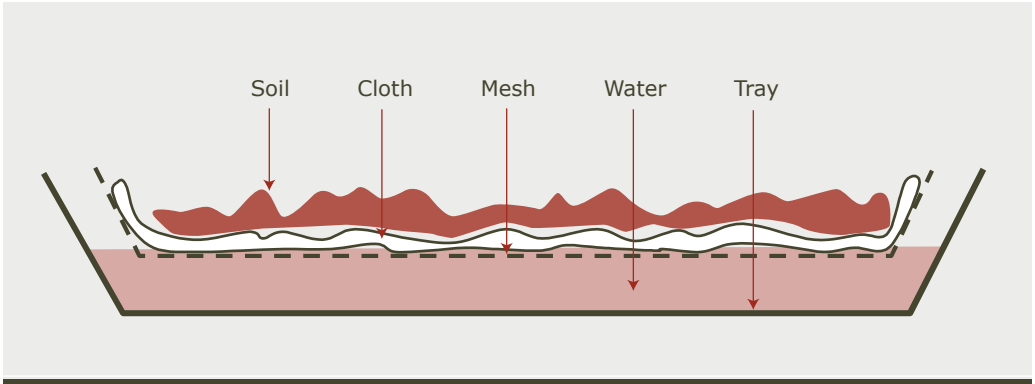


Figure 5.3 Whitehead tray for extraction of nematodes from soil and plants

Before collecting the nematodes, carefully remove the mesh support taking care to minimise contamination of the water with soil. The water is transferred to a suitable container (eg a measuring cylinder) in which the nematodes will settle over several hours. Water is best removed by aspiration leaving only 5–20 mL, and taking great care not to resuspend the nematodes. Alternatively, the nematodes can be recovered by passing the water though a fine sieve (20–38 μm). Finer sieves are expensive and may be difficult to obtain. Nematodes can be lost through sieves, but losses can be overcome by catching and resieving the filtrate through several cycles.

Baermann funnel

Soil is placed on tissue or finely woven cloth on a mesh support in a funnel with a clamped plastic tube attached to the neck, and the apparatus is filled with water to saturate the soil (Figure 5.4). Only relatively small volumes of soil can be processed, but the method has the advantage that the nematodes accumulate in the tubing above the clamp and are readily collected by directly releasing a small volume of water (5–10 mL) into a vial.

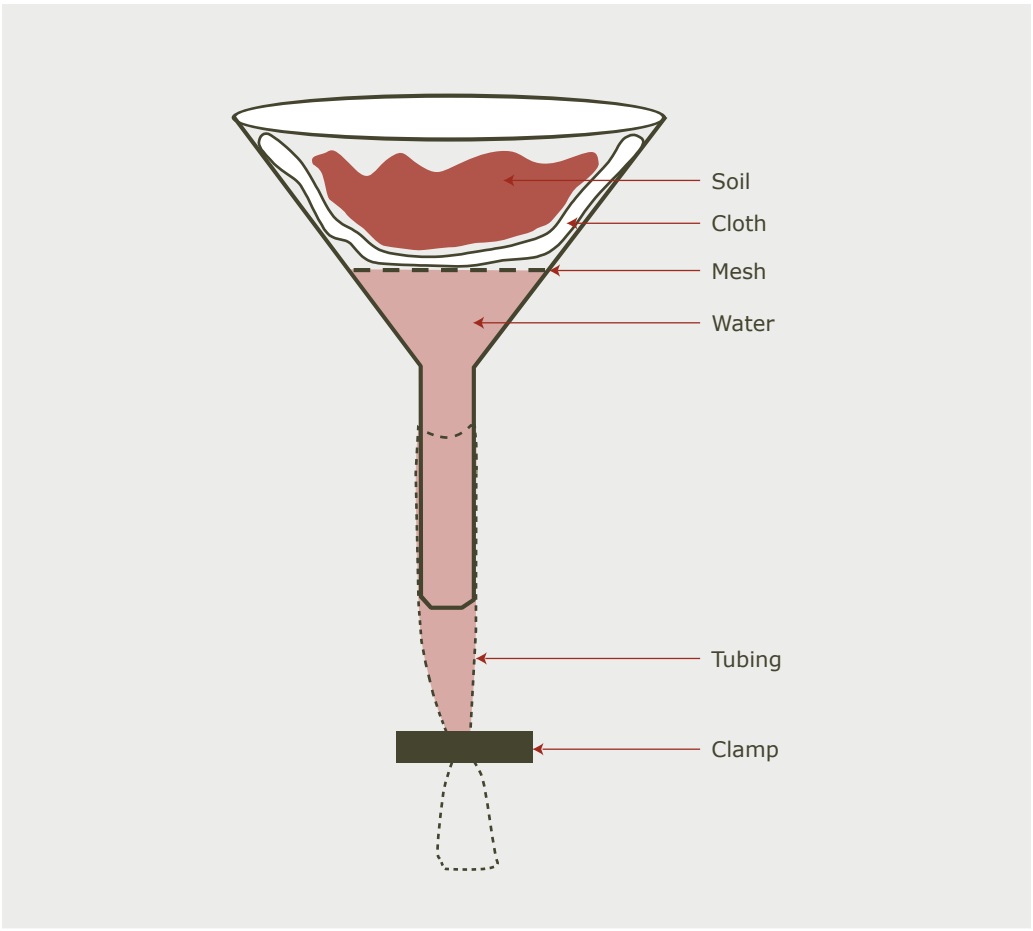


Figure 5.4 Baermann funnel extraction of nematodes from soil and plants

Wet sieving and differential centrifugation

Soil is suspended with running water in a 4 L bowl, avoiding overflow. The larger soil particles are allowed to settle for 10 s, the water is passed through a 38 μm sieve, and the filtrate is collected in a second bowl. Any captured nematodes are washed from the sieve into a centrifuge tube in a very small volume of water. Because some nematodes can pass through the sieve, the filtrate is resieved to recover those that have escaped. This step can be repeated a third time. To improve overall recovery, the whole process, starting with washing the soil, is repeated twice more. The nematodes are then spun down in a centrifuge and the supernatant carefully decanted leaving a small volume of water. The nematodes are then suspended in a dense sugar solution (450 g/L) and again centrifuged. Floating organic matter is removed from the surface and the sugar solution, containing suspended nematodes, is washed through the 38 μm sieve and the nematodes collected. The filtrate can be saved and resieved twice to ensure maximum recovery.

The concentrated sugar solution will desiccate and damage nematodes, so exposure time should be kept to a minimum. Other compounds can be used to give a solution of suitable density, such as NaCl, $MgSO_4$ or colloidal silica (Ludox, DuPont de Nemours).

Extracting cysts

Air-dried soil is suspended with running water in a deep beaker (4 L), avoiding overflow. The soil is allowed to settle for 10 s, and is passed through a coarse sieve (710 μm) over a 250 μm sieve. The soil is washed and sieved twice more. The organic material on the fine sieve is then deposited on filter paper in a Buchner funnel under vacuum before cysts are sought under a dissecting microscope. If a conical funnel is used and the organic matter suspended in 70% ethanol, the cysts will be deposited in a band towards the top of the filter paper.

5.3.2 Plant samples

Nematodes are found in roots, stems, leaves and seeds of plants, so extraction methods will vary with the type of sample and nematode species. Migratory endoparasites are best extracted with a mister, although Whitehead trays or Baermann funnels can be used. Sedentary nematodes may need to be dissected from infested tissue or preserved in situ. The eggs of some sedentary nematodes can be extracted by washing in bleach and sieving; however, eggs are of limited taxonomic value so the method is not described here. The motile juveniles and vermiform adult males of sedentary nematodes can be extracted by misting.

Mist extraction

A misting cabinet contains funnels in which plant tissue (cut into 10 mm lengths if appropriate) is placed in a mesh basket and mist irrigated for approximately 10 s every 10 mins (Figure 5.5). The water is collected in a tube that fills to overflowing, but the flow rate is sufficiently slow that the nematodes settle in the tube and can be collected after 2–4 days. The volume of water is reduced in the tube by careful aspiration. Misting keeps the water well oxygenated and removes toxins produced by decaying plant tissue, so the nematodes remain in good condition. Whitehead trays and Baermann funnels can be used, but have limited use for plant material compared with soil.

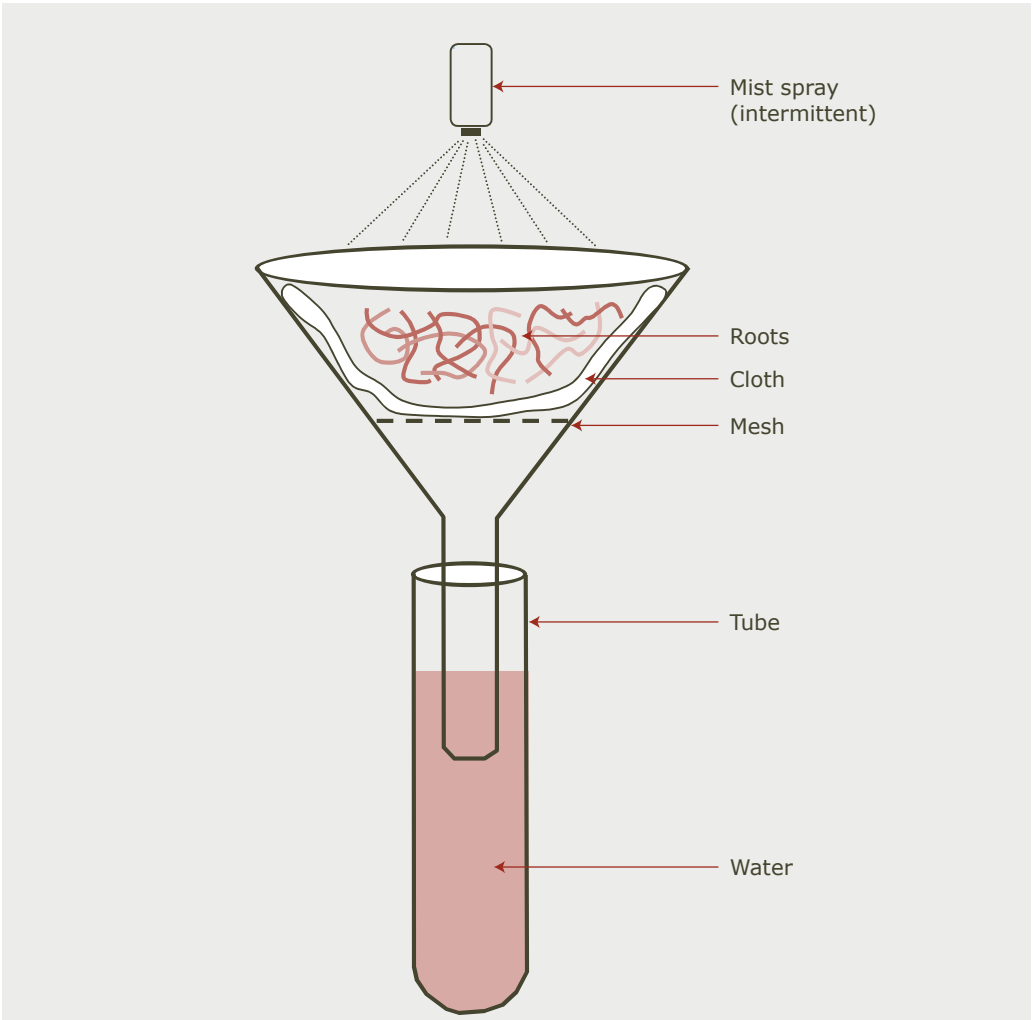


Figure 5.5 Mist extraction of nematodes from plant tissue

Dissection

Plant tissue can be teased apart with needles or fine-pointed scalpels in a Petri dish containing water, under a suitable dissecting microscope. A combination of transmitted and incident illumination is best. Nematodes released from the tissue are readily seen with oblique, below-stage illumination. Leaving the sample to stand for a while allows more nematodes to escape from the dissected tissue. Incident illumination may be helpful for the swollen stages of sedentary nematode, such as cyst (*Heterodera* spp), root-knot (*Meloidogyne* spp) and seed and leaf gall (*Anguina* spp) nematodes.

Plant tissue can be cleared in bleach and stained in various methods to observe nematodes in situ. Although this can be useful for diagnostic purposes, it will not yield specimens suitable for inclusion in collections.

5.3.3 Preservation and mounting

Heat killing

Before fixing nematode specimens with a preservative, it is important that they are killed to maintain structural integrity. This is done by short exposure to a temperature of about 60°C, either in water or fixative. Hot water or fixative can be added to raise the temperature to 60°C, followed by rapid cooling with cold water or fixative. If a water bath is unavailable, to maintain water or fixative at 60°C, boiling water can be added in equal volume to the water containing the nematodes. Alternatively, an oven (55–60°C) can be used to heat the nematodes in shallow water, and observations should be made at 1 min intervals until they are killed. During heat killing, it is important not to overheat or boil the nematodes, because this will disrupt their internal structures.

Fixing

The simplest method for fixing is to add concentrated formaldehyde to give a final concentration of 2–5%, or to heat kill with hot formaldehyde solution at double strength. Specimens should be left in this fixative for at least 12 h, but ideally for 2 weeks, before further processing. Long-term storage in formaldehyde solution is acceptable.

Other fixatives commonly used are TAF (3% formaldehyde, 0.2% triethanolamine in distilled water) and FA4:1 (4% formaldehyde, 0.1% acetic acid in distilled water); however, these are not suitable for long-term storage. Ethanol cannot be used as a fixative for morphological studies, but is used for preserving nematodes for deoxyribonucleic acid (DNA) analysis.

Mounting

For permanent mounts, transfer specimens to glycerol by a slow evaporation method. Place specimens in a few mL of 2–5% glycerol in distilled water in a shallow glass dish. Evaporate the water over a period of 1 week in an oven at approximately 40°C, or in a desiccator. place a loose lid on the dish to slow the rate of evaporation.

To transfer specimens of interest to a microscope slide, pick out individual nematodes under a dissecting microscope. This may be done with a fine needle, sharpened feather, or an eyelash glued to a needle. Tease the nematode gradually to the surface and lift it through the surface tension by a final, sharp movement of the needle. Do the operation in a shallow dish and refocus the microscope regularly. Practice is needed to become skilled at this process, but it can be readily mastered for efficient recovery of individual nematodes.

Transfer approximately 10 nematodes of representative stages to a drop of anhydrous glycerol on a clean glass slide. Use the needle to centre the specimens and ensure that they are not floating. To prevent the nematodes being squashed, arrange several pieces of glass fibre, broken cover slip (No. 0) or wax shavings around the specimens. Lower

a cover slip (circular preferably) onto the drop, avoiding glycerol flowing beyond the cover slip. If wax shavings or a ring of wax (applied molten and allowed to set) are used, the slide needs to be placed on a warm hotplate to just melt the wax, which then seals the cover slip. Glyceel was routinely used for this process, but is no longer readily available, so alternatives such as a two-part epoxy resin (eg 5 min Araldite) or DePeX (various scientific suppliers) can be used. Although nail vanish may be used, it is not durable in warmer climates.

5.4 Growth media

Most growth media can be made from basic ingredients or obtained commercially. Commercial sources have a more consistent quality than those prepared from basic ingredients.

Bacteria are suppressed by adding antibiotics to the agar. As most antibiotics are denatured by heat, they are usually added to sterilised molten agar (just above the setting temperature).

Penicillin (50 units/mL), streptomycin (50 units/mL) and tetracycline (30 units/mL) may be used alone or in combination. Chloramphenicol (50 mg/L) can be autoclaved and is added during preparation.

The amount of agar added to media in the following recipes may differ depending on the brand of agar. Enough agar should be added to produce a gel that is firm enough to streak. If the local tap water is known to contain toxic impurities it should be replaced with distilled water.

Potato dextrose agar (PDA) is a good medium for growth and isolation of fungi.

Ingredients for potato dextrose agar	
Potatoes	200 g
Dextrose (glucose)	20 g
Agar	20 g
Tap water	1 L

Clean the potatoes (but do not peel). Cut them into pieces and boil for approximately 1 h, and then strain the potatoes and liquid through a sieve (or gauze) and dispose of coarse fragments. Alternatively, blend the entire mix with an electric blender. Dissolve the dextrose (glucose) and agar, and make up to 1 L with tap water. Autoclave at 121°C for 20 min.

This recipe can be modified using other vegetables and cereals. Half- or quarter-strength PDA can be made by reducing the amount of potatoes and dextrose proportionally.

Tap water agar (TWA) is a good medium for the isolation of many fungi. Sterilised wheat straw, cereal seeds or other plant material can be placed onto the medium to induce many fungi to sporulate.

Ingredients for TWA	
Agar	20 g
Tap water	1 L

Mix the agar in water and autoclave at 121°C for 20 min.

V-8 juice agar (V-8J) is a good medium for the growth of fungi, particularly *Phytophthora*.

Ingredients for V-8J	
V-8 juice, clarified	200 mL
$CaCO_3$	3 g
Agar	20 g
Distilled water	800 mL

Clarify V-8 juice by straining. Mix ingredients and sterilise by autoclaving at 121°C for 20 min. If V-8 juice is not available, it can be substituted with a blend of tomato juice, carrots and celery.

King's B agar is a good medium for general isolation of plant pathogenic bacteria, particularly fluorescent pseudomonads and *Erwinia* spp.

Ingredients for King's B agar	
Proteose peptone	20 g
Glycerol	10 g
K_2HPO_4 (anhydrous)	1.5 g
$MgSO_4.7H_2O$	1.5 g
Agar	15 g
Distilled water	1 L

Adjust the pH to 7.2 and sterilise by autoclaving at 121°C for 25 min.

Nutrient agar is a good general-purpose medium for bacteria.

Ingredients for nutrient agar	
Yeast extract	3 g
Peptone	5 g
NaCl	5 g
Agar	15 g
Distilled water	1 L

Adjust the pH to 7.4 and sterilise by autoclaving at 121°C for 15 min.

Sucrose peptone agar is a good medium for growth and isolation of many plant pathogenic bacteria.

Ingredients for sucrose peptone agar	
Sucrose	20 g
Peptone	5 g
K_2HPO_4 (anhydrous)	0.5 g
$MgSO_4.7H_2O$	0.25 g
Agar	12 g
Distilled water	1 L

Adjust to pH 7.2–7.4 and sterilise by autoclaving at 121°C for 15 min.

Malt extract agar is a good medium for growth and isolation of many fungi and yeasts.

Ingredients for malt extract agar	
Malt extract	2–20 g
Agar	20 g
Distilled water	1 L

The amount of malt extract can be varied. Autoclave at 121°C for 20 min.

Glycerol agar (GA) is used to preserve dried agar cultures as herbarium specimens.

Ingredients for GA	
Glycerol	25 mL
Agar	20 g
Tap water	1 L

Autoclave at 121°C for 20 min. Agar cultures that have partially dried are floated culture-side upwards onto GA that has been poured into the inverted lid of a Petri dish. These plates are then dried for 2–3 days. Dried GA plates are more pliable and rubbery than most other dried agar media.

How to preserve and store specimens 6

6.1 Herbarium specimens

A herbarium specimen is typically a piece of dried plant or fungal material in a labelled packet. The specimen may also comprise dried cultures, microscope slides, photographs and illustrations. The specimen packet may also contain annotation slips, correspondence and other relevant notes.

6.1.1 Dried specimens

Plant and fungal material is pressed and dried by placing it between sheets of blotting paper or newspaper. The blotting paper or newspaper containing the specimen is then placed between two pieces of corrugated cardboard. Wooden boards (or other stiff material) are put on each side of the entire stack and straps are used to apply pressure, thus pressing the plants. The plants dry but they should not shrivel, and high-quality specimens can be obtained in a few days. To speed the drying process (a necessity in humid areas, such as the tropics), a source of heat or forced air may be necessary. The blotting paper or newspaper will need to be changed regularly.

Plants should stay in the press until they are completely dry. The press can be placed in the sun, in an airconditioned room or near a heat source, to accelerate drying. The time necessary will vary depending on the type of plant material and the humidity. Removing plants too soon from the press will result in wrinkling or the growth of superficial fungi on the specimen. Most plants will be dry after 5–10 days. Before specimens are deposited in the herbarium, they should be fumigated or put through the freezer to prevent the introduction of mites and insects.

The easiest way to preserve the larger members of the Agaricales and the larger fleshy Ascomycetes is to dry them rapidly. Drying will affect colour, shape and size, although dry specimens are much easier to manage and maintain than specimens preserved in liquid. Macrofungi can be freeze-dried, which will preserve their colour and shape, but they become brittle and must be handled carefully. As the macrofungi often do not fit into herbarium packets and are quite easily damaged, small cardboard boxes or zip-lock plastic bags with self-indicating silica gel are necessary for long-term storage.

Dried herbarium specimens and dried cultures should be stored in acid-free (archival quality) paper or card packets of a standard size (approximately 10–15 cm wide × 10 cm long). The specimens may be first inserted into packets made from comparatively thinner, acid-free paper. Ideally all of the annotation slips, labels, glues, inks and plastics used in the herbarium should be acid-free to prevent damage to the specimens. There are pH test pens available that can be used to determine if paper is acid-free.

Each packet should be labelled on the front (Figure 6.1) with the:

- herbarium accession number
- scientific name of the pathogen
- substrate or scientific name of the host of origin
- locality of collection, including country, province, latitude and longitude
- collector's name and number
- date of collection
- name of the person who identified the specimen
- reference to any published papers in which the particular specimen was cited.

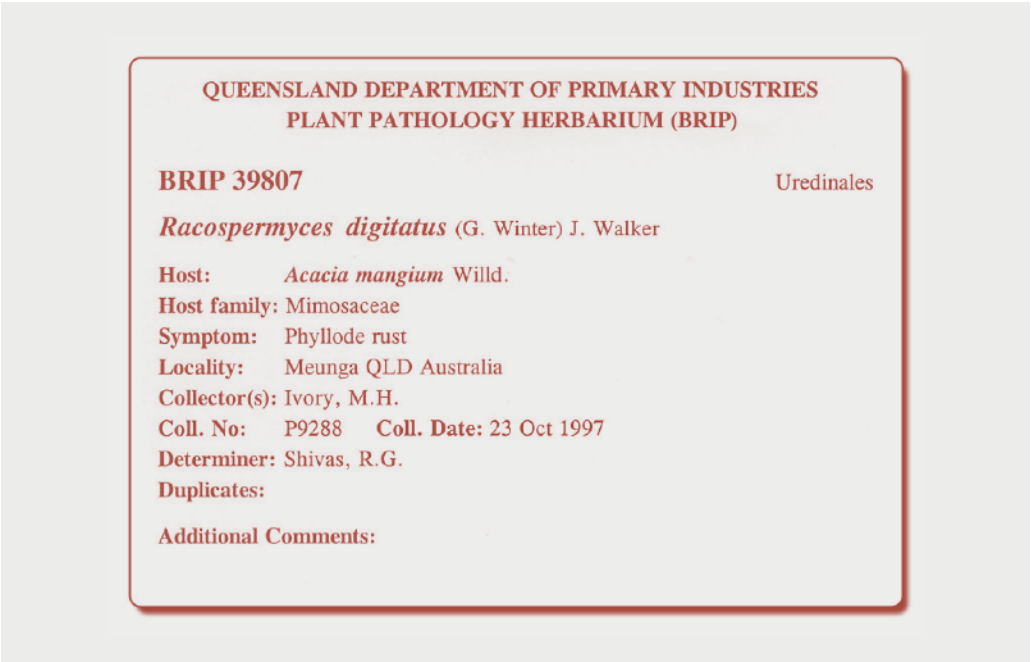


Figure 6.1 Example of a packet label used at Queensland Department of Primary Industries and Fisheries Plant Pathology Herbarium (BRIP)

Each specimen in the herbarium is assigned a unique accession number that distinguishes it from all other specimens in the collection. This unique number is found on the herbarium specimen packet label (Figure 6.1). The combination of the accession number and the institution code from *Index Herbariorum* allows any specimen to be identified uniquely from any other specimen in the world. If a specimen is found to yield more than one taxon, a suffix (a, b, c, etc) is placed after the accession number to represent each particular taxon. The same accession number is used in the herbarium for dried plant material and cultures derived from it.

If it is necessary to divide the specimen into a number of packets, duplicate packets should be prepared. Each duplicate packet should bear the same information and herbarium accession number as the packet from which it is taken. Duplicate packets may be deposited or exchanged for specimens in other herbaria.

The majority of herbarium specimens in a plant pathology collection are fungi. The physical arrangement of these specimens is mostly in groupings above the rank of order in a manner that reflects evolutionary ancestry (phylogeny). Most specimens fall into either the Ascomycotina or the Basidiomycotina, with further arrangement within these groups alphabetically by fungal genus. Some large groups of plant pathogens are often kept separately; for example, rusts (Uredinales), smuts (Ustilaginomycetes) and powdery mildews (Erysiphales), with further arrangement within these groups alphabetically by host genus (because many species of rust, smut or powdery mildew are host-specific).

Anamorphic fungi (Deuteromycetes, *Fungi Imperfecti*, asexual fungi, conidial fungi) are best placed under their teleomorphic names, if known. Furthermore, some fungi may have two or more names of anamorphs (synanamorphs) belonging to the same teleomorph. The specimen should still be housed under the teleomorphic name, even if that state of the fungus is not present in the specimen. In this case, the absence of the teleomorphic stage should be noted on the specimen label.

Specimens that are too large to locate in their correct (phylogenetic) position in the herbarium should be cross-referenced with a card (or empty herbarium packet) that clearly indicates where the actual specimen can be found. Similarly specimens that are on loan should be cross-referenced with a card located in the position that the specimen would normally occupy.

6.1.2 Type specimens

A type specimen is a single specimen (or illustration) on which a scientific name is based. When a new fungus is described and named, a herbarium specimen must be designated as a fixed reference for the name. Type specimens of fungi must be permanently preserved and dried. Living cultures cannot be designated as type specimens; however, cultures of fungi preserved in a metabolically inactive state by freeze-drying or deep-freezing can be type specimens.

Type material is traditionally placed in red packets or packets bordered or marked in red, to make them easily recognisable in the herbarium collection. Types include the following:

- Holotype — the one specimen or illustration designated by the author as the type for the new name.
- Isotype — any specimen that is a duplicate of the holotype.
- Lectotype — a specimen or illustration designated from the original material as the type if no holotype was indicated at the time of publication, or if it is missing, or if it is found to belong to more than one taxon.

- Isolectotype — any specimen that is a duplicate of a lectotype.
- Syntype — any specimen cited in the original place of publication when no holotype was designated, or any one of two or more specimens simultaneously designated as types.
- Paratype — any specimen cited in the original place of publication that is neither the holotype nor an isotype, nor one of the syntypes.
- Neotype — a specimen or illustration selected to serve as a nomenclatural type if all of the material on which the name of the taxon was based has been lost or destroyed.
- Epitype — a specimen or illustration selected to serve as an interpretive type when the holotype, lectotype or previously designated neotype, or all original material associated with a validly published name is demonstrably ambiguous and cannot be critically identified for purposes of the precise application of the name of a taxon.
- Topotype — any specimen collected at the same location as the holotype.

6.1.3 Dried cultures

The morphological characteristics of fungal cultures are important in mycological studies. Dried cultures are the only way that permanent specimens of isolates from soil, water or animal hosts can be retained. Dried fungal cultures are also useful for supplementing dried host material on which sporulation may be sparse. Fungal cultures can be dried and stored with specimens in herbarium packets.

Dried cultures are prepared by growing the fungus on a suitable agar medium in a Petri dish until the desired stage is attained. The culture is then dried in the Petri dish until the colony is loose in the dish. Cultures in Petri dishes can be dried in a laminar flow for 2–3 days. After drying, the culture can be removed from the Petri dish and placed in a herbarium packet. Cultures with delicate fungal structures can be stored in thin Petri plates.

Cultures dried as described may curl, crack and be impossible to remove without damage. This outcome can be avoided by removing the partially dried culture from the plate and floating it on hot glycerol agar (GA) (see Chapter 5) that has been poured in the lid of a Petri dish. The same lid that has covered the specimen during its growth can be used, because it will bear the culture details. The culture is then left uncovered to dry completely. The result is a dry, pliable disc that can be peeled off and placed in a herbarium packet.

Drying may not kill the fungus. Killing the culture is often necessary as some fungi produce large numbers of airborne conidia that can contaminate other cultures in the laboratory. Placing these in a desiccator containing formalin will kill the cultures. Alternatively, cultures can be killed by inverting the Petri dish containing the culture and placing approximately 1.5 mL of formalin in the lid and leaving it in a fumehood for 1–3 days.

6.1.4 Microscope slides

The edges of microscope cover slips on lactic acid mounts can be sealed with two or three coats of nail varnish. This should prevent the slide from drying out for several years. Alternatively, a solution of polyvinyl alcohol (in powder form) in lactic acid can be used as a mountant. This gradually sets to make a permanent mount.

Dehydration procedures can be used to produce permanent mounts. This requires the immersion of the plant or fungal specimen in a series of progressively stronger ethanol solutions (eg 40%, 50%, 60%, 70%, 80%, 90% and absolute ethanol) for a few minutes each so that all water is removed. The specimen can then be cleared in xylene and mounted in a medium, such as Canada balsam.

Ideally, microscope slides should not be kept in herbarium packets. Instead, they should be kept in special purpose microscope slide drawers. Each slide should be cross-referenced with its herbarium specimen accession number.

6.1.5 Packing and transport

All plant disease specimens to be lodged in the herbarium should be filed in acid-free paper or card packets (Figure 6.2). Very large specimens are best kept in cardboard boxes to avoid damage. Each item inside the herbarium packet (eg collection slip, annotations, drawings, microscopic slides) should be clearly labelled with the accession number of specimen. Dried cultures should be placed on filter paper in transparent paper packets and kept with the dried plant material. Microscope slides are best stored inside cardboard or plastic slide cases to protect them from being broken.

Figure 6.2 Herbarium packets used by Herbarium Ustilaginales Vánky and Queensland Department of Primary Industries and Fisheries Plant Pathology Herbarium (BRIP)

Herbarium specimens should remain inside their paper or card packets when transported and bundled together, wrapped in newspaper or bubble wrap, and enclosed in a larger cardboard box for protection. Freeze-dried living cultures, whether stored in glass ampoules or vials, should be wrapped in cotton wool and placed inside cylindrical cardboard mailing packages.

6.2 Cultures

The primary objective of keeping a culture of a fungus or bacterium is to maintain it in a viable state without morphological, physiological or genetic change for as long a period as required. Cultures need to be kept viable at least during study and often indefinitely, especially if the culture is derived from type material or has been listed in a publication. Without identified cultures, particularly of bacteria, it is impossible to perform comparative taxonomy in order to classify and name new taxa.

The Convention of Biological Diversity, which is signed by 180 countries, affects microbial cultures collected since 1995. The convention gives sovereign rights to the country of origin and aims at fair and equitable benefit sharing, especially with regard to the country of origin in the case of successful economic exploitation of the culture. The exchange of cultures between collections often requires a Material Transfer Agreement. The implications of these agreements for culture collections and clients are unclear, and a workable situation for many collections has yet to be developed.

Not all of the following preservation methods are suitable for all species of fungi and bacteria that grow on culture media. Trial and error is often required to find the best long-term storage method.

6.2.1 Growth on agar

Cultures are usually grown in 20 mL McCartney bottles, with a wide neck to ensure more manageable subculturing. A suitable medium, for example, half-strength potato dextrose agar (PDA) for fungi or nutrient agar for bacteria, should be used. Some fungi and bacteria will require special media. The medium is poured into the bottle to approximately half-depth and sterilised, and the bottles are cooled at an angle so that the agar solidifies to form a slope.

Slopes are inoculated with the fungus or bacterium and, after incubation, must be stored in a cool, dust-free environment. A refrigerator or a cold room at 5–8°C is most suitable. Some fungi and bacteria are sensitive to the cold and should be stored at 15°C. The cultures must be monitored regularly, because agar can dry out rapidly in low-humidity environments. When storing fungi, be aware of potential mite infestation. All bottles should be sealed with plastic film or cling wrap before incubation and during storage. Cultures will need to be transferred to fresh media every six months. Vigilance is necessary to avoid losing valuable cultures, although frequent transfer can accelerate morphological change, loss of pathogenicity and decreased sporulation.

6.2.2 Under mineral oil

This method is particularly useful in the tropics, because it prevents cultures from drying out and protects against mite infestation. Cultures are grown as above, but on a short agar slope. It is important to ensure the culture is healthy, and in the case of fungi, sporing abundantly. The culture is covered with mineral oil to a depth of 1 cm above the top of the short agar slope. The oil must be sterile, which can be achieved by autoclaving twice at 121°C for 15 min.

Oil is best added in individual doses to avoid cross-contamination. Plastic capped McCartney bottles can be used, but are likely to leak if the bottle falls over. The technique is simple and does not require expensive apparatus or chemicals. Depending on the species, survival can be from 2–40 years, although a renewal rate of every 5 years is ideal.

6.2.3 Water storage

Agar blocks are excised from the edges of young (1 week) fungal cultures and submerged in sterile distilled water in 5 mL Wheaton vials or 20 mL McCartney bottles. The caps are screwed down and sealed with film or cling wrap. The vials are then stored at room temperature. Good retrieval rates for several years have been obtained. This storage method is particularly suitable for *Pythium* spp and *Phytophthora* spp. Many bacteria can also be stored using a similar method. A sterile inoculating loop is used to transfer 24–48 hour-old bacterial colonies into sterile distilled water.

6.2.4 Freeze-drying

Freeze-drying involves the removal of water (drying) under reduced pressure from cultures in a frozen state by a process called sublimation. Sublimation occurs when a frozen liquid goes directly to the gaseous state without passing through the liquid phase.

The freeze-dried cultures are sealed and stored in glass ampoules or vials. Freeze-drying best suits vigorously sporing fungi. Bacteria also store well using this method. Viability can be maintained for 10 years or more. The freeze-drying apparatus is relatively expensive to buy and maintain. Experienced staff are required to operate the system and prepare the cultures for storage. One of the main advantages of freeze-drying is that the freeze-dried cultures do not need refrigeration and can be stored at room temperature.

After freeze-drying, one ampoule from each isolate in the run should be opened and grown to check for viability and purity, as not all species survive the process. Placing a piece of the freeze-dried culture into a freshly poured agar plate can revive many freeze-dried fungi. Freeze-dried bacteria and yeasts need time (30 min is usually adequate) to rehydrate, either in a broth or distilled water, before streaking.

6.2.5 Soil storage

Soil is sieved and dry-heat sterilised (or double autoclaved at 121°C for 15 min) in 20 mL McCartney bottles approximately half-filled. A spore suspension in sterile distilled water is poured onto the sterile soil and incubated at 20–25°C for approximately 5–10 days. The cultures should then be stored in a refrigerator. These cultures remain viable for several years. Revival is achieved by aseptically removing some of the soil to a suitable agar medium. This method can be used to store *Fusarium* spp, which often mutate if maintained on agar for long periods of time.

6.2.6 Silica gel storage

Pour a 5% (weight/volume) skimmed milk spore or bacteria cell suspension onto sterilised cooled silica gel. Use 6–22 mesh, non-indicating, purified, silica gel, in a small screw cap glass bottle half-filled and sterilised in an oven. Allow the gel to dry at room temperature until the crystals separate after approximately 14 days. Screw the caps down and store the bottles in a refrigerator over indicator silica gel at 4–6°C. Scatter a few crystals onto a suitable agar medium when required. Survival may be maintained up to 11 years, depending on the species. This method is suitable for organisms that survive freeze-drying, including mycelial forms that produce sclerotia or chlamydospores.

6.2.7 Filter paper storage

Place sterile filter paper into a Petri dish and add a few drops (enough to wet the filter paper) of sterile distilled water. Place agar plugs on the filter paper and store the Petri dishes in an incubator at 25°C until the fungus has colonised the entire filter paper. Once the filter papers are completely dry (approximately 2–4 weeks), cut them into pieces aseptically and place them in individual sterile, air-tight, screw-top vials, and store at 4°C. To revive the cultures, transfer one or two pieces of filter paper aseptically onto potato dextrose agar (PDA). Hyphal growth from the pieces of filter paper should be apparent within 2–4 days. This storage method is often used for *Fusarium* spp.

6.2.8 Cryopreservation

The storage of microorganisms in a low-temperature freezer at temperatures from –20°C to –85°C (cryopreservation) is a good preservation method for most fungi, bacteria and viruses. One of the simplest and most popular storage systems for fungi and bacteria involves the use of porous ceramic beads (cryobeads) suspended in a cryopreservative fluid, such as glycerol, in a small plastic vial. Once inoculated, the excess solution should be removed with a sterile pipette and the vials stored in the freezer. To recover the culture, it is simply a matter of removing one of the glass beads and inoculating a liquid medium or streaking it onto the surface of an appropriate solid medium.

6.2.9 Liquid nitrogen

The storage of microorganisms at very low temperatures of –190°C to –196°C in or above liquid nitrogen (also a form of cryopreservation) is the best preservation method for most fungi and bacteria. Cultures, tissue or spore suspensions are treated with a cryoprotectant, such as 10% glycerol (emulsified with a nutrient broth solution for bacteria), before aseptic transfer into sterile ampoules, and frozen to ultra-low temperatures in the vapour phase of liquid nitrogen. The cooling rates are critical and the best revivals are achieved when this is done slowly. At ultra-low temperatures, metabolism is suppressed and, if the organism survives the initial freezing, viability should be indefinite. This technique requires large and expensive equipment and a reliable source of liquid nitrogen. Experienced staff are required to ensure optimum standards are maintained.

How to identify pathogens 7

Taxonomic keys do not exist for many plant pathogens. Furthermore, the literature on plant pathogens is so large and complex that only a specialist is likely to be familiar with all of the available information on the taxonomy of a particular group. Nevertheless, through familiarity with keys, the literature and the examination of specimens, the task of making an accurate identification becomes less arduous. The following sections describe the characteristics of some major taxonomic groups.

7.1 Fungi

Fungi are small, eukaryotic, usually filamentous, spore-bearing microorganisms that lack chlorophyll and have cell walls that contain chitin, cellulose, or both. The body of the fungus is called mycelium, and the individual branches or filaments of the mycelium are called hyphae. Growth of the mycelium occurs at the tips of the hyphae.

Fungi reproduce by spores, which are specialised reproductive bodies consisting of one or a few cells. Spores may be formed asexually or as the result of a sexual process. In primitive fungi, asexual spores are produced inside a sac called a sporangium. Some of these spores are motile (zoospores). Other fungi produce asexual spores (conidia) from special hyphae called conidiophores, while some (eg conidia) produce spores inside thick-walled structures called pycnidia.

Sexual reproduction occurs in most groups of fungi. In some, two cells (gametes) unite to produce a zygote called a zygospore. In others, the zygote is called an oospore. In the group of fungi known as ascomycetes, the sexual spores are produced within the zygote cell, the ascus, and the spores are called ascospores. In another group of fungi known as basidiomycetes, the zygote cell is called the basidium and the spores are called basidiospores.

There are possibly more than 250 000 species of plant pathogenic fungi. Almost all of these fungi spend part of their lives on their host plants and part in the soil or on plant debris.

The organisms traditionally studied by mycologists belong to the kingdom Fungi, but some belong to the kingdoms Protozoa and Chromista. The Protozoa contain the slime moulds. The Chromista contains the Oomycetes and includes the downy mildews, as well as *Pythium* and *Phytophthora*. There are four major phyla of fungi: Zygomycota, Chytridiomycota, Ascomycota and Basidiomycota.

Plant disease caused by a fungal pathogen is often recognisable from the particular plant organ infected and from the types of symptoms produced.Common types of fungal disease are damping-off, root rots, vascular wilts, downy and powdery mildews, leaf spots and blights, rusts, smuts, anthracnose, galls, dieback and post-harvest disease (see Table 3.1).

7.1.1 Root pathogens

Fungal infection of plant roots can interfere with water uptake and nutrient translocation, resulting in stunting of plant shoots, wilting and leaf discolouration (yellowing). Young roots are particularly susceptible to fungal attack, and root damage caused by cultivation or during transplanting can often exacerbate disease. Similarly, low soil nutrient status (P or K), salinity or pH imbalances all increase the vulnerability of plants to root rots.

Root rots are often difficult to diagnose, because they may be caused by a range of fungi or a complex of fungi, for example, *Fusarium*, *Pythium*, *Macrophomina* together with nematodes, or a succession of fungi. *Phytophthora* and *Pythium* are most common in wet soils. *Rhizoctonia* and *Fusarium* predominate in warmer conditions and in soils with high organic matter.

Although *Fusarium* and *Phytophthora* can cause root disease in some woody plants, many root rots of such trees are due to cellulose-destroying Basidiomycetes, such as *Armillaria*, *Ganoderma*, *Rigidoporus* and *Phellinus*.

7.1.2 Stem pathogens

Vascular wilts

Pathogens that cause vascular wilts are mostly confined to the vascular (xylem) system. Symptoms include loss of turgor, leaf wilting, leaf discoloration and, in severe cases, plant collapse and death. Only after the plant is dead, does the fungus move into other tissues to sporulate. The four genera of fungi that cause vascular wilts are *Fusarium*, *Verticillium*, *Ceratocystis* and *Ophiostoma*.

Fusarium causes vascular wilts in many vegetable, flower, fruit and fibre crops. Most of the important species belong to the *Fusarium oxysporum* complex. There are numerous special forms (formae speciales, ff spp), each with a restricted host range and often a number of pathogenic races.

Cankers

Cankers on herbaceous plant stems are produced by fungal pathogens such as *Colletotrichum* and *Phomopsis*, which also attack foliage and fruit. *Rhizoctonia solani* and *Corticium rolfsii* are important causal agents of stem-base lesions on herbaceous crops, especially legumes. Often, visible mycelium can be seen on the host surface. *Phytophthora* and *Fusarium* are often involved in woody plants, although external symptoms may be hard to detect.

Galls

Galls are abnormal growths or swelling caused by the hyperplastic enlargements of plant tissues due to stimulation from insects, bacteria, viruses and fungal pathogens such as *Exobasidium* and *Synchytrium*.

Witches' broom

This symptom is characterised by abnormal, massed, brush-like development of many weak shoots or roots. An example is the fungus *Crinipellis pernicosa*, which causes witches' broom disease of cocoa.

Pink crust or pink disease

The basidiomycete *Corticium salmonicolor* forms a flat, pinkish crust on the twigs and branches of many tropical and subtropical woody plants, causing pink disease or pink crust. It penetrates the bark and wood, killing twigs and branches and causing leaves to shrivel.

7.1.3 Leaf pathogens

Leaf symptoms are particularly important in plant disease diagnosis. Some leaf diseases are caused by saprobes, others by obligate fungal pathogens. Generalised symptoms can be caused by many different fungal pathogens, but specific symptoms tend to be associated with particular groups of pathogens.

On leaves, a wide range of microorganisms can cause necrotic spots and blotches, which are usually characterised by shape or pattern. Other useful diagnostic characteristics are the presence of fungal fruiting bodies, the age of leaves and the size of the area destroyed.

Leaf spots are generally limited to small areas of necrotic tissue; however, in some cases, the tissue may not be dead but merely discoloured by the presence of the causal organism. A wide range of fungal pathogens (and insect pests) can cause leaf spots. Leaf spots may be surrounded by a chlorotic halo.

Target spots consist of a series of concentric rings; ring spots are approximately circular with a dark margin; angular leaf spots are confined by leaf veins; and eyespots are approximately lenticular with a central dark spot. Shot-hole is a term used to describe a leaf spot where the necrotic centre of the spot falls out. Often, this is a byproduct of the host plant's defence reaction limiting the spread of the disease.

Anthracnose

The symptoms of anthracnose are dark, sunken necrotic spots or patches, sometimes with raised borders. Acervuli (Acervular conidiomata) can sometimes be found arranged in rings in the lesion. The symptoms usually occur on foliage and stems, as well as fruit. Severe infections can cause twig or branch die-back. Many plant pathologists use the term anthracnose exclusively for some, not all, diseases caused by *Colletotrichum*.

White rust

The white rusts belong in the Peronosporales. They are characterised by chains of sporangia produced in white, subepidermal sori. Symptoms appear as white blisters just beneath the epidermis. Oospores may be found in host tissue. An example is white rust on *Brassica* (*Albugo candida*).

Blight

The term 'blight' describes sudden extensive shrivelling and death of leaves, flowers, shoots, fruit and even entire plants. Usually the youngest growing tissues are attacked first. Blights are caused by a wide range of fungal pathogens, including *Colletotrichum gloeosporioides* (mango blossom blight) and *Phytophthora colocasiae* (taro leaf blight).

Scald

Scald-affected leaves are characterised by lesions that appear as if they are hot water-damaged. The lesions are mainly bleached, but may be partly translucent. Usually no chlorosis is present. Examples include rice leaf scald (*Gerlachia oryzae*) and barley scald (*Rhynchosporium secalis*).

Blast

Pale necrotic patches on leaves caused by fungi and bacteria are called blast. Blast can be a symptom of disease or stress, or the result of insect injury or adverse weather conditions. An example is *Pyricularia oryzae* on rice and some other grasses.

Scab

Scabs are discrete, superficial lesions with localised severe roughening or pitting. Symptoms involve abnormal thickening of the surface layers, with or without the development of cork. Fungi in the genera *Elsinoë*, *Fusicladium*, *Sphaceloma*, *Venturia* and *Cladosporium* mostly cause scabs. Scabs can also occur on fruit and stems.

Downy mildew

This disease is caused by fungi in the orders Sclerosporales (grass-infecting species) and Peronosporales (dicot-infecting species). Most species are dependent on films of water for their movement on the surface of the host plant and to invade plant tissue. Downy mildews can cause extensive damage to crops under conditions of high humidity. An example is millet downy mildew (*Sclerospora graminicola*). Symptoms appear as a white bloom on the undersides of leaves. The shape of conidiophores distinguishes different genera.

Powdery mildew

The powdery mildew fungi are obligate parasites on cereals and grasses belonging to the family Erysiphaceae, for example, *Blumeria graminis*. Powdery mildews are

characterised by a proliferation of white, superficial surface mycelium and powdery conidia on leaf surfaces. Small, spherical ascomata sometimes develop on the surface of the leaves. Haustoria are formed in the cells of the epidermis; conidiophores are formed laterally on hyphae; and aseptate conidia are formed in basipetal chains. Many species have special forms (formae specialis, f sp) based on the species of host. Powdery mildews are adapted to relatively dry conditions, and are the only group of plant–parasitic fungi with conidia that can germinate in the absence of free water.

Sooty moulds

The sooty moulds are caused by members of the Capnodiales and Chaetothyriales orders. They form black mats on living leaves and stems by growing on plant and insect exudates. Sooty moulds are usually associated with damage by sap-sucking pests, such as aphids and scale insects. Sooty moulds can reduce photosynthesis significantly and have the potential to retard plant vigour and reduce yield.

Black mildews

The black mildews belong to the Meliolales, but are often confused with the sooty moulds. Black mildews are leaf pathogens that are common in tropical rainforests, and the damage they cause is largely cosmetic. The fungi are characterised by coarse hyphae with short, lateral hyphopodia, superficial ascomata and large dark ascospores.

7.1.4 Fruit and seed pathogens

Several common pathogens are often associated with fruit rots. One of the most abundant is *Colletotrichum gloeosporioides* (teleomorph is *Glomerella cingulata*), which is usually associated with anthracnose on fruit. These lesions often crack open with age. *Phytophthora* species are involved in many fruit rots (cocoa and coconut are good examples). *Phomopsis* and *Fusicoccum* are associated with stem-end rot of many tropical fruits.

Fruit are particularly susceptible to post-harvest diseases, which develop during the packing, storage, transportation and handling process. Post-harvest diseases often involve pathogens that were present in the field, but were not expressed. Disease may continue to develop even under refrigeration and symptoms may appear at any stage in the process, once environmental conditions become favourable for the disease. Examples of post-harvest diseases include transit rot (*Rhizopus stolonifer*), blue mould (*Penicillium expansum*), grey mould (*Botrytis cinerea*), Aspergillus fruit rot (*Aspergillus* spp) and stem-end rot (*Phomopsis* spp and *Fusicoccum* spp).

Ergots

The ergots are caused by fungi in the order Clavicipitales and occur on grasses, including most cereals. Conidia of the sphacelia state of the ergot fungus infect the ovaries. Insects mostly spread these conidia, which occur suspended in ‘honey dew’ exuded from the florets of hosts in response to infection. The sclerotia often contain poisonous alkaloids and can contaminate grain for human or animal consumption.

7.1.5 Rust fungi

Rusts are diseases caused by fungi in the order Uredinales. Symptoms include powdery pustules on leaves and stems. The spores can be yellow, orange or brown and have a 'rusty' appearance. Examples include *Puccinia polysora* on maize, *Puccinia purpurea* on sorghum and *Phakopsora pachyrhizi* on soybean.

The rust fungi are of great economic importance as pathogens of plants. More than 7000 species have been described in approximately 160 genera, the largest of which is *Puccinia*. At least 30 of these genera are monotypic. Rust fungi may form up to five types of spore in their life cycle. The types of spore are assigned the Roman numerals:

O spermatia (produced in spermogonia)
I aeciospores (produced in aecia)
II urediniospores (produced in uredinia)
III teliospores (produced in telia)
IV **basidiospores (produced on a basidium by a germinating teliospore).**

Rusts that produce all five types of spore are called macrocyclic. Many rusts have lost the ability to produce one or more spore types (eg microcyclic rusts only produce III and IV). Many rusts have two unrelated host plants, a primary host for II, III and IV, and an alternate host for O and I. Rusts with two unrelated host plants are heteroecious. Rusts that have a single host are autoecious.

This life cycle of heteroecious rust, a typical macrocyclic,includes the following five stages:

1. A germinating basidiospore infects the leaf of a host plant.
2. Spermogonia are produced in the leaf, which release small cells (spermatia) in a droplet that is dispersed by insects. Superficial receptive hyphae grow out from the spermogonium (or through the epidermis), which become fertilised by spermatia of the opposite sex.
3. The resulting hyphae produce aecia and aeciospores that are dispersed by wind or insects. The aeciospores are only able to infect the alternate host.
4. After infection, the mycelium forms uredinia and urediniospores, which appear as a dusty, orange or brown mass. Urediniospores are dispersed by wind. They cause new infections on the same host and several generations of urediniospores may be formed during the growing season.
5. Telia with teliospores are formed at the end of the growing season. Sometimes teliospores and urediniospores may occupy the same sorus. Teliospores usually function as resting spores. Each teliospore cell is able to form a single three-septate basidium that produces basidiospores on sterigmata. The basidiospores are spread by wind.

When examining plant material with a suspected rust infection, look for the following:

- uredinia and telia — which surface of the leaf (or other plant part) they occur on
- urediniospores — their shape and surface ornamentation
- paraphyses (sterile cells) — if present, their shape and size
- germ pores — number and position on urediniospores
- teliospores — their shape, septation and whether they have pedicels.

7.1.6 Smut fungi

Smut is a disease caused by fungi in the class Ustilaginomycetes. The disease is typified by black powdery spore masses (sori). The sori appear in or on roots, stems, leaves, inflorescences, flowers, anthers and ovaries.

The smut fungi are very important pathogens of crop plants and ornamentals. There are approximately 2000 species of smut fungi in approximately 90 genera. The classification of the smut fungi has undergone many changes in recent years, based on ultrastructure and molecular studies (historically classification relied on the way spores germinated).

The most conspicuous feature of smut fungi is the dusty, dark masses of spores (teliospores) in sori in particular organs. The spores of smut fungi are not stalked (pedicellate), in contrast to the teliospores of rust fungi. Smut spores are mostly spread by wind.

The teliospores produce basidiospores (sporidia) that germinate as yeast-like colonies in culture. In nature, two germinating basidiospores fuse to form infection hyphae. Usually, only certain parts of host plants are susceptible to infection (eg ovaries or flowers).

Recent changes in the classification of the smut fungi have meant that many names of smut fungi have changed, such as:

- *Sporisorium* is restricted to grasses and accommodates many smuts previously in other genera, including *Sphacelotheca* and *Sorosporium*.
- *Sphacelotheca* is restricted to hosts in the Polygonaceae and is now classified in the Urediniomycetes (which contain the rusts).
- *Sorosporium* is restricted to the flowers of hosts in the Caryophyllaceae.
- *Ustilago* is restricted to grasses, but species described on other hosts (mainly dicots) belong mostly to *Microbotryum* in the Urediniomycetes.

When examining plant material with a suspected smut infection, look for the following:

- the part of the host that is infected
- hypertrophied tissue
- presence of either spore balls or loose spores

- spore shape, size and surface ornamentation
- presence or absence of sterile cells amongst the spores
- presence or absence of a peridium and columella in the sori.

7.2 Bacteria

Bacteria are single-celled, prokaryotic microorganisms, lacking chlorophyll and characterised by rapid reproduction. Bacteria are ubiquitous and physiologically diverse and they occupy a wide range of ecological niches. Bacterial diseases of plants occur worldwide. As they favour moist or warm conditions, bacteria are of most importance in tropical, subtropical and warm-temperate areas.

Most bacteria can survive on crop residues, in soil, on seeds or on living plants. Bacteria infect plants through wounds or natural openings (eg stomata and lenticels). Infected seeds, infected propagating material, water splash, insects and machinery can all spread bacteria.

Although the names given to bacteria are regulated, no official classification exists. The *International Code of Nomenclature of Bacteria* (the Bacteriological Code) contains the rules that govern the way in which the names of bacteria are used. In 1975, the Bacteriological Code (1975 Revision) introduced the concept of valid publication for names of bacteria. The publication of the *Approved Lists of Bacterial Names* (*International Journal of Systematic Bacteriology* [1980] **30**: 225–420) set a new starting point in bacterial nomenclature. The *International Code of Nomenclature of Bacteria* (1990 Revision) is the cornerstone of bacterial nomenclature. It states that the name of a taxon is published validly, and therefore has standing in nomenclature, if one of the following criteria is met:

- The name is cited in the *Approved Lists of Bacterial Names*.
- The name is published in papers in the *International Journal of Systematic Bacteriology* (IJSB) or in the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM) and conforms to requirements laid down in the Bacteriological Code. Since August 2002, it is also a requirement of the IJSEM that authors of new species, new subspecies and new combinations provide evidence that type specimens have been deposited in at least two recognised culture collections in two different countries.
- The name is validly published by announcement in a Validation List. Validation Lists are lists published in the IJSB or in the IJSEM validating bacterial names published elsewhere.

The *Approved Lists of Bacterial Names* contain 2212 names of genera, species or subspecies, and 124 names of higher taxa. The *Approved Lists of Bacterial Names* recognises the names of bacterial species for which there are modern descriptions. In 2002, 5806 bacterial species in approximately 1094 genera were recognised. Plant pathogenic bacteria are recorded in 132 species in 29 genera. The major genera of

phytopathogenic bacteria are *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas*, *Streptomyces*, *Xanthomonas* and *Xylella*. There are several hundred pathogens in these taxa, including many recognised as pathovars that are specific to their host plant species and genera. For example, *Pseudomonas syringae* has more than 40 different pathovars, while *Xanthomonas campestris* has more than 123 pathovars.

Many plant pathogenic bacteria have a disease cycle during which a host is colonised as an epiphyte before the pathogenic phase. Many plant pathogenic bacteria do not enter the cell directly, but multiply in the intercellular spaces. These bacteria gain entrance through natural openings, such as stomata, hydathodes and wounds. A series of virulence factors that are secreted from the bacterium, have evolved, includingextracellular enzymes, toxins, phytohormones and extracellular polysaccharides.

Diagnosis of bacterial plant disease involves careful examination of all symptoms and consideration of other factors that may be important. Bacterial disease may be indicated by the absence of more visible pathogens and pests, such as insects and fungi. The presence of ooze and the microscopic observation of bacterial streaming from cut surfaces under water are indicative of bacterial infection.

Dilution methods to isolate bacteria are frequently used. The aim is to obtain single colonies of the suspect pathogen, thus ensuring the isolation of pure cultures containing only one species of bacterium. The medium must be suitable and the agar surfaces must be dry. Ensure the purity of the culture before beginning tests. Work with mixed cultures is wasted effort, giving meaningless results. Many plant pathogens can be identified tentatively using a few tests.

Prior to attempting to identify a bacterial culture, it should be checked for purity. This is done by making a dilute suspension of some of the cells in sterile water or saline and streaking a loopful on to a dry agar plate, as for an isolation streak. The streak is examined daily for several days to check that all colonies are identical. Colonies that appear identical when growing separately sometimes appear different when growing close together. If there is any doubt about the purity of a culture at this stage, single colonies should again be subcultured until purity is certain. It is pointless to work with mixed cultures.

The streak tests for purity should also be used to observe colony morphology. A note of shape, size, texture, colony surface markings, elevation, margin type, consistency, colour, translucency or opaqueness, and rate of growth should be taken. The presence of pigments, precipitates or crystals in the medium should also be noted.

Dichotomous keys (diagnostic keys) were among the earliest forms of bacterial identification. Diagnosis follows a step-wise progression along a dichotomous branching route. Although keys have been largely discredited in the wider field of bacterial taxonomy, they are still used successfully by the diagnostician interested in bacterial plant pathogens. It has to be stressed that great care must be employed using a key. Care must be taken throughout the identification procedure, but especially on the initial tests. A number of alternative techniques are used to facilitate the identification

of plant pathogenic bacteria, such as fatty acid analysis, phage-typing, monoclonal antibodies or nucleic acid probes.

Morphological characters are of limited value for bacterial identification. Colony size, rate of growth, colours, texture and opacity do not provide enough information to identify a bacterium. Identification of bacteria depends on a series of tests, which usually indicate the presence, or absence of certain enzymes.

7.3 Phytoplasmas

Phytoplasmas, previously known as Mycoplasma-like organisms, are prokaryotes in the class Mollicutes. They are similar to bacteria, but lack a rigid cell wall and cannot live freely in the environment. They have not been grown in culture. Phytoplasmas are found in the sieve tube cells of plant phloem tissue and most are transmitted by phloem-feeding leafhoppers and plant hoppers. Phytoplasmas are obligate parasites and complete their life cycle within host tissues. They cause disease in a wide range of plant hosts. Symptoms commonly caused by phytoplasmas are leaf discolouration, stunting, dieback, reduced leaf size ('little leaf'), witches' broom, phyllody, virescence and floral gigantism ('big bud').

Until recently, the main methods used to identify and distinguish phytoplasma diseases were symptomology, host range, vector specificity and transmission electron microscopy of ultrathin sections of diseased tissue. The development of deoxyribonucleic acid (DNA)-based molecular techniques, particularly phytoplasmas-specific polymerase chain reaction (PCR) primers designed on the basis of the highly conserved 16S ribosomal ribonucleic acid (rRNA) gene sequences, have greatly enhanced the capacity to detect and identify phytoplasmas.

7.4 Viruses and viroids

Viruses are extremely small obligate parasites that are only visible with the aid of an electron microscope. Viruses, unlike bacteria and fungi, are not comprised of cells, but are instead comprised of a protein coat or shell called a capsid, surrounding a genome of ribonucleic acid (RNA) or DNA. Viruses can only multiply in living cells by taking command of the genetic processes of an infected plant. The energy resources of the plant are thereby redirected to reproduce viruses. Viral infection impairs the plant's normal functions, such as photosynthesis and growth.

Sap sucking insects such as aphids and leaf hoppers often spread viruses. Infected vegetative propagation material is a major concern in the movement of viruses. Viruses often survive in alternative weedy hosts.

Viroids are low-molecular weight, circular RNA molecules lacking a protein coat, which infect plant cells, replicate and cause disease. Viroids are transmitted mechanically during pruning, by seed transmission and by vegetative propagation such as grafting.

7.4.1 Virus species and records

Virus species are not obligatorily linked to specimens, as are species of cellular organisms. Plant virus species, as recognised by the International Committee on Taxonomy of Viruses,[10] are described and identified by possession of a unique combination of several characters that include:

- the plant species naturally infected by the virus
- symptoms on naturally infected plants at different stages of infection
- modes of transmission (eg contact, seed, pollen, vectors)
- the range of species that are susceptible to experimental infection
- the shape of the virus particles
- biochemical characteristics of the proteins and nucleic acids of the virus
- comparison of gene sequences and organisation to other known viruses
- serology.

Often, plant virus records are not tied to voucher specimens, because until recently, it has not been possible to store specimens of viruses in a viable state. Most viruses are unstable even when freeze-dried. Now there are sophisticated methods for the long-term storage of viruses, including cloning virus genomes in bacteria.

Consequently, virus records have rarely been specimen based, but are insteadoften based on a suite of recorded characters, including descriptive text, photographs, experimental data, gene sequences and serology. Information on most species of viruses known to infect plants can be found on the Virus Identification Data Exchange database.[11]

Rod-shaped viruses

Rod-shaped viruses, which include tobacco mosaic virus, are generally 3–25 nm in diameter and 150–2000 nm in length, depending on the length of the RNA. Virus particles can be straight, bent or curved. The general structure of these viruses consists of both RNA and protein subunits arranged in a helix.

Isometric viruses

Isometric viruses can occur singly or in pairs and are 20–70 nm in diameter. When viewed under the electron microscope, these viruses appear to have a geometric structure with icosahedral symmetry (ie 12 vertices and 20 triangular faces). An example is cauliflower mosaic virus.

Bacilliform viruses

Bacilliform viruses have a shape resembling those of bacteria in the genus *Bacillus*. They may or may not be surrounded by an envelope. Examples include alfalfa mosaic virus and sugarcane bacilliform virus.

[10] http://www.ncbi.nlm.nih.gov/ (Accessed 31 May 2005)
[11] http://image.fs.uidaho.edu/vide/refs.htm#descriptions (Accessed 31 May 2005)

There are several methods that can be used to identify a plant virus. Plant virologists often maintain herbaceous indicator plants in insect-free glasshouses, which can be used for infectivity and host-range studies. These plants express different symptoms when inoculated with different viruses. Symptoms alone are usually insufficient for positive identification. Laboratory techniques, such as serology, electron microscopy, as well as nucleic acid analysis, must be used for virus identification.

7.4.2 Virus symptoms

The visible symptoms of virus infection are often discernable to the experienced diagnostician. There are two main types of virus symptoms: those resulting from primary infection of host plant cells (eglesions); and those caused by secondary or systemic infection (eg mosaic). Viruses, unlike fungal pathogens, are only able to enter plant cells through wounds, such as broken epidermal hairs, small abrasions, or holes in the epidermal layer of cells — often caused by insect feeding.

Initial symptoms that develop at the site of virus entry into plant cells are called local symptoms and often form distinct areas of diseased cells called lesions. These lesions vary in size from small pin-dots to large patches, which may become chlorotic (due to the loss of chlorophyll) or necrotic (if the cells die). Lesions are common after mechanical sap transmission of virus to a leaf surface and sometimes after feeding by virus-carrying insects such as aphids, although this is less common.

In some host–virus interactions, the virus is unable to spread beyond the initial site of infection and local lesions may be the only observed symptoms. This type of restricted response is termed a hypersensitive reaction. If the virus is not confined, it will spread within the leaf mesophyll. Once it reaches the vascular system, it will spread quite rapidly throughout the entire plant, resulting in secondary or systemic infection. Most viruses move via the phloem.

Secondary or systemic symptoms may produce visible changes, including chlorosis and wilting, and internal changes such as the formation of abnormal cell structures, which can only be observed with the aid of a light or electron microscope.

Mosaic symptoms occur when certain cells in the virus-affected plant organ (generally a leaf) are infected and discoloured, while other cells appear normal. Infected cells are generally pale green, resulting from reduced production of chlorophyll. The shape and pattern of mosaic symptoms varies greatly from plant to plant. In monocotyledonous species, this symptom usually takes on a striped or streaked appearance. In dicotyledonous species, when the discoloured portions are round in shape, they are often referred to as a mottle, chlorotic flecking, spotting and blotching.

In some virus–host plant interactions, the whole leaf may become yellow because of reduced chlorophyll production and the breakdown of chloroplasts. This is the main symptom associated with ‘yellowing’ viruses, beet yellows and barley yellow dwarf. Yellowing is generally first observed as interveinal chlorosis, and sometimes the areas adjacent to the vascular tissues can remain green in contrast to the rest of the leaf.

However, certain viruses cause vein yellowing and vein clearing, including lettuce big vein disease and turnip mosaic virus.

Ringspotting is a symptom that generally occurs when the diseased area is restricted to a ring of infected cells. These infected cells may become chlorotic or necrotic. The rings may occur in concentric circles (Figure 7.1). Ringspots are most common on leaves, although they may also occur on stems and fruits. Some examples of viruses that cause ringspots include tomato spotted wilt virus and papaya ringspot virus.

Cell necrosis can occur in localised lesions around the point of infection or systemically in other parts of the plant, such as fruits and seeds or protected leaves. For example, turnip mosaic virus causes necrosis of internal leaves in cabbage.

Reduced plant size (stunting, dwarfing) is a frequent symptom of virus infection and is likely to be found in combination with other symptoms. Stunting can occur throughout the plant or be confined to specific plant parts, such as the apical meristems. This symptom may be difficult to observe unless virus-affected plants are growing side by side with healthy plants.

Bean common mosaic virus and strawberry latent ringspot virus are just two of the many viruses that cause abnormal growth (leaf and stem distortion) of infected host plants. These two viruses cause the leaves of beans and celery, respectively, to become strap-like. Growth abnormalities come about because of a hormonal imbalance within the leaf. Other distortions and abnormalities include cell proliferation, as is the case with cacao stems infected with cacao swollen shoot virus. An increase in the number of cells is called hyperplasia; an increase in the size of cells is called hyerptrophy. An example of hypoplasia is stem pitting of citrus caused by citrus tristeza virus.

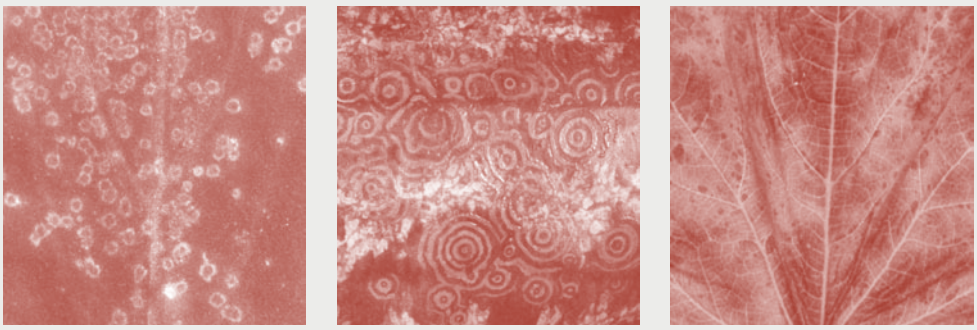


Figure 7.1 Virus symptoms (from left to right), chlorotic circles, ringspot and mosaic

Some viruses cause tumour-like outgrowths (enations and tumours) on leaves and roots. Outgrowths on leaves are referred to as enations. They have a wart-like appearance and can appear on the upper and lower leaf surface. Enations can be found in pea plants infected with pea enation mosaic virus. Like leaf and stem distortions,

tumours are the result of virus-induced hormonal imbalances, which cause abnormal cell proliferation.

Colour break in the petals and flowers of tulips was one of the first described virus diseases in the seventeenth century. This disease, caused by the tulip mosaic virus, resulted in colour variegation in the tulip flowers. Bulbs infected by the virus were highly prized by Dutch growers and the disease is still exploited to this day. Viruses such as turnip mosaic virus and bean yellow mosaic virus can also cause colour break in stocks and gladiolus, respectively.

Virus infection can result in fewer, smaller or misshapen fruit. For example, cucumber mosaic virus can result in deformed gherkin fruit. Similarly, infection of lettuce plants by lettuce mosaic virus can greatly reduce seed production. In addition, pollen from infected plants is frequently sterile, or its viability somewhat impaired.

Breakdown of cell chloroplasts and abnormal cell proliferation have been mentioned above. However, there are other cytological and histological changes that take place, such as virus-induced inclusion bodies. Several viruses have been observed in the nuclei of plant cells. Many viruses can cause changes in the chloroplasts, most of which result in biochemical and structural degradation (ie loss of colour and shape). Other histological changes include a reduction or increase in cell numbers, internal cell necrosis, lignification of xylem elements, and degeneration and death of phloem cells.

Viruses may accumulate in large numbers within the cell to form inclusion bodies, which may be composed almost entirely of virus particles. Inclusion bodies may occur in the nucleus, but are most common in the cytoplasm. Particles can be arranged randomly, side by side, end on end or in a three-dimensional lattice.

Just because a plant does not display visible symptoms of virus infection does not mean that the plant is virus free. Viruses are able to infect certain hosts and multiply within the host cells without causing visible symptoms. Latent infection is quite common in wild plants and weeds. Viruses may survive in these alternative hosts and be re-introduced to horticultural and agricultural crops at a later time by sap-feeding insects.

Symptom development can often vary depending on the virus strain and the virulence genes it possesses. The host plant itself may be resistant, tolerant or susceptible to virus infection. Similarly, the age of the plant and the timing of infection play an important role in symptom expression. In general, younger plants are more susceptible to infection, and older plants are quite tolerant. Therefore, earlier infections tend to result in much greater yield losses than late infections.

The development of virus symptoms is often slow at higher temperatures, because virus replication is generally slowed. However, high temperatures can also reduce the ability of the host plant to resist infection and, once the temperature drops, infection can progress rapidly. Plants grown under high light intensity are less susceptible to infection than plants growing under low light. In addition, plants that grown in

nutrient-rich soil are often more susceptible to virus infection. High nitrogen, for example, can increase susceptibility.

Disease symptoms associated with virus infection can often look similar to nutrient deficiencies or chemical toxicities, such as herbicide damage. There are two ways to rule out a nutrient disorder or chemical imbalance:

1. Look at the distribution of affected plants. Generally, in the case of nutrient disorders, the plants will be affected in a pattern associated with soil type or the application of chemicals. Viruses, which are most often spread by vectors, generally have clumped distributions or show a gradient from the source of infection (eg weeds).
2. Demonstrate transmission of the symptoms using grafting or mechanical sap transmission from a plant suspected of being infected to a healthy plant under experimental conditions. This is the first step in applying Koch's postulates to determine the cause of the disease.

7.5 Nematodes

The long-established method of identifying nematodes is to compare the morphological features of the specimens with the published descriptions, often with the aid of keys. This requires specimens to be preserved and mounted for observation and measurement under high-power light microscopy. For confident identification of many species, 5 to 10 adult females and/or males may be needed, because some distinguishing features are quantitative, and intraspecific variation is common.

In many cases, identification of plant parasitic nematodes to genus can be based on general morphological features and knowledge of the host and nematode fauna of the area of collection. In some cases, an adequate working identification of live specimens to species using such information is possible.

However, some nematode species are difficult to identify, even with expert examination of morphology and precise morphometrics. Therefore, taxonomic and diagnostic nematology is moving increasingly towards molecular and biochemical methods. For some nematode genera, DNA sequence information is now required for description of new species. Chemotaxonomic studies have demonstrated the occurrence of cryptic species (ie species not distinguishable morphologically), although it is possible to find retrospectively morphological differences to support the chemical data.

7.5.1 Need for identification

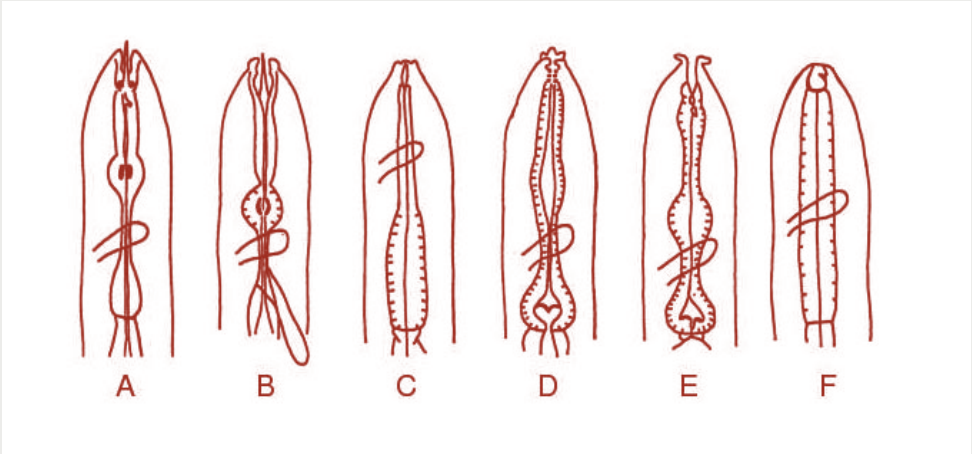
The approach and expertise needed for nematode identification will depend on the purpose. Specimens lodged in national and international collections should be determined, or at least confirmed, by an experienced nematode taxonomist; however, it must be recognised that for difficult taxon, experienced taxonomists may provide differing determinations, or may not be able to provide certain identification.

For survey purposes, it may be sufficient for diagnostic nematologists to make identifications, with expert taxonomic advice sought only for new records or unidentified species. New records should be lodged in a national collection for re-examination as needed. If quarantine or trade implications are likely, independent confirmation is advisable before publication.

For management or ecological studies, determinations on live specimens (taking into account the local fauna and the hosts under study) may be appropriate. However, confirmation by a taxonomist of representative material is advisable to strengthen such studies. Often for such work only the genera is determined.

7.5.2 Distinguishing plant parasitic nematodes

For crop production and regulatory purposes, it is initially important that plant-feeding nematodes are distinguished from species that feed on other substrates. Nematodes that feed on plants have stylets (protursile hollow feeding structures) in their oral cavity. Classification of live specimens into presumptive trophic groups is possible with examination under a good-quality dissecting microscope. Figure 7.2 illustrates variation in anterior morphology associated with feeding behaviour.



A, B, C: tylenchid, aphelenchid, dorylaimid — styletbearing nematodes; feed on plants, fungi and alage; some predators; D, E: rhabditid, cephalobid — bacterial feeding; and F: mononchid — predator

Figure 7.2 Comparative anterior morphology of some groups of nematodes

Rather than feeding on plants, some nematode species with stylets feed on fungi, algae and lichens and others are predatory on soil microfauna. Thus, morphology and host interactions need to be considered to confidently place the specimen in a plant feeding genera.

7.5.3 Species identification

Morphological identification

Identification is based on the shape, presence and number of anatomical features including sexual dimorphism (morphology) and measurements and ratios of measurements (morphometrics). Published keys and descriptions will indicate features that are diagnostic for genera and species. Computerised keys are available and two examples using different programs are Genera of Plant Nematodes[12] and Nematodes of Australia.[13]

Molecular and biochemical identification

DNA methods based on sequence, probes, restriction fragments and the like have been developed to resolve particular identification and diagnostic challenges. Some rely on extraction and amplification of DNA from individual nematodes, while others can detect and quantify particular species in soil samples. A significant constraint is that the identification can be restricted to a single or small group of species, and validation may have been undertaken with limited sampling of intraspecific variation. Nevertheless, the application of this technology is likely to increase rapidly.

Other chemotaxonomic methods have been developed, including isozyme analysis, protein profiles and serological assays, but relatively few have been adopted widely. Distinguishing species of root knot nematodes by isozyme analysis has proven to be a practical approach, because the diagnostic morphology is equivocal. DNA and chemical approaches require more sophisticated facilities, but circumvent the need for a skilled taxonomist in routine diagnostic processes. However, further development of these approaches must use reliably identified material in order to provide robust solutions.

7.6 Diagnostic techniques

7.6.1 Scanning electron microscopy

Unlike the compound microscope, which uses light to view the sample, the scanning electron microscope (SEM) uses electrons to generate an image. The SEM has a much greater resolution than the compound microscope, because the wavelength of the electrons is approximately 100 000 times smaller than the wavelength of light. The SEM is useful for examining the surface structures of fungal spores.

The best resolution of the light microscope is 0.2 µm or 200 nm. The resolution of a SEM is 3–6 nm, which is almost 100 times better than the light microscope. Scanning electron microscopes provide much more surface detail than light microscopes and have a larger depth of field, allowing more of the sample to be in focus at one time.

The SEM uses a beam of electrons to scan the surface of a sample to build a three-dimensional image. Electrons are very small and easily deflected by gas molecules in

[12] http://www.lucidcentral.org (Accessed 31 May 2005)
[13] http://www.ento.csiro.au (Accessed 31 May 2005)

the air. Therefore, to allow the electrons to reach the sample, the column through which the electron beam passes and the specimen chamber are held under a vacuum.

To preserve the structure of biological samples under vacuum, samples must be carefully dried using liquid carbon dioxide in a machine called a critical point dryer. The samples are generally mounted on metallic stubs using special double-sided tape (carbon tabs) and then coated with a thin layer of metal, such as gold, to make them electrically conductive. Thick-walled spores of rusts and smuts do not need to be critically point dried and can be coated directly after mounting on the stub.

7.6.2 Biochemical and molecular techniques

The absence of visible disease symptoms does not necessarily indicate that a plant is free from pathogens. It is necessary for plant pathologists to use biochemical or molecular techniques for detecting the presence of some pathogens. Indexing is a term used for any procedure that tests for the presence of known pathogens, particularly viruses, in plants. Indexing allows rapid implementation of control strategies and reduces the possibility of an epidemic developing. Indexing is also important in implementing quarantine strategies to keep countries free from exotic diseases and certification schemes producing disease-free planting or propagating material.

7.6.3 Serology (immunology)

In serology, specific antibodies made to antigens on the pathogen are exploited for diagnosis. These antibodies may be polyclonal (mixed population of antibodies made by immunising an animal with an extract of the pathogen and harvesting the blood) or monoclonal antibodies (the single antibody-secreting spleen cells of an immunised animal are cloned and propagated in tissue culture).

One of the most commonly used serological diagnostic tests is enzyme-linked immunosorbent assay (ELISA). The antibody is allowed to adsorb to the wells of a microtitre plate. A test solution is then added to the wells and if antigens are present they will bind to the antibody. The wells are then washed, enzyme conjugated antibody is added, the wells are washed again and finally the enzyme substrate is added. If antigen is present, the bound enzyme–antibody conjugate catalyses the conversion of the chromogenic substrate to a coloured product.

Pathogen diagnosis using serological methods has many advantages. Although antibodies may take several weeks to produce, they are stable for long periods if stored correctly, and produce results quickly. Serological methods can be adapted for both laboratory and field conditions.

7.6.4 Nucleic acid-based methods

Although many genes are shared by living organisms, genes with the same function generally vary in sequence from one taxon to another. This variability may be exploited in diagnostics using techniques such as nucleic acid hybridisation and the polymerase

chain reaction (PCR). Modern molecular techniques for analysing nucleic acids are so sensitive that under ideal conditions, picogram quantities of DNA can be detected.

A typical PCR involves heating a mixture of DNA, a heat-stable DNA polymerase, DNA primers and dNTPs in a suitable buffer to more than 90°C to denature the DNA, cooling to a temperature of approximately 50–60°C to anneal the primers to the separated strands of DNA, and then heating to 72°C — the optimal temperature for DNA polymerisation — to allow a complementary strand of DNA to be made, extending from the DNA primer. After each cycle of denaturation, annealing and extension, the amount of DNA is doubled, leading to an exponential increase in the amount of DNA, which after 30 cycles, can be easily visualised in an agarose gel by staining with ethidium bromide.

Nucleic acid-based methods of diagnosis have the advantage of speed, and the sensitivity of detection exceeds that of immunology by several orders of magnitude. Disadvantages of nucleic acid based techniques include the expense of the equipment, reagents and facilities, a lack of robustness when compared with serological tests, and enhanced sensitivity can mean that contamination of samples is more of a problem.

8.1 Databases

Entering plant disease records into a database is important because it allows herbarium staff to rapidly access information, without the need to sort through numerous physical samples. Data are stored in an organised manner that can easily be searched, retrieved, analysed and updated, when required.

The information stored in a database can be used to map the distribution of plant pathogens and is important for quarantine and pest risk analysis. A database may be as simple as a table set up in a spreadsheet program, such as Microsoft Excel, or it can involve a more sophisticated database program, such as Microsoft Access, Oracle, BioLink or KE EMu. These programs allow the management of multimedia, such as digital images, and also feature built-in reporting tools, which enable the rapid exchange of information.

Databases such as KE EMu (Figure 8.1) are invaluable, because they allow the user to track changes. This is especially useful when it comes to constantly changing variables, such as pathogen and host plant nomenclature.

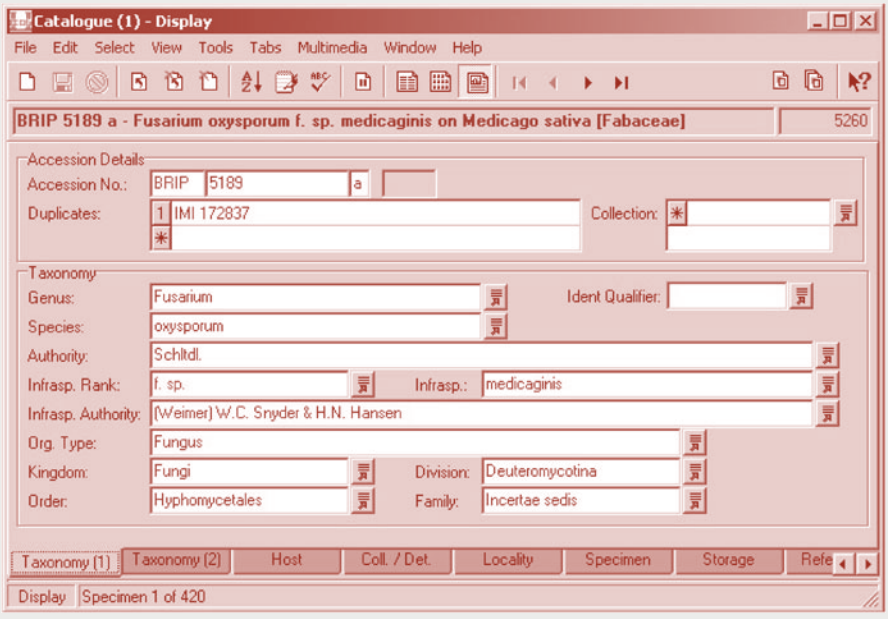


Figure 8.1 Catalogue module of the KE EMu database

Database store not only digital images, but also many types of multimedia, including text files, word documents, PDFs, html and videos. Detailed information can be kept about collectors, determiners and growers; for example, address, telephone, facsimile, email, biography and references. Task templates can also be set up to alert herbarium staff about overdue loans, culturing and other important events.

Regardless of the complexity of the database being used to house information about the herbarium specimens, it is important to ensure that the data stored in the database are accurate. This is now becoming easier as many taxonomic databases can now be found on the internet (Figure 8.2).

CABI Bioscience Database of Fungal Names: www.indexfungorum.org
International Plant Names Index: www.ipni.org

Figure 8.2 Internet addresses and home pages of two useful taxonomic databases

On a national scale, the databases of dispersed collections held by different agencies can be linked by a web-based system to create a virtual herbarium. An example is the Australian Plant Pest Database (APPD),[14] which provides the framework for a virtual, national, plant pest database. The APPD integrates existing specimen records from more than nine nodes distributed throughout Australia, enabling rapid location of voucher specimens and efficient retrieval of detailed data.

On an international scale, the Global Biodiversity Information Facility (GBIF),[15] has a searchable database that aims to make the world's primary data on biodiversity freely and universally available via the internet. The GBIF uses its own portal to enable questions against biodiversity databases throughout the world. The GBIF portal has access to some databases of plant pathogens.

[14] http://www.planthealthaustralia.com.au/APPD/legal.asp (Accessed 31 May 2005)
[15] http://www.gbif.org (Accessed 31 May 2005)

Care of collections 9

9.1 Herbarium facilities

A herbarium should provide a safe and permanent place to store specimens. It should be insect-proof, fireproof and waterproof. A moderately large (up to 50 000 specimens) plant pathology herbarium can be housed in a single room (9 m^2), especially if compactus storage is used for filing specimens. Metal storage shelving and cabinets are more insect resistant than wooden ones.

The herbarium should be kept in a room with a temperature- (20–23°C) and humidity- (40–60%) controlled atmosphere. This provides an extremely effective way of managing damaging insect pests, especially when combined with the freezing of new specimens. Airconditioners should be used to control temperature, and dehumidifiers to reduce humidity. Windows and doors should be kept shut to prevent insects from entering. Solar tinting and films can be placed on windows to reflect heat.

9.2 Control of herbarium beetles

Conditions in tropical regions with high temperatures and high humidity suit the rapid development of damaging insect pests. Some insects, particularly herbarium beetles, consume dried plant material and can rapidly destroy herbarium collections in the tropics. Deep freezing herbarium specimens at —20°C or below, for a minimum of 7 days — is the most useful technique for dealing with potentially damaging insects. It is essential that all new specimens are frozen for one week prior to being placed in the herbarium. Existing herbarium specimens should be rotated through the freezer periodically.

Specimens must be wrapped in plastic bags or placed in styrofoam boxes with tight lids to prevent condensation of moisture on the specimen during deep freezing. After freezing, the specimens should be allowed to warm gradually by leaving in the airconditioned herbarium until they reach room temperature.

Fresh plant specimens are often sent to the herbarium for disease diagnosis. This material should not be stored near the herbarium and, ideally, should be examined away from the herbarium collection. Herbarium specimens removed (for examination or loan) from the temperature- and humidity-controlled herbarium environment should be returned to their position only after having been frozen for seven days.

Many herbaria employ professional operators to fumigate the collection once a year with an approved fumigant; for example, methyl bromide, carbon bisulphide, carbon tetrachloride, ethylene dichloride, hydrocyanic gas, lindane, dichlorvos strips or

paradichlorobenzene. Care should be taken with fumigants, because they are harmful to people and many are highly flammable. Fumigation alone is not a satisfactory way to control insects in the herbarium, because insect eggs and pupae are often not killed.

9.3 Control of culture mites

Mites can be a chronic problem in fungal culture collections. These fungus-eating culture mites can be brought into the laboratory on fresh plant material, shoes and clothing, the bodies of insects and the cultures from other laboratories. They thrive in tropical conditions. Not only do they eat the cultures, but they also carry fungal spores and bacteria on their bodies and in their guts, which contaminate cultures.

If undetected, mites can cause a major problem in a laboratory in a matter of days, because they rapidly move from plate to plate in an incubator or on a bench, feeding on fungal cultures. They can be seen in fungal cultures as minute, white dots, just detectable to the naked eye. Mites are often first detected by the trails of contaminant fungi and bacteria they leave on plates, or by the trails they leave in droplets of condensation on the lids of Petri dishes (Figure 9.1). Often their first detection signifies a major outbreak.

Prevention through sound hygiene is better than having to control an outbreak. Sound hygiene includes:

- examining, as soon as possible, all cultures that come into the laboratory for mites — if the culture is to be retained, a subculture should be taken and the original plate discarded by autoclaving
- having separate incubators for clean cultures and primary isolation plates
- autoclaving old cultures and surplus plant material as quickly as possible
- regularly cleaning all surfaces with 70% alcohol and, once a week, swabbing workbenches and the interior of incubators with a non-fungicidal acaricide
- keeping culture plates sealed with plastic film or cling wrap at all times; however, mites will eventually penetrate cultures stored in this way
- destroying infested and contaminated cultures by autoclaving, or if a culture is valuable and cannot be replaced, placing it in a freezer for 24 h to kill the adult mites and eggs, and subculturing it onto fresh medium and destroying the original plate (some fungi will not survive freezing).

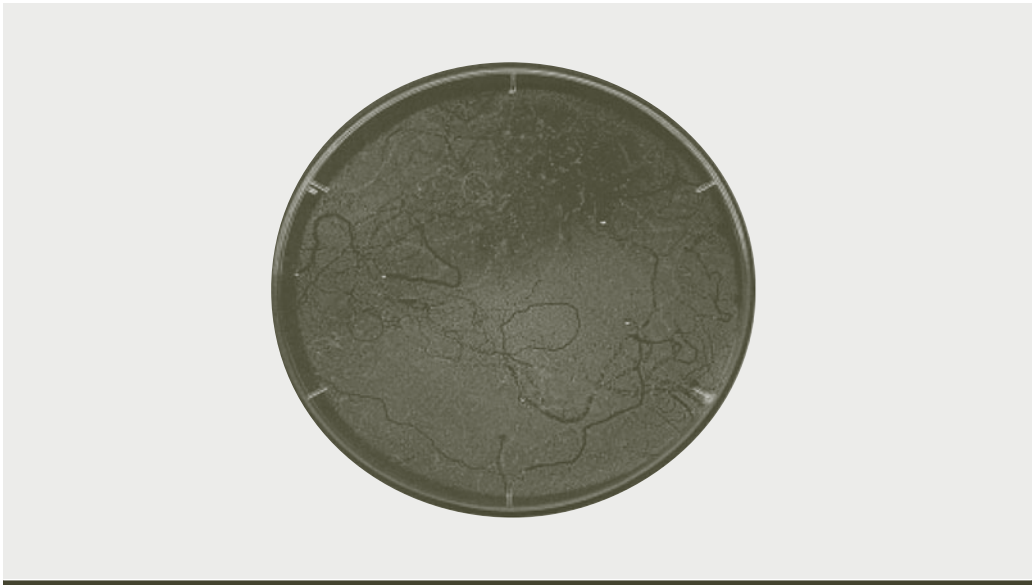


Figure 9.1 Mite trails left in droplets of condensation on the lid of a Petri dish

9.4 Loans

Herbarium specimens are generally made available via short-term loans for the purposes of scientific research. Protocols are necessary to ensure the safety and security of the specimens. It is advisable to lend material only to other herbaria where safe and secure transport and storage arrangements exist. Loan requests should be declined, in whole or in part, if the curator of the herbarium considers the request excessive, disruptive or incompatible with existing or proposed herbarium research programs, or feels that specimens might be subject to misuse, damage or deterioration.

Specimens sent overseas are sometimes subject to quarantine treatments on arrival. These treatments may include heat treatment, fumigation and gamma irradiation. These treatments may damage the specimens or affect their deoxyribonucleic acid (DNA). It is the responsibility of the curators of collections to take every measure to ensure that specimens lent and borrowed are properly looked after. If there is any doubt about how a specimen will be treated or whether a particular quarantine treatment is damaging, then the specimen should not be lent.

The normal loan period is between 6 and 12 months, but loan extensions may be granted upon request. The herbarium lending the specimens should request that loans are returned as soon as possible after the completion of study. Partial returns of loans are generally acceptable, but must be negotiated with the curator of the herbarium.

A condition of the loan must be that the material is stored under secure conditions. Herbarium packets should not be bent, folded or handled in any way that causes deterioration to the specimens. Except during actual examination, specimens should

remain in their original packets. The original packets must also be returned with specimens. All specimens should be carefully packaged to prevent damage during transit.

Loans are made for non-destructive examination of herbarium specimens and microscopic observation of pathogen morphology for taxonomic research purposes. No material or extracts may be removed from loan specimens for permanent retention, or for transmission to any third party, without written permission from the curator of the lending herbarium. The removal or dissection of parts of loan specimens and extraction of DNA for research purposes is sometimes allowed. Removal of material from a specimen must be done carefully and only when the host organ of interest is in sufficient quantity to leave similar material intact. Particular restraint must be exercised when dissecting type specimens.

Annotation slips should be used to record relevant information, including nomenclatural and taxonomic information. All annotation slips should be on archival quality paper.

Below is a list of the all the information required for a valid loan record. A copy of this loan record should be sent with the herbarium material lent and the curator of the herbarium should retain a duplicate copy.

1. Loan number (unique loan identifier).
2. Name and contact details of the scientist requesting the loan.
3. Loan purpose (the nomenclatural and taxonomic scope of the research).
4. Commencement date and return date of the loan.
5. Objects on loan (a list of all the individual herbarium specimens borrowed).

All loans must be authorised by the curator of the herbarium, and the loan record should be signed and dated by this person before sending the material.

9.5 Security

There are two aspects to providing security for a collection. One is ensuring its physical security and the other concerns the ethical and legal responsibilities of the collection's custodian.

9.5.1 Physical security

To ensure the physical security of plant disease collections, it is advisable that they are not open to the general public. The collection should be housed in a secure, weatherproof building, preferably surrounded by a lockable fence with security patrols at night.

Herbarium specimens should be stored on robust metal shelves or in metal cabinets. The herbarium should be fitted with an automated fire-extinguishing system, preferably

gas (water can do as much damage as fire to dried herbarium specimens). Hand-held fire extinguishers should also be situated throughout the building, and staff should be trained in the use of these systems.

Security of data is also important. It is essential that data stored in an electronic format (eg spreadsheets and databases) be backed up at regular intervals (each day). Sophisticated database programs such as KE EMu (discussed more fully in Chapter 8) can control the ability of particular users to view data and edit records.

9.5.2 Ethical and legal responsibility

Any institution that maintains a collection of value to the scientific community has an ethical and legal responsibility to ensure that the collection in its care is protected, secured, cared for and preserved. It is therefore essential that the institution minimise the use of scientifically unsound techniques, bad environmental conditions and poor handling in order to protect the specimens for present and future needs.

The institution responsible for the collection should develop policies and procedures that provide a written framework for collection management, care and use. It is essential that the institution provide the resources, including qualified technical and professional staff, money, appropriate space and equipment, needed for the long-term preservation and documentation of the collection in its care. In some cases the value of plant disease collections has been recognised by governments who have enacted specific legislation to protect them. For example, the New South Wales Plant Pathology Herbarium (DAR) is protected under the *Agricultural Scientific Collections Trust Act 1983* (NSW).

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