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Guide to Delivering Phytosanitary Diagnostic Services



Ministry for Primary Industries
Manatū Ahu Matua





Guide to Delivering Phytosanitary Diagnostic Services

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We would appreciate your feedback through a fast and easy two-question survey here: <https://www.surveymonkey.com/r/diagnosticsguide> This will help the IPPC Secretariat and Capacity Development Committee strengthen this and other training resources.

This paper presents a guide to establishing a plant pest diagnostic laboratory and was created as a component of the IPPC National Phytosanitary Capacity Building Strategy, which was adopted by the fifth session of the Commission on Phytosanitary Measures (CPM) (2010) of the IPPC. This work has been developed by selected experts and reviewed by the IPPC Capacity Development Committee (including phytosanitary experts from the seven FAO regions), the technical consultation among regional plant protection organizations (RPPOs) and the IPPC Secretariat. The elaboration of this manual was possible thanks to the financial contribution of the Standard and Trade Development Facility (Project STDF 350).



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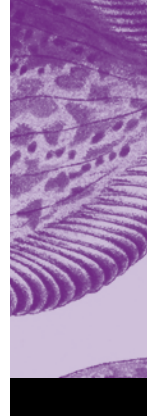


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Acronyms and Abbreviations

APS	American Phytopathological Society
AQIS	Australian Quarantine and Inspection Service
BSPP	British Society for Plant Pathology
DAS	Double-antibody sandwich
DIC	Differential phase contrast
DMZ	Data management zone
DOI	Digital object identifier
DPV	Descriptions of plant viruses
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPPO	European and Mediterranean Plant Protection Organization
EU	European Union
Fera	Fera Science Ltd
FTP	File Transfer Protocol
GMO	Genetically modified organism
HTTP	Hypertext Transfer Protocol
HVAC	Heating, ventilating and air conditioning
IAA	Isoamyl alcohol
ID	Identification
IDCR	Investigation and Diagnostic Centres and Response
IgG	Immunoglobulin G
IP	Internet Protocol
IPPC	International Plant Protection Convention
ISO	International Organization for Standardization
ISPM	International Standards for Phytosanitary Measures
ISPP	International Society for Plant Pathology
IT	Information technology
LAMP	Loop-mediated isothermal amplification
LAN	Local area network
LFD	Lateral flow device
LIMS	Laboratory information management system(s)
M-MLV	Moloney murine leukemia virus
MPI	Ministry for Primary Industries, New Zealand
MSDS	Material Safety Data sheets
NASH	Nucleic acid spot hybridization
NCBI	National Center for Biotechnology Information
NPPO	National plant protection organization
PBST	Phosphate-buffered saline with Tween
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PHEL	Plant Health and Environment Laboratory

PQR	EPPO Plant Quarantine Data Retrieval system
ProMED	Program for Monitoring Emerging Diseases
PVP	Polyvinylpyrrolidone
QIF	Quality improvement
QMS	Quality management system
R&D	Research and development
RPPO	Regional plant protection organization
RT	Reverse transcript
SDS	Sodium dodecyl sulfate
SDW	Sterile distilled water
SOPs	Standard operating procedures
SPS	Agreement on the Application of Sanitary and Phytosanitary Measures of the WTO
TAF	Triethanolamine fixative
TAS	Triple-antibody sandwich
TE	Tris EDTA buffer
UV	Ultra violet
WAN	Wide area network
WTO	World Trade Organization

Introduction

The Secretariat of the International Plant Protection Convention (IPPC), under the supervision of the IPPC Capacity Development Committee, has developed a series of technical resources to support national-level implementation of the IPPC. These resources are intended to provide information and options that support implementation of national rights, responsibilities and obligations under the IPPC.

This guide provides information to support the establishment, operation and maintenance of diagnostic laboratories and services in order to support national phytosanitary systems.

Why a guide to diagnostic laboratories?

Phytosanitary systems are important to prevent the introduction and spread of plant pests.

The IPPC is the international legal agreement that is the foundation of phytosanitary measures. The IPPC has been adopted by 182 contracting parties, who cooperate to safeguard food security, protect the environment and facilitate international trade.

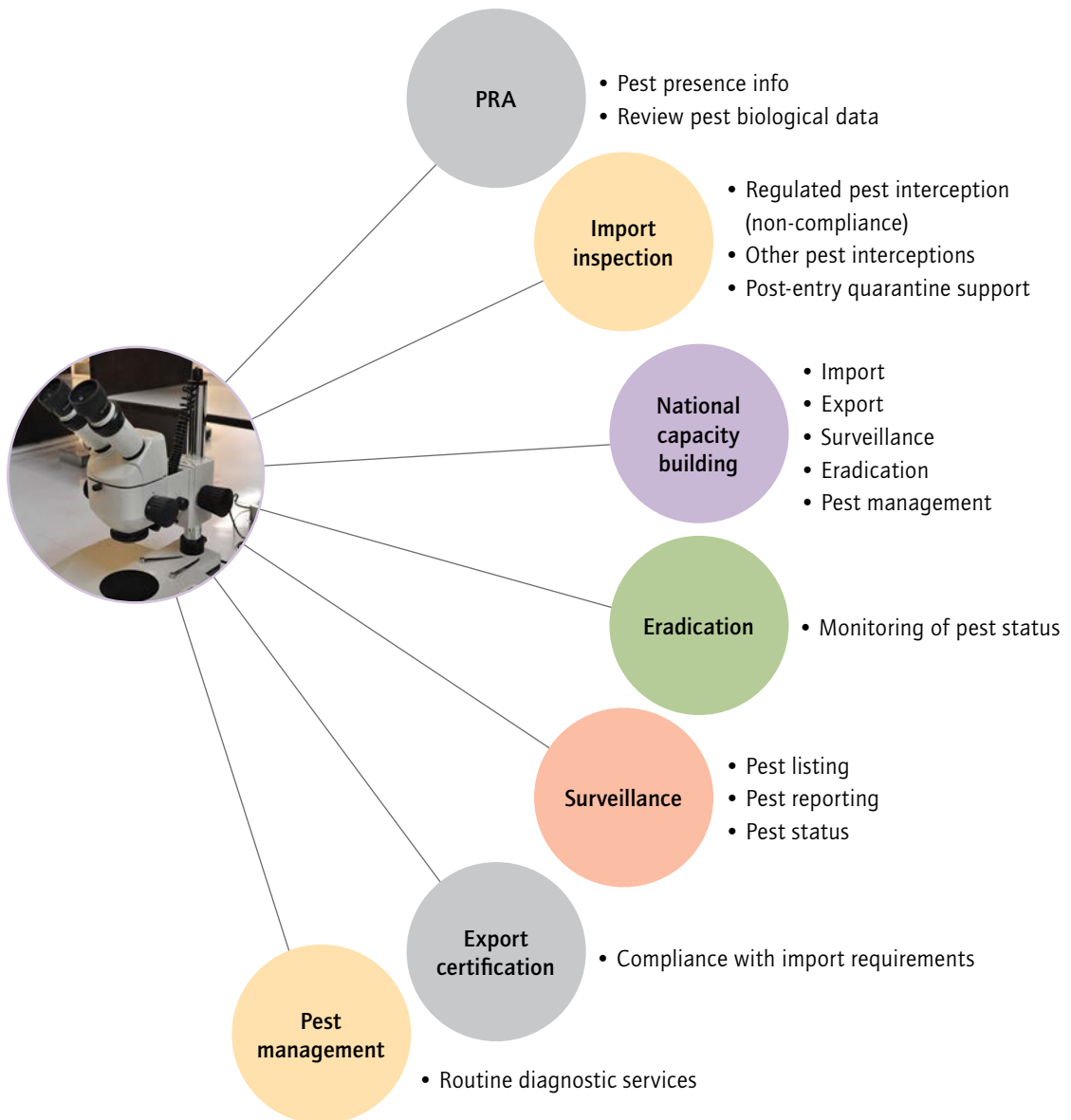
The IPPC adopts International Standards for Phytosanitary Measures (ISPMs) that set out requirements for different kinds of actions. These ISPMs provide the basis for countries enacting plant health measures that can have far-reaching consequences, and they are recognized by the World Trade Organization's Agreement on Sanitary and Phytosanitary Measures (WTO-SPS). According to the Convention text, plant health measures must be technically justified and consistent with the risk presented. In order to be technically justified, many of the phytosanitary procedures and systems required under the IPPC rely on accurate diagnostics. Diagnostic services are therefore essential for the fulfilment of obligations and responsibilities under the IPPC, as well as under the WTO-SPS agreement.

NPPO responsibilities and obligations that rely on diagnostics include (Figure 1):

- ◆ pest risk analysis (IPPC Article IV.2(f); ISPMs 2 and 11), because diagnostics can provide essential information to clarify which specific pest risks need to be analysed
- ◆ establishment of appropriate phytosanitary import measures (Article VII; ISPM 20), because these measures should be based on pest risk analysis, which should be supported by diagnostics
- ◆ import verification (Article VII; ISPM 20) and inspection (Article VII; ISPM 20) and notification of non-compliance (Article VII; ISPM 13), because diagnostics are essential to ensure the accurate identity of the pest intercepted
- ◆ surveillance (Article VII.2(j); ISPM 6), because diagnostics provide essential information on organisms collected through specific surveys, in order to provide accurate information on pest status (Article VII.2(j); ISPM 8), which contributes to regulator actions such as inclusion of pests on lists of regulated pests (ISPM 19) and pest reporting (ISPM 17).

Diagnostics are thus fundamental to science-based phytosanitary measures. Accurate pest information requires diagnosis of pest identity; the ability to offer accurate and timely diagnostic services and to report on the results of such diagnoses is thus crucially important to the functioning of a national plant protection organization (NPPO) and the implementation of the IPPC. While many NPPOs undertake to operate a diagnostic laboratory themselves, others outsource some of the work required. This guide discusses the physical, financial and personnel requirements that NPPOs need to be able to call on in order to fulfil their obligations and also offers suggestions for how to conduct various activities.

Figure 1: The range of phytosanitary programmes to which diagnostic information and expertise may contribute



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How to use this guide

This guide is intended for managers of technical programmes within NPPOs in order to set up and sustain a functional diagnostic laboratory system. It brings together, in one place, some of the operational and functional considerations that lie behind providing a diagnostic service. These include establishing a diagnostic laboratory and some guidance on the kinds of policies and procedures that need to be in place, based on the experience of established laboratories. The contents have been compiled from information and experiences provided by experts from different disciplines and countries, with input from the IPPC Capacity Development Committee and the IPPC Secretariat. However, because each laboratory and service should be designed with its specific purposes and context in mind, readers should use this guide as a source of general information only. There are additional sources of information and specific practical experience (some of which are listed in section 3). NPPOs are encouraged to explore different approaches.

This guide covers the work of official laboratories providing diagnostic services to NPPO procedures such as surveillance, pest risk analysis, export certification and import verification. Some NPPO diagnostic laboratories serve multiple functions. For example, a national policy may give a diagnostic laboratory the mandate to provide official phytosanitary diagnoses for regulatory purposes in addition to diagnostic services for other purposes, such as domestic programmes that may include walk-in services for producers and others who need pest status certification. Furthermore, within a country there may be other laboratories that do not provide phytosanitary diagnostics, but that offer other diagnostic functions, for example, related to research, education and walk-in services for producers. This guide does not cover authorization of those laboratories for NPPO services.¹

Regarding diagnostic procedures and the specific methods discussed, the guide is not intended to be exhaustive or prescriptive. The reader should keep in mind that there may be other relevant methods that can be used. However, regardless of

the methods selected, the diagnostic staff should follow relevant “best practice” necessary to achieve intended results.

Note that this guide does not:

- ◆ discuss details on options for the authorization or supervision of external laboratories or service providers – an IPPC ISPM on this topic is being developed
- ◆ provide detailed guidance on the use of specific diagnostic techniques
- ◆ provide prescriptive guidance on how diagnostics should be performed or quality management procedures used.

In cases where technical information is provided, it is an illustration of functional procedures that have been used elsewhere, but should not be interpreted as the only correct procedure to use.

Guide structure

The guide is organized in two main sections that deal with setting up and managing a diagnostic laboratory and sample workflow, respectively. The first section considers the operational basis of the laboratory and the hard and soft infrastructure needed to deliver diagnostic services. The second section considers the stages through which a sample progresses through the diagnostic process, from initial reception through to the final fate of the sample. In section three, the guide suggests sources of information and expertise that NPPOs may wish to use.

Inputs are the required foundations for delivery of the service and are the basis of section 1.

¹ ISPM 5 (*Glossary of phytosanitary terms*) contains the definition of “official”, which covers what activities a national plant protection organization (NPPO) can perform to address any component of the phytosanitary system of a contracting party under its own responsibility and authority.

Official is: established, authorized or performed by a national plant protection organization (ISPM 5).

On many occasions the use of the term “accreditation” has been discussed in the IPPC framework, to refer to the authorization of entities other than NPPOs to perform phytosanitary actions on behalf of NPPOs but always under the responsibility of the NPPO.

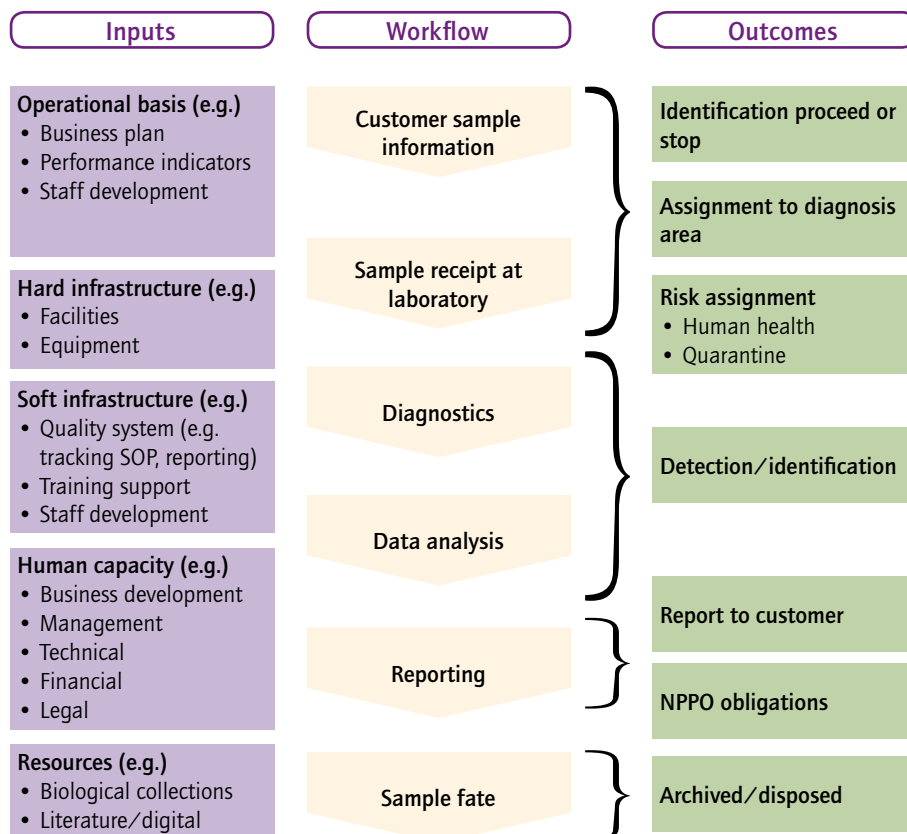
These discussions have arrived at the conclusion that NPPOs are not legally entitled to act as accreditation bodies at national level and that the preferred language should follow the terminology used in the definition of “official”: using “authorization of entities other than NPPOs to perform phytosanitary actions on behalf of NPPOs”.

Chapter 1 explains the need for a sound operational basis for the diagnostic service, including the necessary staff and skills and practical business plan that secures the future of the laboratory. Hard infrastructure (Chapter 2) covers not only the kinds of facilities and equipment that are required, either within the NPPO’s laboratory or at an outside supplier, but also the management of those facilities. Soft infrastructure (Chapter 3) includes quality assurance systems and the tracking systems and standard operating procedures (SOPs) on which such systems depend. Included here too are the reporting systems that ensure that information about specific diagnoses reaches the stakeholders who require this information. Some of this reporting is a formal obligation, some is matter of good practice and transparency. A final input element is the additional information needed to verify diagnoses.

Section 2 mirrors the workflow along which many of the samples received by the laboratory will

move (Figure 2). The process begins (Chapter 4) with the arrival of a sample, along with information about the context in which it was obtained. The sample needs to be registered and examined in order to determine what to do with it. Chapter 5, the bulk of the section, focuses on diagnosis: what methods are available, which disciplines need to be involved, the need to record useful images of the sample, the potential for remote diagnosis and the use and maintenance of reference collections. Chapter 6 offers an introduction to creating useful images of specimens, which is an essential element in remote diagnostics (Chapter 7). Reference collections, essential for good diagnoses, are covered in Chapter 8. A verified diagnosis needs to be reported to all interested parties (Chapter 9) and decisions made about the final fate of the sample (Chapter 10), which may need to be destroyed, archived as evidence or for reference, or in some cases returned to its owner.

Figure 2: The operation of the diagnostic laboratory is divided into two main areas – inputs and outcome – that are mirrored in the structure of the guide



Section 1 – Diagnostic Laboratory

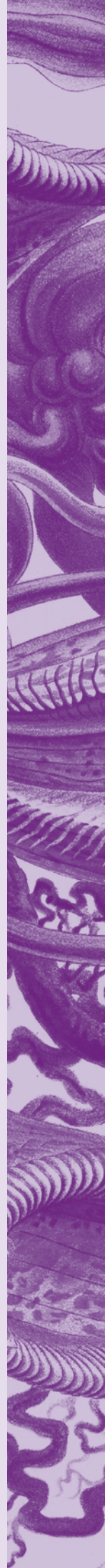
Introduction

The NPPO, as required by ISPM 27 (*Diagnostic protocols for regulated pests*), is responsible to “perform” or otherwise “authorize” plant pest identification services that support national plant pest surveillance or surveys. In fulfilling this obligation various models of operation can be envisaged, from in-house services to out-sourcing to authorized independent entities, with any combination between these operational models.

This guide sets out the types of capabilities that allow identification of all pests as if these capabilities were housed within a single NPPO. It is recognized that this presents an idealized model and that cooperation and coordination of finite

technical resources among NPPOs, particularly within a regional plant protection organization (RPPO), is a necessary activity and a core spirit of the Convention.

While this document is a “How to technical guide” for pest identification, it is important to recognize the operational background that enables technical capabilities. Set out in the chapters that follow are some of these operational considerations that should be given high priority in establishing, operating and maintaining a pest identification capability. An NPPO will not be effective if it neglects these areas through an overemphasis on acquiring technical skills and infrastructure.





1. Operational Basis of Diagnostic Laboratory

Introduction

The NPPO should always strive for excellence in the delivery of services. It will therefore need to identify priority programmes and activities and ensure that adequate resources are available. NPPOs need a stable and adequate funding base. In many cases, government budgetary provisions to NPPOs are insufficient and can change from year to year with changing government priorities. NPPOs should ensure that the national legal frameworks make adequate provision for charging fees for services where applicable. They should also negotiate an arrangement where part or all of these fees are retained for the operation and constant improvement of the NPPO.

Note that the NPPO is not required to possess all the required competencies and facilities, but it certainly needs to have access to them. Collaborating institutions and service providers should be identified for all phytosanitary programmes, including surveillance, diagnosis, treatment and import regulation. External services can be engaged through collaboration or authorization systems, where services are provided but the ultimate responsibility remains with the NPPO.

1.1 Sustainability considerations

The role of the NPPO's phytosanitary laboratory in contributing to national development relies on the sustainability of its services. Sustainability should therefore be considered in the NPPO's establishment to ensure that it functions effectively and predictably. The NPPO should ensure that its phytosanitary diagnostic laboratory has the conditions that contribute to its sustainability in order to support the various NPPO phytosanitary programmes that rely on its services. The conditions to consider are as follows:

- ◆ adequate numbers and appropriately trained staff with the required levels of competency

- ◆ good staff development and retention
- ◆ access to resources and secured sources of funding
- ◆ resources for dealing with phytosanitary emergencies and crises
- ◆ access to additional expertise via third parties (national or international) if the laboratory does not possess them.

In many countries, diagnostic services are provided at no cost to the users. However, even in such cases, the NPPO should ensure that the necessary resources are factored into its budget to ensure long-term provision of services at the quality and dependability expected by its user base.

1.1.1 Competencies and shared resources

In order to provide the range of services that will have been determined by national priorities, the phytosanitary diagnostic laboratory needs various competencies and specialized skills. For example, subject specialists may be needed to perform routine or specialized pest diagnostics in entomology, plant pathology, nematology, weed science and others. While the NPPO is not required to possess all the required competencies, it needs to have access to them. This can be through collaboration or authorization systems, but the responsibility lies with the NPPO. The use of an authorization system should be supported by a considered cost-recovery mechanism. When establishing (and operating) a phytosanitary diagnostic laboratory, the contracting party should be aware of national, regional and international bodies that may be able to provide additional resources beyond those of the NPPO. National institutions such as universities, relevant research institutions, regional organizations (e.g. RPPOs), centres of phytosanitary excellence, private companies and international organizations all represent resources that may be tapped.

Prerequisites for successful sharing of resources will include the following.

- ◆ The NPPO or the contracting party should establish instruments of collaboration (e.g. authorization, letters of agreement, contracts, memoranda of understanding) with these collaborating institutions and service providers to ensure its laboratories are served in a timely manner.
- ◆ Collaborating institutions and service providers should be made aware of the national obligations to be fulfilled under relevant international conventions.
- ◆ Protocols, manuals or SOPs should be developed for the collaborating institutions and they should be trained on these to ensure that the integrity of their inputs is not compromised.
- ◆ The collaborating institutions acting on behalf of the NPPO should be authorized, monitored, audited and held to approval requirements established by the NPPO.
- ◆ Performance and instruments of collaboration should be reviewed, as necessary.

1.2 Strategic plans

A strategic plan sets out where an organization is going over the medium to long term, how it is going to get there and how it will know whether it got there. The planning process provides an opportunity for partners and staff to establish a common language and to engage in the NPPO's work to achieve its goals. The plan will include a clear vision, mission statement, strategic objectives and organizational culture, as well as detailed action plans.

The strategic objectives of the NPPO should be defined for a set time (e.g. five years) and guided by the desire to fulfil its mandate, as defined by the IPPC. The ISPMs provide the basis for the application of appropriate phytosanitary measures. Within the context of national development, the broad objectives may be as follows:

- ◆ to protect national plant resources by implementing appropriate phytosanitary measures in imports
- ◆ to facilitate market access and safe international trade in plants and plant products by a robust export certification system

- ◆ to reduce threats to national food security and the environment by protecting plant resources.

Each strategic objective should be supported by defined and achievable activities and results. Achieving the strategic objectives depends largely on the resources available and the support of stakeholders.

The provision of phytosanitary laboratory diagnostic services should be reflected in the NPPO strategic plan and it is therefore important that staff of such services are involved from the early stages of development of the plan. The phytosanitary diagnostic laboratory itself may develop its own strategic plans. These plans, however, should be compatible with the strategic framework developed by the NPPO. Particular attention should be given in all strategic plans to address operational issues, such as having a sound policy on staff deployment (required number, competency and specializations of staff, plus attention to training and competitive salaries) as well as the provision of operational resources necessary on a timely basis for the laboratory to perform the required tests to the required level of confidence.

1.3 Mechanisms for funding

The mechanisms for funding the operations of a phytosanitary diagnostic laboratory are based largely on the institutional arrangements in which the NPPO is embedded and its level of autonomy. Phytosanitary diagnostic laboratories are most efficient and effective when they have an adequate and stable funding base. NPPOs in many countries are funded by government allocations and collected fees. This section describes the principal sources of funds.

1.3.1 Government budget

When NPPOs depend solely on government funding, they may compete with other national institutions. Since government priorities sometimes change, reallocation of funds may have a negative effect on NPPO programmes including the operation of the phytosanitary diagnostic laboratory. Furthermore, allocations may change from year to year and this will affect the ability of the NPPO to pursue its strategic goals. Government funding is usually linked

to approved work plans and may limit flexibility to allocate emergency funding. Staff numbers may be constrained by government approval policies and allocations.

1.3.2 User fees

User fees allow the NPPO and, by extension, the phytosanitary diagnostic laboratories to recover the costs of services in full or in part. A cost-recovery system supports the continuous improvement of phytosanitary services. In many countries, however, user fees go straight into the government treasury and government priorities determine what portion, if any, is allocated to the NPPO for further improvement of its services. There appears to be a growing trend in which part or all of the user fees collected are transferred to the NPPO. In these cases it is important that funds are allocated to cover staffing and operational resources of the laboratory.

User fees should be:

- ◆ fair, uniform and related to the cost of delivering the service
- ◆ reasonable and not presenting a barrier to trade imports and exports
- ◆ reviewed at regular intervals.

1.3.3 Contingency and other emergency funds

The NPPO needs access to extraordinary financial resources so it can respond to phytosanitary emergencies. These include the provision of diagnostic services to cover the increased demand for such services during containment or eradication of an introduced quarantine pest or other pest outbreak. In an ideal situation, the NPPO would have a contingency plan which factors in the required pest diagnostic support and funds supplied by government and industry donors. Without such an emergency fund, the NPPO may be unable to respond to the spread of a quarantine pest, thus making eradication difficult or impossible.

1.3.4 Grants, aid and other contributions

The NPPO may secure significant investment for improved services and infrastructure from extraordinary allocations or special line items from the general treasury, co-funding and partnership

arrangements with the private sector and donations or funding support from international or regional organizations. Investment loans and grants may be obtained by the government or an autonomous NPPO from a developed country or lending institution in cases where clear targets can be met that result in significant benefits to the country.

1.3.5 Securing funds

The NPPO needs to have a good ability to access financial resources to ensure its sustainability. Government's allocation of funding is influenced by its priorities. The NPPO has to be positioned properly in the list of priorities in order to secure adequate funding. It therefore needs to educate all stakeholders, including politicians and consumers, about the role of the diagnostic services in the following:

- ◆ the specific national obligations and functions of contracting parties, as outlined by the IPPC
- ◆ the costs and benefits of conducting these functions
- ◆ problems in accessing or maintaining export markets due to lack of credibility in export certification or in establishing equivalency recognition agreements as alternative sanitary and phytosanitary measures
- ◆ the implications and consequences of the introduction of a quarantine pest on the national economy, food security and environment, and the potential impacts of inadequate pest exclusion on livelihoods.

Consequently, the diagnostic laboratory, supported by its NPPO, should articulate and advocate for an acceptable level of resources based on the importance of its services. Advocacy by the NPPO should be reflected in its strategic plan in order to strengthen its ability to secure the necessary funds and resources it needs to provide satisfactory services to its client base.

1.4 Legal support

The work of the NPPO must operate from an informed legal basis that is cognizant of the full implications that information it releases to the public domain may carry. This is especially true in the context of plant pest identification, where

conclusions reached on the identity or detection of a pest may carry substantial trade consequences and concomitant financial losses accruing with affected commercial sectors. Accordingly, the NPPO must have access to appropriate professional legal advice, either through in-house staff, or via third-party arrangements.

1.5 Human resources

The staff of an NPPO are its primary resource and there is a shortfall of appropriately skilled individuals across the world. Human resources are thus a key element, working with management to provide effective opportunities for staff training, professional development and succession planning. See also sections 3.2 and 3.3 in Chapter 3 Quality Systems.

1.5.1 Roles and responsibilities of laboratory staff

The following section describes the generic skill sets important to the operation of a diagnostic laboratory. The number of staff in each of the roles will depend on the overall demand for the diagnostic services. Apart from basic financial constraints, the human resource capacity and degree of specialization within staff is driven by the origin, diversity and volumes of samples being processed, expected turnaround times and the need to quickly expand capacity to support critical events such as an incursion response.

1.5.1.1 Technical support

Technical support staff are involved in a variety of laboratory-based activities.

The key responsible areas include:

- ◆ receiving samples and logging samples into database (may also be done by administrative support)
- ◆ preparing and processing samples for identification
- ◆ disposing of samples or preparing specimens for inclusion in a reference collection
- ◆ imaging specimens for image library
- ◆ preliminarily identifying pest
- ◆ maintaining reference and image collections.

1.5.1.2 Diagnostic expert

Diagnostic experts provide diagnostic and advisory services for exotic and emerging pests and diseases affecting plants or the terrestrial environment in surveillance, investigation, border and post-border identification and post-entry quarantine programmes.

The key responsible areas include:

- ◆ definitive specimen identification
- ◆ scientific analysis, reporting and advice via critical examination of data, interpretation of results, and trend analysis and implications
- ◆ advice on national diagnostic standards
- ◆ project management
- ◆ preparation of images for the image library.

1.5.1.3 Facility management and administrative support services

The technical nature of plant pest diagnostic services requires strong facility management and administrative support, especially in the areas of equipment, facilities and maintenance. Having staff who have the skill to maintain equipment and to organize contract repairs is essential for the day-to-day operation of laboratories. It is also critical to have an understanding of the costs associated with depreciation and replacement of equipment and facilities. Building such costs into the business plan is a necessary part of the NPPO operation.

The key responsible areas include:

- ◆ management of financial resources
- ◆ procurement and inventory of equipment and supplies
- ◆ management of information technology (IT) systems
- ◆ building maintenance (HVAC, plumbing, etc.)
- ◆ maintenance of proper functioning of biowaste systems and biosecurity systems.

1.5.1.4 Laboratory manager

The role of management must be set out and this role is often best assigned to personnel with designated management functions. These individuals take responsibility for a variety of non-scientific tasks, but mostly ensure that the technical capability operates with maximal efficiency. It would be the

role of management to monitor the operation of the service against key performance indicators. Critical deliverables of professional development and staff succession planning also fall within the role of management.

The key responsible areas include:

- ◆ functional leadership
- ◆ people management (staffing, training needs, etc.)
- ◆ business and strategy
- ◆ finance and contracts.

1.6 Training laboratory personnel

Under Article IV.2(h) of the IPPC, the responsibilities of the official NPPO shall include "training and development of staff". Effective training, the opportunity to build experience and demonstrate competency will all ensure that successful and robust diagnosis is achieved.

A number of training sessions may be required until the trainer and trainee agree that the trainee is competent to undertake the task unsupervised, and all training sessions should be documented. All stages of the training must be recorded and shall cover:

- ◆ Stage 1 – Reading relevant instructions (e.g. SOPs)
- ◆ Stage 2 – Observing the task being performed by a trained member of staff
- ◆ Stage 3 – Carrying out the task under supervision

- ◆ Stage 4 – Assessment of competence to carry out the task unsupervised.

Wherever possible, evidence or experience used as part of the assessment of competence should be recorded. Competence is assessed using at least one of the following, where possible:

- ◆ spiked recovery experiments
- ◆ repeat analysis of previously analysed samples
- ◆ analysis of reference or proficiency test materials
- ◆ comparison of results of trainer and trainee.

Criteria of acceptance are documented and are normally set at the quality control acceptance limits for the method. The date of authorization to carry out a task unsupervised is recorded on the form along with the confirmation by the line manager. Line managers should ensure that the evidence presented and documented is correct and appropriate.

It is acceptable that staff may self-train in certain procedures (e.g. administrative procedures or simple experimental techniques) by reading the SOP. In these cases, the individual will indicate on the training record that self-training took place.

Laboratories will often try and participate in proficiency testing schemes. Proficiency testing determines the performance of individual laboratories for specific tests or measurements and is used to monitor laboratories' continuing performance. This testing will also provide ongoing evidence of an individual's competency.

2. Hard Infrastructure of the Plant Diagnostic Laboratory

Introduction

The recommendations in this chapter are mainly based on the existing Plant Health and Environment Laboratories (PHEL) established in the Investigation and Diagnostic Centres and Response (IDCR) group of the Ministry for Primary Industries, New Zealand. The following references were also considered:

- ◆ **Burgess, L.W., Knight, T.E., Tesoriero, L. & Phan, H.T.** 2008. *Diagnostic manual for plant diseases in Vietnam*. ACIAR Monograph No. 129. Canberra, Australian Centre for International Agricultural Research. 210 pp. Available at http://aciarc.gov.au/files/node/8613/mn129_diagnostic_manual_for_plant_diseases_in_viet_13726.pdf (last accessed 17 September 2015).
- ◆ **WHO Regional Office for South-East Asia.** 2008. *Guidelines on establishment of virology laboratory in developing countries*. New Delhi, World Health Organization Regional Office for South-East Asia. Available at http://apps.searo.who.int/PDS_DOCS/B4249.pdf?ua=1 (last accessed on 17 September 2015).
- ◆ Setting up a diagnostic laboratory. In **M.A. Connolly, ed.** *Communicable disease control in emergencies – a field manual*. Geneva, World Health Organization, pp. 253–267. Available at <http://www.unhcr.org/456c3ce92.pdf> (last accessed 17 September 2015).
- ◆ **Skoglund, L.G. & Blunt, T.** 2012. *The plant diagnostic lab experience*. St Paul, MN, American Phytopathological Society. APSFeatures 2012-05. DOI: 10.1094/APSFeature-2012-05. Available at <http://www.apsnet.org/publications/apsnetfeatures/Pages/diagnostician.aspx> (last accessed 17 September 2015).

2.1 Functions of the diagnostic laboratory

Rapid and accurate diagnosis of plant health problems is the main service of the plant diagnostic

laboratory, which is essential to maintain healthy farms, crops, forests, landscapes and public spaces, as well as to protect countries from exotic pests and diseases across borders.

The diagnostic laboratory is responsible for:

- ◆ providing rapid and accurate diagnosis of pests and diseases
- ◆ recording and maintaining data on pest occurrences
- ◆ detecting and tracking new and invasive pests
- ◆ facilitating responses to clients
- ◆ delivering timely and cost-effective services.

The diagnostic laboratory can support extension, research and training at the state or country level and may include crop survey work and phytosanitary regulatory services.

The diagnostic laboratory can provide diagnostic services in the following disciplines: bacteriology, botany, entomology, mycology, nematology and virology. The following tests can be carried out in the laboratory.

- ◆ Bacteriology and mycology: Isolation and culturing of fungi and bacteria allows their identification by morphological, biochemical and molecular analyses. The maintenance of a culture collection of exotic fungi and bacteria (positive controls) is required to allow comparative tests to be made for accurate identification.
- ◆ Botany:² Identification of weeds and diseased host plants for other disciplines.
- ◆ Entomology: Invertebrates are identified using morphological examination, comparison with identification keys and reference specimens, and molecular methods. Immature invertebrates may be reared through to later stages or adults so that a more accurate taxonomic determination can be made, or to collect reference material;

² Botany can be integrated into other disciplines or the service can be provided by local herbaria, museums and universities.

if the organism is suspected to be exotic, this should be undertaken in a containment rearing room. All reared invertebrates should be killed on completion of the work.

- ◆ Nematology: Extraction of nematodes from substrates and identification by morphological and molecular analyses.
- ◆ Virology: A variety of methods can be used to identify and characterize plant viruses, viroids and non-culturable organisms such as phytoplasmas and *Liberibacter*. Depending on the diseases, the following tests may be carried out: transmission electron microscopy, herbaceous and graft indexing, serology and molecular methods.

The diagnostic laboratory should comply with the following principles:

- ◆ it should be able to undertake the types of test required
- ◆ it should be large enough to handle required sample throughput
- ◆ it should be safe and comfortable for the staff
- ◆ it should be sustainable for the long term.

To meet these needs the laboratory should have:

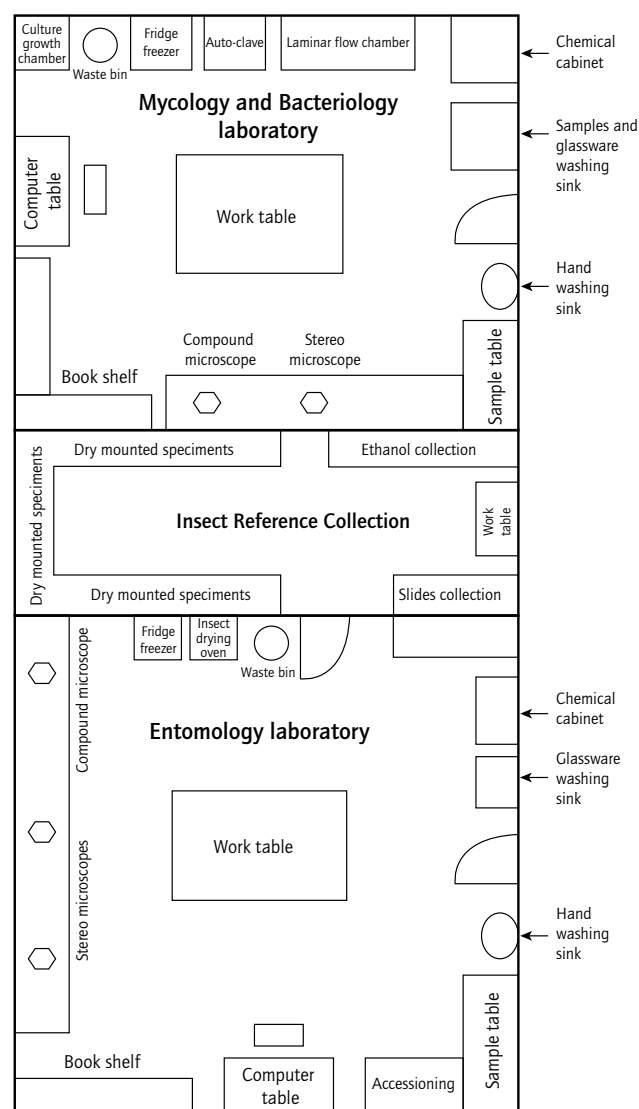
- ◆ a suitable building or room(s), appropriately laid out and furnished
- ◆ adequate number of staff who are trained and competent in the tests to be undertaken
- ◆ internal and external quality control to ensure consistency and accuracy of output
- ◆ a safety policy based on the tests undertaken and the risks posed by the organisms
- ◆ technical and logistic support.

2.2 Laboratory site

The diagnostic laboratory should be established in a structurally sound permanent building. The internal walls and floors should be sealed with paint or suitable sealant so that these can easily be cleaned or disinfected.

Preferably, the diagnostic laboratory should be separated into bacteriology and mycology, entomology and nematology, and virology laboratories (Figures 3 and 4). If this is not feasible, all the disciplines could be in the same room with well-designated areas. Areas for

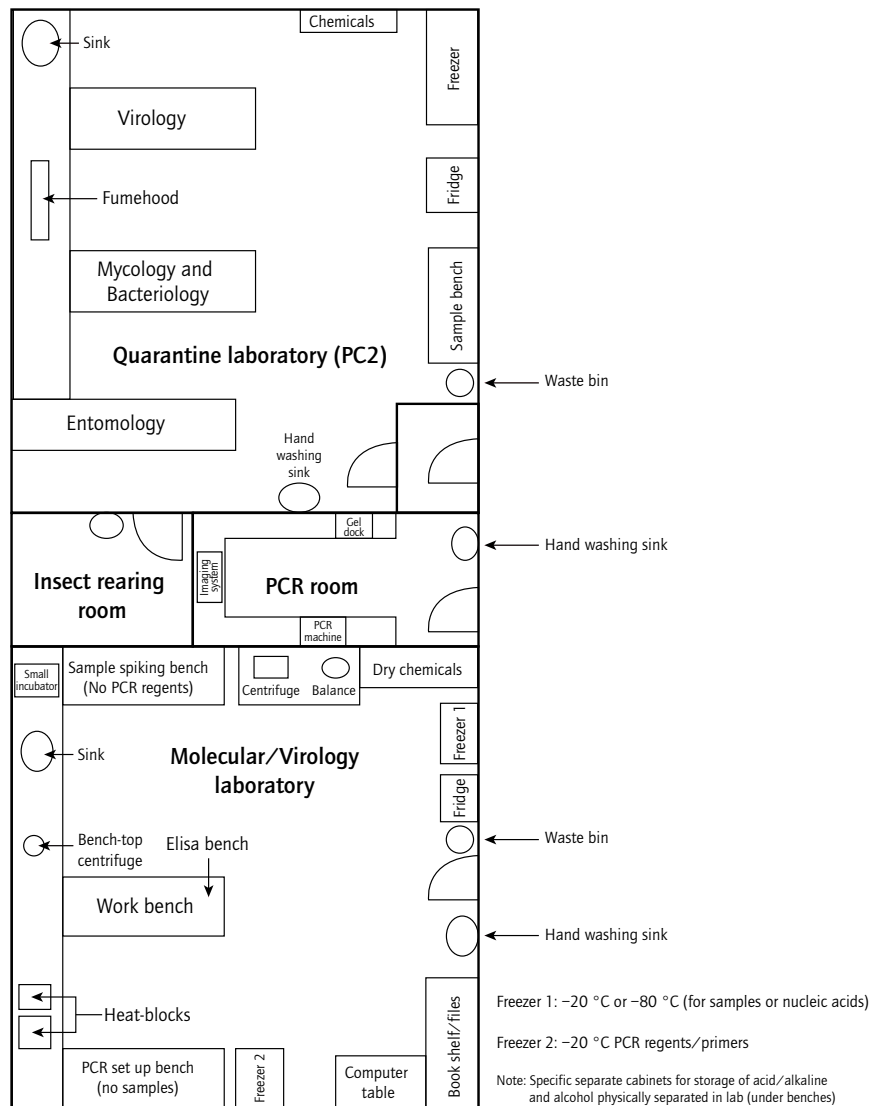
Figure 3: Indicative laboratory plan, showing separation of work areas for different disciplines



sample preparation, polymerase chain reaction (PCR) reagent preparation and DNA amplification should be physically separated from each other to prevent cross-contamination of DNA. If this is not feasible, these areas should be at least well apart from each other within the same room to avoid cross-contamination and staff should follow strict laboratory practices (e.g. changing gloves between the two areas; no movement of pipettes and tips between the different areas).

The laboratory may work with samples varying in potential biosecurity risk, such as samples from interceptions at the border and from transitional facilities. Processing of these high risk samples should be performed in a separate

Figure 4: Separation of molecular and virology units within the diagnostic laboratory



containment area. Suspect exotic invertebrate specimens can be processed into a non-viable state before identification or testing outside the containment area.

2.3 Laboratory facilities

2.3.1 Diagnostic laboratory

- ◆ Work benches, seats and other laboratory surfaces are designed for ease of cleaning with surfaces that are smooth, impervious to water and resistant to acid, alkali and organic solvents. Benches should be of a suitable height (90 cm for work benches and 75 cm for microscope benches).
- ◆ Laboratory seating should be of adequate height to allow comfortable working posture at the bench (stools or height-adjustable chairs) and should be able to be decontaminated with disinfectants when required.
- ◆ Open spaces between and under benches, cabinets and equipment should be accessible for cleaning.
- ◆ Storage space within or outside the laboratory area must be adequate to hold supplies, thus preventing clutter on bench tops and in aisles.
- ◆ An autoclave is required for sterilizing media and buffer.
- ◆ Windows and doors should have locks. If provided with open windows, these should be

fitted with fly-screens. All internal doors should have a vision panel.

- ◆ External doors should not open directly into the laboratory but into a service corridor.
- ◆ Facilities for personal items, outer garments, as well as tea and lunch rooms and restrooms should be provided outside the laboratory working area.
- ◆ Hand-washing basins with running tap water should be provided in each laboratory room, preferably near the exit door.

2.3.2 Containment laboratory

- ◆ A biological hazard symbol with laboratory access restrictions should be prominently displayed near the entrance of the laboratory.
- ◆ Where the laboratory is mechanically ventilated, a directional airflow into the laboratory is maintained by extracting room air.
- ◆ A containment laboratory should not have opening windows.
- ◆ Containment laboratories should be equipped with a basin for hand washing, positioned near the exits.
- ◆ Emergency drench shower should be provided in the laboratory for chemical decontamination.
- ◆ Eyewash station should be provided.
- ◆ An electric insect trap should be positioned in the containment laboratory and be operational at all times.
- ◆ A can of fly spray should be kept in the containment laboratory to knock down flying insects should any escape during the processing procedure. Fly spray should not be used in the insect rearing room.
- ◆ Furniture should be ergonomically suitable for the use for which it is intended.
- ◆ Benches, floors, walls, seats and other laboratory surfaces should be designed for ease of cleaning with surfaces that are smooth, impervious to water and resistant to acid, alkali and organic solvents.
- ◆ Containers for infectious materials and a supply of labelled disinfectants for decontamination purposes should be provided.
- ◆ A freezer or fridge-freezer should be located inside the containment laboratory for the purposes of killing or relaxing invertebrates,

storing diseased or infected plant materials, storing reagents and chemicals, and interim storage of infested host material and packaging, before being placed in the quarantine waste bin for destruction.

- ◆ A quarantine waste bin should be double lined with bin liners and the inner liner (i.e. the one into which waste is placed) must be biohazard marked.
- ◆ An autoclave should be located inside the containment laboratory for sterilizing infectious material prior to disposal in the quarantine waste bin.
- ◆ Where significant quantities of infectious aerosols or spores are likely to be produced, a Class II Biohazard (Biological Safety) cabinet should be used.

2.4 Contingency plans – quarantine laboratory

All accidents and incidents involving loss or escape of exotic organisms (including genetically modified organisms (GMOs) developed for diagnostic purposes) will be reported to the relevant organizations promptly after the event is noticed (e.g. within 24 h of the breach being detected).

The following will be reported immediately to the Laboratory Manager or Operator of the Facility:

- ◆ any accident or incident involving exotic organisms
- ◆ any loss or escape of exotic organisms
- ◆ any loss or breach of containment
- ◆ any sabotage or suspected sabotage of the containment facility.

Manage spills that may involve micro-organisms or other biohazardous substances. Biohazard spill management is dependent on the risk group of the material and the volume of material spilt. Generally, biohazardous spills in the containment facilities are of a minimally hazardous nature and the potential spill volume is small.

Work shall be planned to minimize the chances of a spill. Clean-up materials and equipment should be kept in appropriate locations and include: "Do Not Enter" and "Biohazard" signs; suitable disinfectant supplies; absorbent materials; protective clothing (e.g. gloves, coats) and appropriate containers

2.4.1 Procedure in event of invertebrate escape

- ◆ Advise any other users in the facility.
- ◆ Ensure any live specimens are secure before leaving the Quarantine Laboratory.
- ◆ Seal off the area, closing doors.
- ◆ Prevent people moving through the area, locking doors if necessary.
- ◆ Search the adjacent area for the escaped invertebrates.
- ◆ Discharge the appropriate amount of fly spray and allow a saturation period of five minutes before re-entering the adjacent area.
- ◆ Widen the search in nearby areas if the invertebrates cannot be located.
- ◆ Advise the Laboratory Manager as soon as possible.
- ◆ Review procedures and adopt corrective action to prevent the incident occurring again.

2.4.2 Procedure following a spill involving new micro-organisms

- ◆ Advise any other users in the facility.
- ◆ Put on protective clothing such as gloves and gowns if not already being worn.
- ◆ If the spill is of a liquid nature and the volume is such that spread is a danger, limit the spread by dropping absorbent materials such as paper towels onto the spill.
- ◆ Use disinfecting agents with a final concentration of 1 percent sodium hypochloride or other commercially available disinfectant.
- ◆ Leave disinfecting agent on spill for at least 30 min for effective disinfection, before mopping up spill.
- ◆ Use the same disinfectant solution to wipe over any surrounding area likely to be contaminated.
- ◆ Transfer all contaminated material and disinfectant solution for disposal into the laboratory quarantine waste stream.
- ◆ Remove any protective clothing to autoclave bags (or quarantine waste bins if disposable) and wash hands thoroughly.
- ◆ Advise the Laboratory Manager and Operator as soon as possible.
- ◆ Review procedures and adopt corrective action to prevent the incident occurring again.

2.4.3 Procedure for personal decontamination

- ◆ Immediately put on (if not already being worn) or change (if necessary) protective clothing such as gloves and gowns.
- ◆ If clothing, skin or hair is contaminated with spore or aerosol release, change into fresh protective clothing and wash the contaminated skin or hair.
- ◆ Advise others working in the same area to take protective measures.
- ◆ Take steps to prevent further spore or aerosol release and clean up the area.
- ◆ Place protective clothing and contaminated outer personal clothing and footwear into separate bags for appropriate secure cleaning (may need to autoclave prior to laundering).
- ◆ Report incident to the Laboratory Manager.

2.4.4 Procedure in the event of fire

- ◆ The lab is required to have fire alarm system.
- ◆ Use a fire extinguisher if it is safe to do so.
- ◆ Follow fire and emergency evacuation procedure immediately.

2.4.5 Procedure to prevent theft and sabotage of containment room

- ◆ Unauthorized removal of viable exotic organisms is not permitted.
- ◆ Only authorized users will be allowed access to the containment facilities.
- ◆ The containment laboratories are key locked after hours.

2.5 Hygiene

Routine cleaning of benches, floors and laboratory equipment will ensure the facilities maintain a high standard of cleanliness.

The cleaning equipment used in the quarantine laboratory must be used only for that purpose and must remain in the facility.

- ◆ **Wet mopping:** Wet mopping with a detergent-based solution will be used for the cleaning of floors. The bucket will be of a type with a wringer attached. Such equipment will be dedicated to and stored within the containment facility.

- ◆ **Dry mopping:** Dry mopping, if used, will be carried out with a mop that has dust-retaining properties.
- ◆ **Vacuum cleaning:** A vacuum cleaner will *not* be used.
- ◆ **Sweeping:** Brooms will *not* be used, as they produce airborne dust that can increase contamination of work in the laboratory.

2.6 Reference collections

Reference collections, where pest organisms are preserved and kept for long term for future reference, are an invaluable resource in the diagnostic process. The reference collection should be established in a structurally sound permanent building with solid walls, roof, floor, ceiling and a door. No windows or skylights should be installed as sunlight could damage the specimens. The reference collection requires protection from atmospheric conditions such as humidity and pests capable of destroying specimens and the following conditions are desirable.

Recommended temperature:

- ◆ 18–19 °C for entomology and herbarium collections
- ◆ –80 °C freezer for DNA extractions, live cultures and infected plant material that serves as positive controls.

Recommended relative humidity:

- ◆ below 50 percent (for entomology and pathology collections).

No vibration effects.

See Chapter 8 on reference collections for more detailed specifications.

2.7 Laboratory services

- ◆ Adequate water supply is essential and a purification system may be required if the water supply could be contaminated. The laboratory will also need to have mineral-free water.
- ◆ Adequate electricity supply is required. If local electricity supplies are intermittent or inadequate, a generator may be needed. The capacity of the generator will be governed by the anticipated load and whether it is required for continuous or occasional use. If

all the laboratory electricity comes from its own generator and some of the laboratory equipment needs to run continuously, a backup generator is essential. If town supplies are intermittent, an automatic system to switch to the generator is required for when the supplies fail.

- ◆ Gas supply is required for Bunsen burners.
- ◆ Adequate drainage system must be provided. If the public drainage system is used, adequate trapping must be fitted to the laboratory waste system to allow for the trapping of any chemical or biological spills to avoid contamination of the public system.
- ◆ Adequate system for disposal of quarantine waste (e.g. incinerator); check your local authority.
- ◆ Insects, rodents and any other pests must be kept out of the laboratory area to avoid secondary contamination.

2.8 Environmental controls (ventilation, temperature and humidity)

- ◆ Ventilation and airflow in a basic diagnostic laboratory can usually be provided using windows and doors. All windows should be fitted with a means of shading them from the sun.
- ◆ In laboratories where pathogens are handled, a unidirectional airflow across and out of the laboratory should be maintained when the facility is in use as to protect the laboratory staff.
- ◆ The laboratory functions cannot be followed easily in extreme temperatures (>30 °C) and humidity. It should be also noted that some diagnostic assays perform unpredictably above 28 °C. The entire laboratory should be air-conditioned to maintain a dust-free environment and ambient temperature of 22–25 °C. Re-circulating air-conditioning is not suitable for microbiological laboratories due to possible recirculation of pathogens.
- ◆ Fans should not be used to avoid dissemination of micro-organisms.

2.9 Standard work practices

- ◆ Access to the facility is limited to specified personnel.
- ◆ Laboratory staff to advise maintenance and service personnel of the special microbiological or other hazards in the laboratory.
- ◆ Facility doors are kept closed at all times.
- ◆ The containment laboratories are also key locked outside of working hours.
- ◆ Authorized users receive instruction in containment procedures and requirements, and training in handling exotic organisms. All authorized users are given annual refresher training.
- ◆ Rearing of exotic organisms should be done in a separate containment rearing room.
- ◆ Laboratory coats and closed footwear must be worn at all times in containment facilities. The laboratory coats are to be removed when leaving the laboratory and left on provided hooks.
- ◆ No drinking, eating or smoking is allowed in the laboratories.
- ◆ Laboratory staff should always wash their hands when leaving the laboratory.

2.10 Laboratory equipment

The equipment required for the laboratory depends on the functions, volume of work, type of specimens, budget, etc. The equipment lists provided below are only guidelines and not comprehensive. In addition, it is possible to share equipment for different applications in the lab across disciplines.

2.10.1 Morphological identification

2.10.1.1 Entomology

To identify insects, the laboratory requires:

- ◆ a compound microscope fitted with $\times 5$, $\times 20$, $\times 40$, and $\times 60$ and $\times 100$ (oil immersion) objective lenses and with a built-in scale in one eye piece for measuring purposes
- ◆ a dissecting microscope (preferably 2–3 depending on the number of users) with $\times 50$ magnification and with a built-in scale in one eye piece for measuring purposes
- ◆ a fridge–freezer for storing materials and killing insects

- ◆ a bookshelf containing text books, manuals and research papers
- ◆ a computer with Internet access for information search, accessing image libraries and e-mail communication.

2.10.1.2 Bacteriology, mycology and nematology

To identify bacteria, fungi and nematodes, the laboratory requires:

- ◆ a dissecting microscope for examining plant samples for fungal structures and nematode extractions, with a built-in scale in one eye piece for measuring purposes
- ◆ a compound microscope fitted with $\times 10$, $\times 20$, $\times 40$ and $\times 100$ (oil immersion) objective lenses and with a built-in scale in one eye piece for measuring purposes
- ◆ a laminar flow chamber for pouring media and isolating from plant tissues
- ◆ a fridge–freezer for storing media in bottles, and petri dishes with media
- ◆ electronic balances with accuracy of 0.1 g and 0.001 g are recommended
- ◆ a water bath
- ◆ a pH meter
- ◆ autoclaves – one for sterilization and one for decontamination
- ◆ hot air oven for sterilizing glassware
- ◆ large work benches, for sample processing, microscopic examination and culture maintenance
- ◆ a bookshelf containing text books, manuals and research papers.

2.10.1.3 Virology

To identify viruses, the laboratory requires:

- ◆ a fume hood (if doing virus purification)
- ◆ fridge or freezers ($-20\text{ }^{\circ}\text{C}$; $-80\text{ }^{\circ}\text{C}$ for storage of plant tissue)
- ◆ a pH meter
- ◆ a magnetic stirrer and hotplate
- ◆ an electronic balance for weighing chemicals (with accuracy of 0.001 g)
- ◆ a bench-top centrifuge
- ◆ pipettes (1 000 μl , 200 μl) and non-filtered tips
- ◆ glassware such as volumetric flasks, measuring cylinders, storage bottles (50 ml, 100 ml,

- 250 ml, 500 ml and 1 000 ml)
- ◆ a water purification or distillation system
- ◆ an autoclave (for sterilization)
- ◆ an ice-making machine
- ◆ grinding equipment: ELISA grinder or mortar and pestle or plastic bag and roller
- ◆ a hot air oven for drying glassware (optional)
- ◆ micro-centrifuge tubes (1.5 ml or 2 ml)
- ◆ shelving and cupboards for chemicals
- ◆ chemical storage cabinets: one for flammable and one for corrosive solutions
- ◆ laboratory coat, gloves, masks and goggles
- ◆ waste bins (quarantine and non-quarantine)
- ◆ tissue and disinfectant wipes
- ◆ a transmission electron microscope with camera
- ◆ forceps and grids
- ◆ stains (uranyl acetate or phosphotungstic acid)
- ◆ high- and ultra-high-speed centrifuges (optional; for virus purification only)
- ◆ a refrigerated bench-top centrifuge
- ◆ plasticware for centrifuge.

2.10.1.4 Immunology testing

To conduct immunology testing, the laboratory requires:

- ◆ a fridge and freezers (–20 °C and –80 °C)
- ◆ a pH meter
- ◆ a magnetic stirrer and hotplate
- ◆ an electronic balance for weighing chemicals (with accuracy of 0.001 g)
- ◆ a bench-top centrifuge
- ◆ pipettes (1 000 µl, 200 µl, 20 µl, 10 µl) and non-filtered tips
- ◆ glassware such as volumetric flasks, measuring cylinders, storage bottles (50 ml, 100 ml, 250 ml, 500 ml and 1 000 ml)
- ◆ a liquid nitrogen container and isothermal gloves (optional; useful for grinding very hard tissue)
- ◆ a water purification or distillation system
- ◆ autoclaves: one for sterilization and one for decontamination
- ◆ an ice-making machine
- ◆ grinding equipment: ELISA grinder or mortar and pestle or plastic bag and roller
- ◆ a hot air oven for drying glassware (optional)
- ◆ micro-centrifuge tubes (1.5 ml, 2 ml)

- ◆ a vortex mixer (optional)
- ◆ shelving and cupboards for chemicals
- ◆ 2 chemical storage cabinets: one for flammable and one for corrosive solutions
- ◆ laboratory coat, gloves, masks and goggles
- ◆ waste bins (quarantine and non-quarantine)
- ◆ tissue and disinfectant wipes
- ◆ an incubator (optional; depends on temperature at which the ELISAs need to be incubated)
- ◆ an ELISA plate reader
- ◆ an ELISA plate washer or squeeze bottles.

2.10.1.5 Molecular testing

To conduct molecular testing, the laboratory requires:

- ◆ a fume hood (may not be required if using commercial kit for extraction)
- ◆ a fridge and freezers (–20°C; –80°C for nucleic acids and some parts of cloning kit storage)
- ◆ a pH meter
- ◆ a magnetic stirrer and hotplate
- ◆ an electronic balance for weighing chemicals (with accuracy of 0.001 g)
- ◆ a bench-top centrifuge
- ◆ pipettes (1 000 µl, 200 µl, 20 µl, 10 µl) and filtered-tips; dedicated pipettes and tips for PCR reagents and nucleic acid spiking
- ◆ glassware such as volumetric flasks, measuring cylinders, storage bottles (50 ml, 100 ml, 250 ml, 500 ml and 1 000 ml)
- ◆ a liquid nitrogen container and isothermal gloves (optional; useful for grinding very hard tissue)
- ◆ a water purification or distillation system
- ◆ autoclaves: one for sterilization and one for decontamination
- ◆ an ice-making machine
- ◆ grinding equipment: e.g. mortar and pestle or plastic bag and roller
- ◆ a hot air oven for drying glassware (optional)
- ◆ micro-centrifuge tubes (0.2 ml, 0.5 ml, 1.5 ml, 2 ml)
- ◆ a vortex mixer (optional)
- ◆ shelving and cupboards for chemicals
- ◆ 2 chemical storage cabinets: one for flammable and one for corrosive solutions
- ◆ laboratory coat, gloves, masks and goggles

- ◆ waste bins (quarantine and non-quarantine)
- ◆ tissue and disinfectant wipes
- ◆ dry heat blocks (at least 2, to be set at different temperatures)
- ◆ a microwave oven
- ◆ a spectrophotometer (sensitive enough to read nucleic acid quantity and quality)
- ◆ a PCR machine (conventional and real-time)
- ◆ gel electrophoresis apparatus
- ◆ UV transilluminator or blue-light box (safer than UV light)
- ◆ a water bath (for cloning)
- ◆ an incubator-checker (for cloning)
- ◆ biosafety cabinets Class II (for cloning).

2.11 Safe working practices

The safety of the laboratory staff should be a prime consideration when the laboratory is set up. Safe working in the laboratory depends on the performance of basic safety precautions and on good training of staff both in safety and in good work practice. The laboratory should have a written document on safe practice and this should be followed at all times.

2.11.1 General safety principles

- ◆ A first-aid box must be provided and at least two or three staff members should be trained in first aid and should be present at all times when the laboratory is working.
- ◆ Only laboratory staff and approved users should be allowed to enter the laboratory working area.
- ◆ Laboratory staff should wear protective clothing, which should be removed when leaving the laboratory. It should not be worn in laboratory support areas, such as offices of staff members.
- ◆ Covered shoes should be worn. Open-toed shoes are not suitable in the laboratory.
- ◆ Gloves, goggles and masks should be provided and worn when working with hazardous materials and when working in the molecular laboratory. Gloves and masks are not to be reused and are to be discarded with laboratory waste.
- ◆ All contaminated material (e.g. glassware) should be decontaminated before washing.

Appropriate containers (sharps bins, plastic bags, disinfectant containers) and disinfectant must be provided.

- ◆ Eye-wash facilities and drench shower must be provided.
- ◆ Eating, drinking and smoking should be forbidden in the laboratory.
- ◆ Benches should be cleaned and disinfected after every use.
- ◆ Laboratory staff should wash their hands before leaving the laboratory (even when they use gloves while working).
- ◆ All spills and other accidents should be reported to the laboratory supervisor.

2.12 Quarantine waste disposal

2.12.1 Micro-organisms

Micro-organisms (e.g. bacteria, fungi, viruses, viroids, phytoplasmas) for disposal are classified as infectious material. These will be collected in a robust plastic bag displaying the biohazard symbol and pressure steam sterilized (i.e. autoclaved) in a solid container.

Once autoclaved, the neck of the bag is twisted and tied off with tape and placed into a heavy-duty plastic bag. When this bag is considered full (don't allow to get too heavy to be easily moved), the neck is twisted and tied off with tape, folded over and taped off again ("swan neck"). The bag is placed in a hard, lockable, lined quarantine waste bin and transported to quarantine waste treatment facility for disposal.

Note: this includes chemical and medical (as well as quarantine) waste. Using a registered waste management company does not require the raising of Transfer Permits.

2.12.2 Plant material

Plant material for disposal will be contained in a robust plastic bag displaying the biohazard symbol. The neck of the bag should be twisted, tied off with tape, folded over and taped off again ("swan neck"). This bag is placed in a heavy-duty plastic bag. The neck of this second bag is again twisted, tied off with tape, folded over and taped off again and then the double-bagged waste is placed into

a hard, lockable, lined quarantine waste bin. When the bin is full of double-bagged waste, the liner bag is tied off in the same manner. The locked bin is transported to a quarantine waste treatment facility for disposal.

2.12.3 Cell lines, etc.

Cell lines, bacterial vectors, material derived from cell lines, media and other aqueous solutions that have come in contact with bacterial vectors or cell lines are treated similarly for disposal.

All waste involving GMOs will be classed as infectious material and needs safe disposal. The following protocols could be followed.

- ◆ **Liquids** are autoclaved or chemically sterilized. Due to the variable length of time taken to attenuate solutions by autoclaving, it can be preferable to chemically sterilize solutions rather than use an autoclave.
- ◆ **Glassware** is chemically sterilized if it has been contaminated with a GMO or micro-organism, otherwise washed in a normal manner with laboratory detergent and then autoclaved before use (if required).
- ◆ **Syringes, needles, glass Pasteur pipettes and razor blades** are to be high heat treated or

steam sterilized via medical waste disposal. This waste must go into approved hard plastic pails, which must be sealed when full and transported to a quarantine waste treatment facility for disposal.

- ◆ **Plastic ware** (plastic dishes, tubes, pipette tips and flasks), gloves, agar plates and contaminated wipes, agarose gels containing recombinant DNA and hard disposable plastic pipettes are to be high heat treated or steam sterilized via medical waste disposal.

Waste material must be placed in small plastic bags, which are tied off securely before leaving the laboratory or hood in which the waste has been generated.

These plastic bags are placed in a larger plastic bag, marked with the "Biohazard" symbol. When this bag is full, the neck should be twisted, tied off with tape, folded over and taped off again ("swan neck"). This bag is placed in a heavy-duty plastic bag, the neck of which is twisted, tied off with tape, folded over and taped off again ("swan neck") and placed in a hard, lockable, lined quarantine waste bin and transported to a quarantine waste treatment facility for disposal.

3. Soft Infrastructure

Introduction

Soft infrastructure refers to all the supporting services that are required for the functioning of a diagnostic laboratory. It gives direction in the form of standard work procedures (SOPs) and an information management system to shape the diagnostic laboratory. It is the most important element to make the laboratory a productive, innovative, risk-free environment.

3.1 Quality system

3.1.1 Importance of quality system

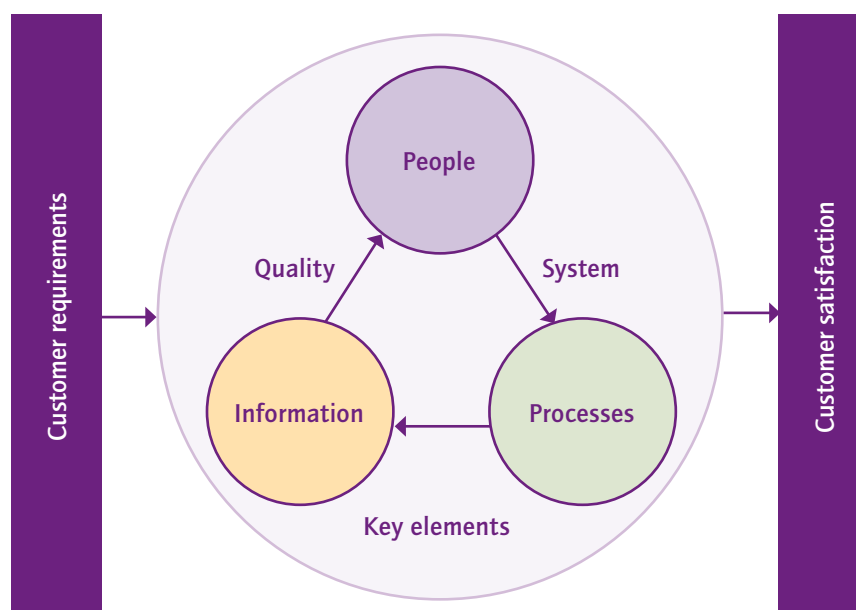
There are many drivers for an organization to start implementing a quality management system, such as:

- ◆ requirements to meet customer expectations
- ◆ needs of a regulatory body
- ◆ demands from internal and external business markets
- ◆ identification and management of critical elements (risk management) to boost business growth.
- ◆ providing improved consistency and reliability of processes to produce services and products
- ◆ complying with the requirements to meet the demands of customer-applicable regulatory bodies, etc.
- ◆ addressing customer satisfaction through the effective application of the system, including

The key elements of a quality management system are people, processes and information (Figure 5). The interaction of these elements creates a well-run quality system to meet the needs of customers and hence the organization's quality objectives.

There are costs involved with the development and implementation of a quality management system by investing in more staff, equipment, facilities, the way the organization operates and, finally, training staff to adapt to the new quality requirements. However, the benefits of incorporating the quality management system principles outweigh the cost. The advantages of implementing quality system are:

Figure 5: Key elements of a quality management system



processes for continual improvement and the prevention of nonconformities

- ◆ improving the business with the implementation of quality systems
- ◆ improving effectiveness and productivity of testing and increasing credibility of results and products nationally and internationally
- ◆ maintaining a high level of service in ever-changing, technologically complex and fast-paced environments
- ◆ providing enhanced traceability of records, reference materials, etc.
- ◆ establishing uniform and better training and induction programmes for staff through a thorough introduction to the system
- ◆ using technically validated methods that are assessed by independent technical experts in the field.

When the organization's quality system has been certified, this formal recognition approves the organization's technical competency after an assessment of its processes, resources, facilities, staff and other key factors that relate to, and impact on, the quality of the testing service provided.

Moreover, the full potential of a quality management system will be evident when the organization is faced with a sudden challenge to combat an unexpected situation, for example, in a laboratory where testing is required for an outbreak of a new disease or response – such as *Pseudomonas syringae* pv. *actinidiae* (bacterial kiwifruit vine disease) or fruit fly incursion – and thousands of samples are coming through in rapidly moving situations. The above situation can be handled easily with smooth and successful laboratory testing supported by documented systems in place for testing, recording, traceability of samples and training of new staff.

A systematic approach to day-to-day activities needs little management intervention, thereby freeing management to focus on the areas where initiative and change or improvements are needed. In other words, quality management systems can enhance the 80 percent of what we do so that we do not need to use our initiative for every decision. We can then use our time on the other 20 percent

of activities, where real problems need to be solved and real improvements identified and enacted.

3.1.2 Structure of quality system

There are two major requirements to be considered when establishing a quality system: general quality management system (QMS) requirements and technical requirements. Management requirements are primarily related to the operation and effectiveness of the quality management system within the laboratory or business. Technical requirements address the competence of staff, methodology, test and calibration equipment, and the test methods.

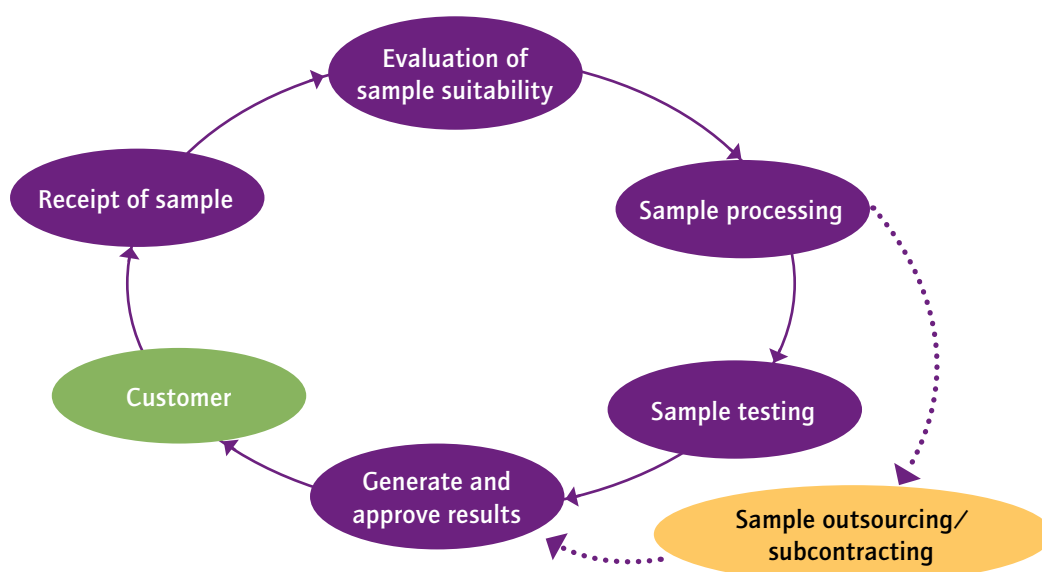
3.1.3 Quality management system

3.1.3.1 General requirements

When an organization wants to develop a quality management system in compliance with general requirements for the competence of testing and calibration laboratories (ISO/IEC 17025), or any other ISO standards, the international standard recommends having a detailed working knowledge of all the requirements of the standard. The international standard also encourages a "process approach" to develop and implement a QMS, where the interaction between the individual processes and their linkages within the overall system processes can be controlled. For an organization to function successfully, it has to establish, document, implement, maintain and continuously improve its QMS through:

- ◆ clearly identifying and managing activities or processes for the QMS (Figure 6)
- ◆ documenting the processes identified
- ◆ understanding sequences or interaction of these processes and describing how the output from one process forms an input to another
- ◆ determining the criteria to ensure the effective operation and control of the processes involved (e.g. sample acceptance criteria before it can be taken for testing, critical equipment performance levels for obtaining desired results)
- ◆ planning for the management and control of outsourced processes that can affect the planned results

Figure 6: Guide to the core processes within a testing laboratory



- ◆ establishing specific methods required for the operation and control of each process involved in the QMS – these can be written as work instructions, manuals, set-up checklist, etc.
- ◆ ensuring resources and information necessary to support the operation and monitoring of these processes are available – the resources include facility, equipment, staff, chemicals, reagents
- (Figure 7); information includes worksheets, work instructions, schedules
- ◆ monitoring, measuring and analysing these processes to oversee the performance of the QMS using internal audits
- ◆ implementing actions necessary to achieve the required results and to continually improve these processes.

Figure 7: Guide to identify support process required in the testing laboratory



3.1.3.2 Documentation requirements

Developing documents for an organization's QMS depends on its size, the types of activities it carries out and the complexity of its activities. The QMS needs to include:

- ◆ quality policy and quality objectives statements
- ◆ quality manual detailing how the QMS operates
- ◆ documented procedures for a minimum of the following six areas:³
 - control of documents
 - control of quality records
 - internal audit
 - control of nonconformity
 - corrective actions
 - preventive actions
- ◆ other documented procedures at the discretion of the organization's requirements to demonstrate the effective implementation of QMS
- ◆ documented procedure for the management of records which can be either in hard copy or electronic form – this procedure details how quality records are identified, stored, controlled, maintained, disposed of and altered.

It is important that all the documents and records that are part of the QMS are appropriately controlled.

3.1.3.3 Control of documents

A document is information that is written or recorded either on paper or electronically. A document may specify requirements (e.g. a drawing or technical specification), provide direction (e.g. quality plan) or show results or evidence of activities performed (e.g. records).

All documents that are a part of a QMS processes must be controlled. The organization needs to have its own documented procedure for both hard copy and electronic documents, covering the following.

- ◆ How a document is compiled (style, template, etc.) and reviewed – documents must also be readily identifiable as to their purpose and scope.

- ◆ How the documents are approved for use and by whom – only approved documents will be in use.
- ◆ How the documents are issued or made available for use – if you determine to keep certain documents at various locations, implement some form of distribution control so that everyone uses the current version of the document.
- ◆ How the documents are revised and updated – periodically determine if any updating or revisions of any QMS documentation is needed and, if the documents are changed, they must be reapproved for adequacy.
- ◆ How the document revision information is recorded on the document – identify changes made to documents, so users know exactly what has changed.
- ◆ How legibility and retrievability of documents are ensured – regularly review the condition of frequently used hard copy documents to determine whether they need to be replaced.
- ◆ How the documents are retained and stored – obsolete documents can cause many problems if not controlled; if computerized documents are in use, make only the current version accessible at workstations in read-only mode; obsolete documents need to be removed immediately; in case of hard copy documents, remove obsolete documents through distribution control.
- ◆ How documents are archived – make sure that all such documents are properly identified, indexed and filed, and preferably have controlled or restricted access to them.
- ◆ A master index of all current documents and their issue and dates of amendments.

Note: Nonconformities against the document control process are one of the most frequent audit findings.

3.1.3.4 Document writing guidelines

When writing:

- ◆ keep it simple, practical and flexible
- ◆ have "just enough" information and no more
- ◆ write clearly and simply
- ◆ use plain English and language that your people know and use

³ Procedures for several activities can be combined into a single documented procedure (e.g. corrective action and preventive action) or to document a given activity by using more than one documented procedure (e.g. internal audits).

- ◆ use flow charts or pictorial representations, if appropriate
- ◆ keep every document as clear and as short as possible
- ◆ only write a document (whether a procedure, form, checklist, etc.) if it is needed.

3.1.3.5 Control of quality records

A record is a special document that provides evidence of results achieved or activity performed, test worksheets, calibration records, training sheets, etc.

- ◆ Ensure that a documented procedure for control of quality records is available detailing authority, how records are identified, stored, protected, retrieved, retained and disposed of. These need to be defined for records that are in hard copy or electronic form.
- ◆ Ensure that the records are legible, easily identifiable and retrievable.
- ◆ Ensure that unauthorized alteration of the records is not allowed and that changes need to be initialled and dated. The original information still needs to be visible.
- ◆ Ensure that records are kept in such a way as to minimize damage, deterioration or loss.
- ◆ Ensure that the records are stored in an orderly manner to aid easy retrieval (indexing and filing of hard copy or electronic records).
- ◆ Keep a list of all the different categories of records and define the retention times associated with each category (inspection and test, sales and purchasing, management review, calibration, training, etc.). Retention times are determined by customer, regulatory, industry or organizational requirements and policies.
- ◆ Dispose of records at the end of the required storage time. Disposal could range from permanent destruction of records by incineration or shredding, to permanent storage in a secure on-site or off-site archive.

3.2 Responsibilities of management

3.2.1 Quality policy and quality objectives

The quality policy and quality objectives are relevant to the organizational goals and the expectations

and needs of the organization's customers. This policy statement must provide the focus for the quality efforts of an organization. Therefore, it should:

- ◆ be current and suitable for the organization's work and the services it provides to its customers
- ◆ be based on the organization's values, vision, mission and existing business strategy
- ◆ comply with the law and be relevant to national and international standards
- ◆ give a framework for setting and reviewing quality objectives
- ◆ be developed and fully supported by senior management
- ◆ be continually reviewed at fixed intervals (e.g. annually) at senior management meetings to ensure the policy and objectives meet business objectives
- ◆ provide awareness to all those who are involved in the development, implementation or maintenance of the quality system.

Note: The policy is a dynamic document and should change as the organization's needs, directions and business activities change.

Prepare quality objectives from the organization's quality policy to make sure that the commitments made by the organization in its quality policy are achieved. The objectives are then documented and reviewed by senior management. These objectives should be:

- ◆ meaningful
- ◆ measureable (things you want to achieve and how you measure that)
- ◆ assigned with responsibilities for establishment, achievement, performance and monitoring.

Senior management is required to identify the action plans that are necessary to achieve these objectives. They need to show their commitment to continual improvement, then document the objectives for communication to all those involved in the QMS.

3.2.2 Quality manual

The quality manual demonstrates and documents an organization's commitment to maintaining a high level of quality and operating services. The

organization must establish and maintain a quality manual. The quality manual describes a QMS and says how it should work. It is required to contain:

- ◆ the scope of the QMS with details of any exclusions claimed by the organization
- ◆ the documented procedures established for the QMS to run the business (procedures, policies, forms, checklists, etc.) or reference to such documented procedures)
- ◆ a description of the interaction between the processes of the QMS.

The quality manual may be combined with other management system manuals (business manuals), but it is recommended that it be kept simple and separate from business manuals. A single manual may be sufficient for a small business's needs whereas a large organization may need several.

It is advisable to ask a third party to review the QMS and its manual before applying for certification.

3.2.3 Management responsibilities and authorities

The senior management of an organization has to define business functions and their interrelations within the organization, including responsibilities and authorities.

- ◆ Who has overall responsibility for the management of the organization or business?
- ◆ Who has overall responsibility for ensuring that the QMS is maintained, improved and that system issues are resolved in a timely manner?
- ◆ Who has responsibility for maintaining quality and implementing the QMS within the team or group?
- ◆ Who is responsible for the technical operation of the business and who are their deputies to take on that role in their absence?
- ◆ Who is responsible to develop, maintain, oversee, report on and facilitate the continued management of quality systems through guidance, training, teamwork and assistance?

An important role of senior management is to appoint a member of management as the Quality Representative, who has sufficient authority to effectively carry out QMS responsibilities. The

individual should preferably be a member of senior management, but need not necessarily be so.

Senior management may have to show evidence of the appointment of the Quality Representative through an appointment posting, attendance at QMS review meetings and provision of support, authorization and resources to QMS activities. The management representative may be a full-time or subcontracted person.

3.2.3.1 Role of quality representative

- ◆ Communicate, delegate, empower, report, oversee and interact with individuals at all levels within and external to the organization.
- ◆ Assist process owners, developing their processes and applying the relevant standard requirements.
- ◆ Report QMS performance to top management at management review meetings or other meetings.

The organization has to ensure that the responsibility, authority and interrelationships of personnel who manage, perform and verify work that affects quality is defined and documented. These responsibilities need to be communicated to staff in order to facilitate effective quality management.

The structure, position and role of the staff within the organization can be shown as an organizational chart.

3.2.4 Management review

Management reviews are internal review processes by senior managers who review the organization's QMS to ensure its continuing suitability and effectiveness and to introduce any necessary changes or improvements including to the organization's quality policy and quality objectives. A schematic of the review process is shown in Figure 8.

3.2.4.1 Quality representative

The Quality Representative will report on QMS performance to senior management at management review meetings. The information for the report will come from the results of the measurement and monitoring requirements (e.g. internal audits and

Figure 8: Schematic of management review process



customer satisfaction feedback). This information is compiled from all the process owners.

3.2.4.2 Review input

The inputs to the management review include, but are not limited to, current performance and improvement opportunities.

3.2.4.3 Review output

The outputs from the management review include, but are not limited to, decisions and actions.

3.3 Management of resources

Senior management has the responsibility to ensure that resources are available to develop and maintain the QMS for an organization. It is important to have adequate resources to address customer requirements, otherwise there is potential for nonconformities (i.e. departures from procedural requirements) to arise as a result of insufficient or improper use of resources. For example, it is necessary to check that adequate personnel, materials and equipment are on hand to ensure timely production and delivery of products, tests and services.

Resources can include:

- ◆ people
- ◆ equipment
- ◆ reagents and materials
- ◆ information (can be procedures or work instructions)
- ◆ facilities
- ◆ work environment
- ◆ finances.

It is good to start by identifying the nature of resource requirements for each process and determine the availability of resources in the business planning. The actual amount of resources needed may vary day to day and over longer periods, so senior management needs to review QMS performance at regular intervals.

Consider developing performance indicators for each major category of resources used, such as machinery and equipment, human resources, facility and environment, transport and communication systems, to determine the effective use of such resources.

3.3.1 Human resources

Competent personnel are required to produce products, tests and services in accordance with product document or service requirement. They need to be competent with appropriate education, training, skills and experience.

Senior management, when planning for resource requirements, has to ensure the:

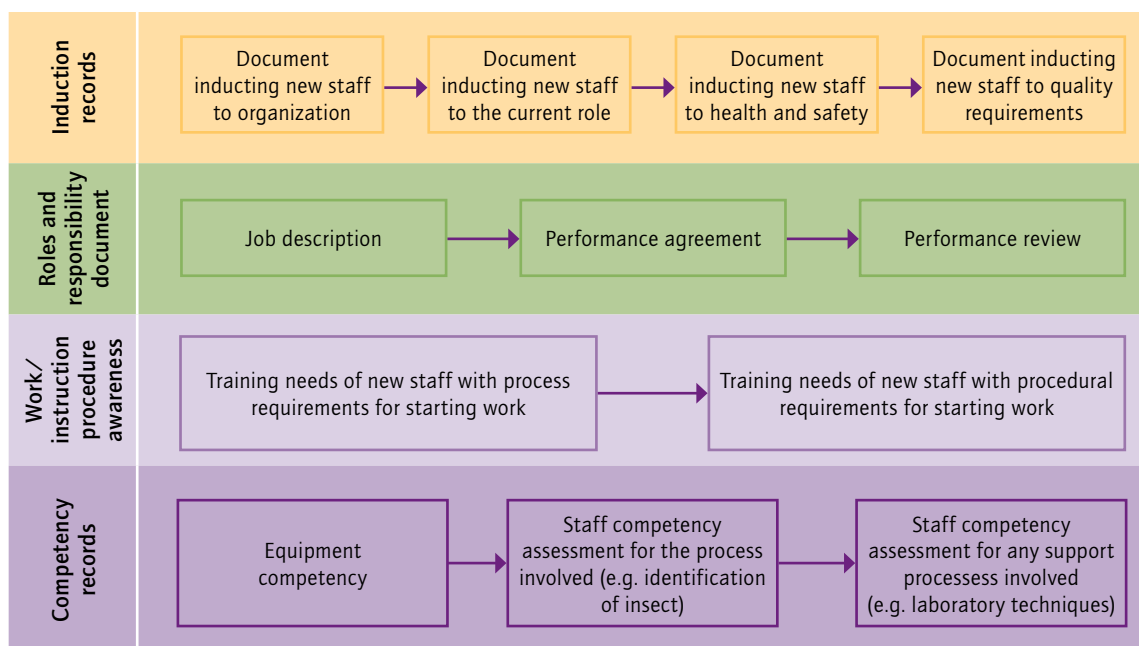
- ◆ determination of competency criteria, skills evaluation and identification of training needs for personnel working in each process that affects the quality of the product or service
- ◆ development of the individual's training requirements as part of the annual performance planning process and assess the effectiveness of this training at the performance review – it is useful to have a training spreadsheet where competency and training undertaken by individuals can be recorded and referred to for future planning
- ◆ awareness by personnel of their roles and responsibilities in undertaking the work – this will be captured in the individual's job description and performance plan
- ◆ awareness by personnel of the quality requirements involved in their work area

- promote quality awareness through team meetings and involvement in quality planning
- ◆ development of checklists for induction and competency assessment
- ◆ determination of the records that are to be maintained in terms of education, training, skills and experience – these records must demonstrate the effective operation of personnel working in the QMS, e.g. in a training folder
- ◆ creation of a procedure to describe the induction process required for all new staff, management of internal and external training courses, and maintenance of staff training records and how skills assessment and training occurs, to ensure that all staff receive training as per the requirements so that only competently trained or appropriately supervised staff perform the work.

Example: all staff can be issued with a prepared training folder during their induction training; the folder is:

- ◆ labelled with the staff member's name
- ◆ divided into sections as shown in Figure 9.

Figure 9: Components of staff training folder



3.3.2 Infrastructure, accommodation and environment

Requirements for the types of infrastructure resources needed for an organization may include:

- ◆ building
- ◆ workspace
- ◆ hardware and software
- ◆ support services.

This infrastructure needs to be identified, provided and maintained by senior management. In addition, systems should be in place for reactive and preventive maintenance of infrastructure.

The key factors to be considered for infrastructure planning include:

- ◆ current availability and capacity
- ◆ future needs
- ◆ expansion for growth
- ◆ contingency planning and linkage to current and future programmes.

In order to achieve quality products and services to conform with customer requirements, the organization needs to decide on and manage the conditions of the work environment.

The work environment is related to the conditions under which work is performed, including physical, environmental and other factors, such as noise, temperature, humidity, lighting, weather, ergonomics, hygiene, cleanliness, pollution, adequate facilities (lunchroom, cafeteria, washrooms), health and safety regulations, cleanliness of premises to avoid contamination.

The extent to which the above environmental factors may apply to any organization will depend on laboratory size, risk and other considerations. The focus should be on employee safety, welfare and product conformity. Industry requirements and other legislation will help to provide guidance on acceptable standards for the work environment.

3.4 Measurement, analysis and improvement

The organization needs to plan and implement procedures that measure, analyse and improve the effectiveness of its QMS. The focus of these must be on:

- ◆ whether products and services conform to the customer requirements

- ◆ whether the processes in use conform to the standard requirement
- ◆ continual improvement of QMS effectiveness.

3.4.1 Monitoring and measurement

The organization can start monitoring and measuring with objectives that focus on meeting customer requirements and then slowly develop meaningful objectives for key processes and risk-prone processes, as initial targets are achieved.

Monitoring and measurement for the following areas need to be addressed.

3.4.1.1 Customer satisfaction

The organization needs to monitor the customers' views on the performance of the core services of the organization. The feedback from customers can be gathered from customer surveys, user opinion surveys, feedback on delivered product and services, lost business analysis, compliments, complaints, etc. Senior management needs to decide on how, when, by who and what type of information will be gathered from the customer. An example of a questionnaire that a testing laboratory can include in its customer feedback survey is given in Figure 10.

The organization can also provide customers with an opportunity to access the facility or laboratory to see how the testing is performed. This creates goodwill and offers an additional chance to gather information about customers' requirements.

3.4.1.2 Internal audits

Audits are required to be conducted in accordance with a documented procedure to provide feedback to management on whether the QMS in place is working effectively.

The last stage in the process of implementing the QMS is to undertake an internal quality audit to determine the inconsistencies, if any, within the newly implemented system.

It is important to carry out this type of audit regularly, even after the implementation of the QMS in the organization, to monitor performance against the set goals. The audit outcomes must be reported to senior management. Internal staff need to be trained to conduct such audits. Records of conduct

Figure 10: Customer satisfaction survey form

Note: Use the 1 to 5 scale where 1 is indicative of a very poor standard and 5 is indicative of an excellent standard.

1. How would you rate the laboratory/business on each of the following service features?

Speed at which results are delivered	<input type="text"/>
Technical credibility	<input type="text"/>
Format of reports	<input type="text"/>
Information and advice provided	<input type="text"/>
Approachability and friendliness	<input type="text"/>
Flexibility to meet your needs	<input type="text"/>
Price	<input type="text"/>
Overall performance	<input type="text"/>

2. What do you feel is the best aspect of the services offered by the laboratory/business?

3. Are there any services you feel could be improved? How could they be improved?

4. In what way do you feel the laboratory could improve its communication with you?

5. If you were able to access information via the intranet what sort of information would be useful?

6. Any other comments

Respondent's name: Interviewer:

Respondent's position:

Organization:

Location:

Date:

of audits and results need to be maintained as a key source of input data.

Audit objectives include:

- ◆ evaluation of current system against compliance with standards, organization's own process and procedures to examine performance using clear scope and audit methodologies
- ◆ identification of improvement opportunities for the existing system
- ◆ identification of deficiencies in the QMS
- ◆ show the continual improvement that has been happening.

3.4.1.3 Monitoring and measurement of product (quality control)

The quality of products, tests and services is monitored and measured to make sure the needs and requirements of customers are met. This is done by carrying out inspection and testing in relation to:

- ◆ measuring product and test characteristics
- ◆ checking outcomes of the various stages of the processes
- ◆ providing evidence of conformity with the acceptance criteria by regular use of certified reference materials
- ◆ participating in inter-laboratory comparisons or proficiency testing programmes
- ◆ enabling product to be authorized for release
- ◆ replicating tests using the same or different methods
- ◆ retesting when required
- ◆ completing specified activities before delivery.

Procedures need to be in place to monitor the validity of tests and results, to analyse data to identify trends and nonconforming results, to investigate identified failures and trends, and to prevent incorrect results being sent out.

The information that is produced by the organization's monitoring and measurement activities needs to be collected and analysed with the objective of continually improving the effectiveness of the QMS. Additional data from internal audits, employee suggestions and customer complaints can also be used in this respect.

3.4.1.4 Control of nonconforming product

Despite the best intentions, training and ongoing communications, parts of the QMS and the organization's operations may not be effective or sufficient to prevent product defects, deficiencies in services and complaints.

Identification of nonconformance could occur at various places within the management system and technical operations (e.g. quality control, audits, complaints, equipment calibration, standards, controls or consumables are out of specification). But when these situations arise, it is necessary for the QMS to accommodate the management of these deficiencies. These should be recorded as nonconformances and processes must be implemented to respond to and fix the issues raised.

The procedure has to contain:

- ◆ who is responsible for making decisions when nonconforming work is identified, e.g. halting work?
- ◆ what immediate actions are to be carried out when nonconforming work is identified (e.g. documentation, notification of senior staff)?
- ◆ how to evaluate the significance of the nonconforming work
- ◆ notifying the customer and recalling test results or products
- ◆ corrective action to be taken and identifying the possibility of nonconforming work recurring.

3.4.2 Improvement

Improvement is a proactive process to identify opportunities for improvement, rather than a reaction to the identification of problems (nonconformances) or complaints.

The organization must continually improve the effectiveness of its QMS through the use of the quality policy, quality objectives, audit results, corrective and preventive actions, and management review. Continual improvement of the QMS is an essential requirement of an effective quality management strategy.

3.4.2.1 Corrective action

Corrective action is raised where there is a departure from an approved procedure or policy. The nonconformance is usually identified as a result of an audit,

but there may be instances where nonconformances are identified outside an audit, for example, as a result of complaints or management reviews.

A documented procedure has to be established by the organization to prevent previously occurring nonconformities from happening again.

- ◆ Designate appropriate authorities for implementing corrective action.
- ◆ Identify the nature and the root cause of the nonconformance.
- ◆ Identify and implement the necessary corrective action to prevent recurrence – this may require changing management practices to create, modify or review controls, such as procedures or training, to avoid repetition of the nonconformance.
- ◆ Think what can and will be done to monitor corrective action.
- ◆ Record any changes in documented procedures resulting from the corrective action.

Note: A form or record (Figure 11) can be devised to capture a nonconformance that is being raised and then issued to designated staff to action any necessary corrective actions.

3.4.2.2 Preventive action

A documented procedure is required to prevent potential nonconformities from happening in the first place. This in turn requires the organization actively to identify and deal with the causes of potential nonconformities or improvement opportunities using the Total Quality Management tools.

Establish a procedure that:

- ◆ identifies the staff member to complete the action required for implementing the improvement
- ◆ sets up the agreed time frame for completion
- ◆ monitors the progress of the work through some tracking system
- ◆ determines if any follow-up actions are required and organizes these to be done
- ◆ requires sign off by an authorized person when complete
- ◆ keeps a record of documentation for completed work for filing in the QMS.

Note: A form or record (Figure 12) can be devised to capture an improvement opportunity that

has been identified and then issued to designated staff to carry out the action necessary.

3.5 Technical requirements

Many factors determine the correctness and reliability of the tests performed by the laboratory, including, but not restricted to:

- ◆ purchasing
- ◆ human factors
- ◆ accommodation and environmental conditions
- ◆ test methods and method validation
- ◆ equipment and its measurement traceability
- ◆ handling test items.

3.5.1 Purchasing

The organization needs to reduce and prevent any problem occurring by means of an effective purchasing system to deliver quality products and services. Therefore, anything that is bought in must meet the organization's specified requirements by:

- ◆ establishing methods for evaluating suppliers or contractors
- ◆ establishing selection criteria for the selection of purchased products and suppliers and contractors – the documentation produced during purchasing (e.g. purchase order, credit card or other receipt, packing slip), including data describing the services and supplies ordered, needs to be reviewed and approved for technical content prior to release to reduce risks of the incorrect product being delivered.

When the service or supply is ultimately received, there must be a process to ensure that it meets the organization's needs and requirements.

3.5.2 Personnel

It is necessary for the laboratory management to ensure that all staff working in the laboratory receive training in laboratory techniques and instrument use so that only competently trained or appropriately supervised staff perform tests, evaluate results and sign test reports. Staff who are undergoing training must be appropriately supervised when they undertake testing.

Management is responsible for:

- ◆ identifying the skills required for staff working in their area

Figure 11: Template for recording nonconformance

Nonconformance Number:	Source: Equipment/Quality Control/Complaint/Audit
NONCONFORMANCE <i>Describe nature of nonconformance</i>	TEAM
DATE ISSUED	SIGNED
CAUSE ANALYSIS <i>Identify the root causes of nonconformance</i>	Person who will complete Completion date
DATE COMPLETED <i>Person completing the cause analysis</i>	SIGNED
CORRECTIVE ACTION(S) (what has been done to implement corrective action?) <i>Actions most likely to eliminate the problem and prevent recurrence</i>	
DATE COMPLETED <i>Person taking action nonconformance</i>	SIGNED
FOLLOW UP/CLOSE <i>What has and will be done to monitor corrective action?</i>	Follow up audit required: Yes / No
DATE QIF CLOSED	SIGNED

- ◆ ensuring assessment and training are carried out
- ◆ ensuring the trainer is competent
- ◆ ensuring that the appropriate training modules are selected and training criteria are met to carry out the laboratory procedures that they are expected to perform
- ◆ ensuring that the trainee is not participating in laboratory test methods until their training

- ◆ record shows competence in the basic laboratory skills
 - ◆ ensuring that the trainee is not using equipment described in the major instrument modules until their training record shows competence in the major instruments.
- For details refer to section 1.5 Human resources.

Figure 12: Template for recording Quality Improvement (QIF)

Quality Improvement Number:	Source: Equipment/Quality Control/Complaint/Audit
QUALITY IMPROVEMENT <i>Identify possible sources of improvements</i>	
DATE ISSUED	SIGNED
ACTIONS TAKEN TO IMPLEMENT QUALITY IMPROVEMENT Person who will complete Agreed date of completion	
DATE COMPLETED	SIGNED
FOLLOW UP/CLOSE Follow up audit required: Yes / No <i>What has and will be done to monitor quality improvement (if available include objective evidence with completed QIF)?</i>	
DATE QIF CLOSED	SIGNED

3.5.3 Accommodation and environmental conditions

The laboratory facilities used for testing should enable correct performance of the tests. The technical requirements for accommodation and environmental conditions that can affect the results of tests must be documented in the technical procedures. It is also necessary to monitor, control and record conditions (biological sterility, dust, humidity, temperature, electrical supply, temperature, etc.) as required by relevant specifications where they influence the quality of

tests. Attention to access to certain test areas, good housekeeping and effective separation of incompatible activities is necessary to prevent cross-contamination.

3.5.4 Test method and method validation

The laboratory has to use internationally or nationally accepted standard test procedures or non-standard procedures (in-house methods) that have been appropriately validated and that are performed regularly. When the laboratory is following standard methods, it is required to

maintain current versions of standard methods with reference texts. The laboratory must verify that it can properly operate standard methods to obtain appropriate limits of detection, selectivity, repeatability and reproducibility before introducing the tests.

Non-standard methods are in-house methods that could include:

- ◆ methods developed in the laboratory
- ◆ modified standard test methods
- ◆ methods taken from scientific publications but not validated.

The laboratory must validate these methods to verify that these non-standard methods are fit for the purpose and keep all the documents associated with it for reference. See Figure 13 for general processes for method validation.

3.5.5 Equipment and its measurement traceability

The laboratory should contain all the test equipment required for the correct performance of the tests and the calibrations and measurements made by the laboratory should comply with international standard requirements and be fully traceable. The management is responsible for:

- ◆ ensuring each piece of equipment in the laboratory is uniquely identified
- ◆ ensuring initial validation or calibration for new equipment occurs prior to use, to confirm that the item meets the purchase criteria and demonstrates that the equipment and components perform to the specifications used for the initial acceptance criteria
- ◆ ensuring equipment used for testing is capable of achieving the expected accuracy for the tests
- ◆ ensuring that servicing, calibration and maintenance requirements are scheduled and met in a timely manner for all equipment used for tests
- ◆ ensuring documentation (e.g. user logs, maintenance records) is kept up to date

- ◆ ensuring that the appropriate corrective action is taken when required
- ◆ ensuring that procedure is written to cover the use and maintenance of the equipment and that equipment is operated by authorized personnel using up-to-date instructions
- ◆ ensuring calibration status is indicated on the equipment
- ◆ ensuring calibrations are traceable to International System of Units (SI units) where required.

3.5.6 Handling of test items

The laboratory should develop a documented procedure for receipt, handling, protection, storage, transportation, retention and disposal of items received for testing in the laboratory. This procedure should specify:

- ◆ how to uniquely identify each test item in the laboratory so that this identification will be retained throughout the testing process
- ◆ how to record abnormalities of items, if any, and consultation with the customer for necessary further instructions or action
- ◆ how to avoid deterioration during handling, storage and preparation.

3.5.7 Subcontracting of tests

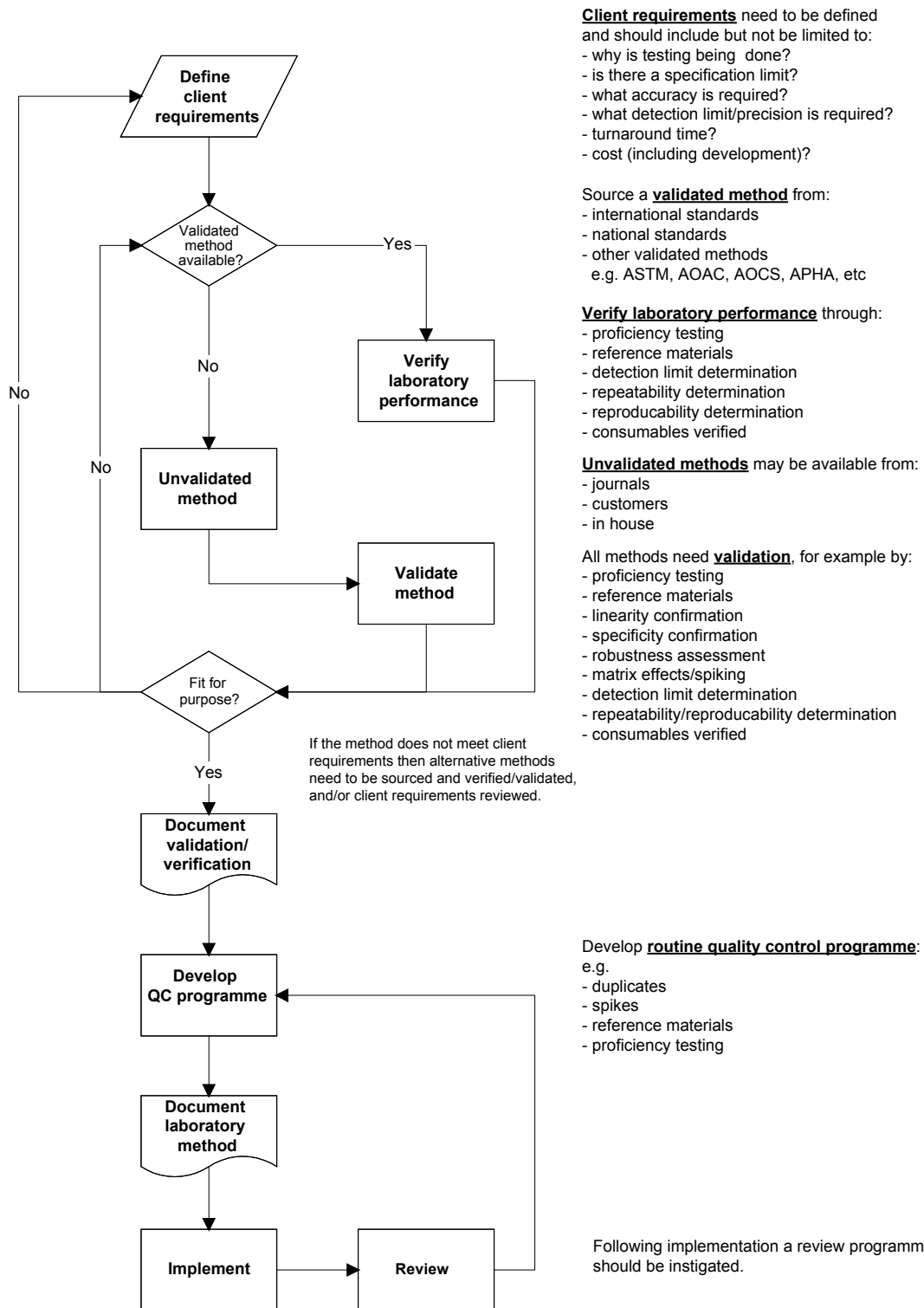
If the laboratory wants to subcontract work, it should consider the following criteria:

- ◆ ensure that the work is carried out by suitable competent subcontractors
- ◆ ensure that the customer is informed of such arrangements
- ◆ ensure that responsibility for the subcontractor's work lies with the laboratory
- ◆ ensure that a register of subcontractors is maintained and evidence of each subcontractor's competency for the work is recorded.

Figure 13: General processes for method validation in biological testing laboratories



Biological testing



Source: International Accreditation New Zealand specific criteria for accreditation – Biological Testing, used with IANZ permission

3.6 Templates

Quality manual

Title for Quality Manual

Record of Document Control Information

Version No.	Issue Date	Description	Writer	Reviewer	Editor	Approver

Table of Contents
[with the following Sections]

Quality Manual of XYZ

1 Purpose
[Describe the reason for the procedure such as quality management system manual to detail about organization’s quality policy, goals, staff responsibilities, management system, etc.]

2 Scope
[Describe to what or whom the procedure applies.]

3 Actions
[Include relevant information for the topic described by text and/or flow chart. It will identify people responsible for the effective control of the activity.]

3.1 Introduction to organization and its functions

3.2 Quality Policy
Signed (by the owner of the organization) Date

3.3 Quality Goals
Signed (by the owner of the organization) Date

3.4 Job Descriptions
[Describe roles and responsibilities of staff including quality requirements. Job descriptions should be signed by the staff.]

Quality manual (continued)

3.5 Performance Agreement

[Include the process by which organization/business develops the performance of its employees and the ongoing assessment of the capability for the organization. This will assist and support managers in improving skills and capability of the staff. It is good to develop annual performance plans for all permanent staff.]

3.6 Code of Conduct

[The Code of Conduct sets out what your organization (as an employer) expects from its employees and what employees can expect from the employer. Write the details in this section.]

3.7 Management System Operating Procedures and its Location

[Describe where to access for controlled versions of business operating manuals and work instructions.]

3.8 Management Review

[Describe who organizes the meeting, what will be discussed, how often it will be conducted, etc.]

3.9 Audits

[Describe all about internal and external audits and how they are handled at the organization, including audit frequency, checklist, reporting, audit follow up, etc.]

3.10 Handling of Compliments and Complaints

[Describe handling of compliments/complaints received from clients, staff or other parties about the activities of the organization, including how to receive a complaint, record-keeping, processing a complaint, complaint review and monitoring, audits, etc.]

3.11 Handling of Corrective Action and Quality Improvement

[Describe handling of nonconformances and improvement opportunities identified from test results/products, any piece of validated equipment is shown to be out of calibration, and when standards, controls or consumables are out of specification, failures from quality control programmes, etc.]

3.12 Document Management System

[Describe how a procedure is compiled, reviewed, authorized, issued, controlled and retained, roles and responsibilities and the associated policies involved in the document management system and the templates for writing a new procedures.]

3.13 Customer Confidentiality

3.14 Contract Review

[Describe how the organization will assess its ability to take on a new project or new work that is not a routine work will get completed.]

Quality manual (continued)**3.15 Service to the Customer**

[Describe an organization's policy on serving its customer and how a customer can request to visit to observe the work will be handled.]

3.16 Customer Survey

[Describe how customer feedback surveys will be undertaken and how often, who organizes and tabulates the survey and results.]

3.17 Management and Staff Responsibilities

[Describe who owns and has overall responsibility for the organization's quality management system, who tracks the maintenance and improvement of the quality system and ensures that system issues are resolved in a timely manner, staff responsibility, organizational chart to depict the structure of the organization, etc.]

3.18 Technical Responsibilities and Deputies

[Describe who has overall technical responsibility for the operation of the laboratory/business and who are their deputies in their absence.]

4 References supporting documents/work instructions

Document control system manual

Title for Document Control System Manual for XYZ

Record of Document Control Information

Version No.	Issue Date	Description	Writer	Reviewer	Editor	Approver

Table of Contents

[with the following Sections]

Document Control System Manual for XYZ

1 Purpose

[Describe the roles and responsibilities and the associated policies involved in the document management system, how a procedure is compiled, reviewed, authorized, issued, controlled and retained, and the templates for various forms.]

2 Scope

[Describe to what or whom the procedure applies.]

3 Actions

[Include relevant information for the topic described by text and/or flow chart. It will identify people responsible for the effective control of the activity.]

3.1 Procedure Description

[Describe the procedures, when they are developed, who is responsible for making sure that procedures exist for smooth operation of the quality system.]

3.2 Roles and Responsibilities

[Describe the roles and responsibilities of staff involved in the Document Management System.]

3.3 Numbering System

[Describe how a unique document number for a procedure will be given and who is responsible and how they maintain the numbering system.]

Control system manual (continued)**3.4 Procedures – Preparation, Style and Format**

[Describe how to make a procedure and then its style and format for the following: Title; SOP Document No.; Page No.; Record of document control information; Header, Footer, Referencing supporting document; template for different type of procedures, i.e. equipment procedure, test methods, etc.]

3.5 Procedure Control

[Describe management of the document control process to meet the ISO 17025 standard including master index of all current SOPs and their issue and/or amended dates.]

3.6 Procedure Issue

[Describe how documents/procedures are issued in the controlled medium, where the controlled hardcopies are kept, etc.]

3.7 Procedure Revision

[Describe policy on the procedure review, criteria and frequency for updating procedures.]

3.8 Procedure Retention

[Describe the policy on how previous versions of procedures are retained and who is responsible for maintaining this.]

4 References to supporting documents/work instructions

Test method

Title for Test Method

Record of Document Control Information

Version No.	Issue Date	Description	Writer	Reviewer	Editor	Approver

Table of Contents

[with the following Sections]

Title for Test Method

1 Purpose

[Test methods relate to laboratory procedures and are internal documents that provide direction to personnel:

- On how to identify the presence of specific analyses or to quantify the amount present
- To describe identification process for samples.

Describe the objective for writing this test procedure.]

2 Principle

[Describe how the procedure works.]

3 Scope

[Describe to what area or whom the procedure applies.]

4 Limitations of Method

[Describe the factors that influence the level of identification achievable by the given test method such as specimen quality, life stage, contamination in case of PCR/RT-PCR, the diagnostic sensitivity and specificity, and how well the (RT-PCR has been validated, taxonomic confusion, time constraints, etc.)

5 Sample Requirements

[Describe what kind of specimen and how this specimen needs to be mounted/extracted for identification using morphological characters or molecular methods. E.g. DNA, RNA or cDNA derived from test samples, positive and negative controls for molecular methods.]

Test method (continued)**6 Quality Control Material**

[Describe specimens that will be used as quality control (reference) materials, e.g. reference collections and other national collection. For molecular methods define the following:

- **Negative Control:** In some circumstances, a sample of the same or similar host species as the test sample that is pathogen free should be included.
- **Positive Controls:** Pathogen-derived DNA, invertebrate-derived DNA or RNA that spans the region to be amplified by PCR or RT-PCR, respectively. This may be extracted from plant, plasmid, bacterial or fungal culture or invertebrate. The positive control will be used at a concentration approximately 1,000 times greater than the PCR's limit of detection.
- **Internal Control:** For each sample, an internal control may be run simultaneously or alternatively where a negative reaction was obtained with the targeted pathogen. Internal controls allow the detection of plant RNA or DNA, or bacterial, fungal, invertebrate or nematode DNA.
- **No template control (water control):** No nucleic acid is added to the reagent mix.]

7 Quality Control Procedure

[Describe calibration of equipment, checking of critical parameters, regular participation in proficiency testing and Estimation of Uncertainty of Measurement (MU).]

8 Equipment

[List all equipment needed for the assay.]

9 Reagents and Solutions

[List all reagents, and solutions derived from them.]

10 Procedure

[Describe the method in detail, up to the stages of detection.]

11 Interpretation and Recording Results**12 Calculations**

[Detail calculation, if required.]

13 Reporting of Results**14 Reference Documents****15 Attachments**

Template for specimen handling

Title for Specimen Handling Procedure
--

Record of Document Control Information

Version No.	Issue Date	Description	Writer	Reviewer	Editor	Owner

Table of Contents
[with the following Sections]

1 Purpose
[Describe the management of specimens coming into the Laboratory that includes:

- How specimens are documented
- How specimens are prepared for processing
- How identifications are carried out
- How identifications are reported
- How specimens are sent away for external testing/validation.]

2 Scope
[Describe to what area or whom the procedure applies.]

3 Actions
[Describe how to handle the specimen that includes:

- How specimens are delivered to the laboratory
- What health and safety instructions to be followed before handling specimens
- The roles and responsibilities of receptionist
- How to store specimens
- How to uniquely identify specimens for traceability
- The roles and responsibilities of diagnostic staff on specimen management
- What to do on receiving unsuitable specimens
- Process and testing of the specimens
- Reporting of results to customer by authorized person
- How to retain or dispose specimens
- How to send specimens for external testing/validation.]

4 Attachments

3.7 Laboratory information management system

3.7.1 Introduction

A laboratory information management system (LIMS) is a method for the laboratory to acquire, analyse, store and report laboratory data. It helps the laboratory to manage and streamline the laboratory's data and its operations. It supports the laboratory in keeping track of its samples, improves accuracy of sample information entered, increases throughput and finally improves operational efficiency.

3.7.2 Need for laboratory information management system

The collection, management and dissemination of information on plant pests is critical for surveillance, incursion investigations, responses and for supporting market access negotiations, etc. It is important to have the diagnostics information available for timely and informed decision-making. Without an appropriate information management system, extraction of sample information and the associated analysis and reporting will be a manual, time-consuming process. Laboratories need to ensure that their results are clean, without error and comply with quality assurance and control standards set by the regulatory bodies (ISO 9001 / ISO 17025). The safety and integrity of data should be taken care of where the laboratory generates a high volume of information, conducting routine tests and being obliged to follow fast sample turnaround times.

A dedicated information management system will assist the laboratory in producing accurate, reproducible results more rapidly and more reliably. The sample information from laboratory testing will be easier to store and track from the moment samples enter the laboratory to when the results are reported. Also, the laboratory can assess pest and disease detections on specified commodities (pest-to-host association), their eradication status, history of observations, etc., for surveillance and research activities.

3.7.3 Management of laboratory information

The laboratory will have to determine what kind of system it needs to have in place for managing the sample information. This decision should be based on an analysis of the needs and the laboratory's practices. The following factors could be taken into consideration for decision-making:

- ◆ size of the organization or laboratory
- ◆ number of routine tests done per week or month
- ◆ total number of samples received
- ◆ regulatory requirements
- ◆ client requirements
- ◆ need for faster sample and result turnaround times
- ◆ how to adapt or cope with challenges in the future, such as a significant increase in volume of data.

3.7.4 Tools to manage laboratory information

3.7.4.1 Paper-based records

It is still possible to use a simple, paper-based record-keeping system. There are certain advantages to using manual record-keeping: it is less expensive to set up, there is no need for a comprehensive or expensive training programme for using specialized software, the risk of data being corrupted is much less, etc. But the main disadvantages are that it is time-consuming to retrieve data, especially when handling a large volume, and sharing of information is not easy.

3.7.4.2 Spreadsheets

A spreadsheet (e.g. MS Excel) is the digital equivalent of a paper worksheet. It can be used to store and manipulate data. Spreadsheets are easy to use but hard to manage when too much information is captured in a single spreadsheet. It becomes more difficult to edit or track down a piece of information. There is no version control and this would create confusion and inaccuracy when spreadsheets are passed around to multiple members of staff. There is no control or hard-and-fast rule on the type of information that the spreadsheets could allow staff to enter in a particular column. Therefore, data can become inaccurate and could compromise the integrity of the results.

3.7.4.3 Databases

A database (e.g. MS Access, Oracle) is generally used for storing large amounts of information. The organization can store any kind of file in a database, including Word files, images and PDFs. Data can be sent to others as PDF or Excel files. A database helps to establish relationships between types of data for quick access to information and simultaneous update. Amendments can be made easily and it is possible for several people to access the same data set using database management systems and at the same time preserve the data integrity. Databases offer a greater range of complexity in terms of data manipulation, but need technical expertise through programming or SQL code.

3.7.4.4 Off-the-shelf software (e.g. LIMS, Q-pulse)

Specialized software is designed to optimize and extend laboratory operations through the data workflow in the laboratory. This kind of software can be used to:

- ◆ receive, log in and label samples
- ◆ assign work (e.g. tests for each sample)
- ◆ check status of work
- ◆ track records and specimens
- ◆ produce test reports after applying quality control.

These systems need to be customized to user requirements. With client/server tools, the system allows processing of data anywhere on the network. In addition, with web-enabled modules in the system, the users may extend operations outside the confines of the laboratory.

3.7.5 What to store

A LIMS allows the organization to store various pieces of information required for laboratory functions. These could include laboratory protocols, list of reagents, images, specimen information, equipment information, with the minimum of the following.

3.7.5.1 Sample registration and accession information

The LIMS is used to store the sample registration and access information:

- ◆ date sample received
- ◆ unique accession number (for sample traceability)
- ◆ tests to be performed
- ◆ discipline (entomology, mycology, virology, nematology)
- ◆ submitter contact details
- ◆ submitter reference number
- ◆ number of vials received
- ◆ sample symptoms, if any
- ◆ urgent or routine testing
- ◆ sample found on host details (soil, variety, container, any plant and its parts, etc.)
- ◆ country of origin and location details.

3.7.5.2 Identification details

- ◆ order
- ◆ family
- ◆ genus
- ◆ species
- ◆ life stage
- ◆ life state (dead or alive)
- ◆ identifier details
- ◆ identification date
- ◆ date of final reply
- ◆ charges, if any
- ◆ new record (new host or new association).

3.8 References

International Organization for Standardization (ISO). 2008. *ISO 9001:2008: Quality management systems – requirements*. Available at http://www.iso.org/iso/catalogue_detail?csnumber=46486 (last accessed on 17 September 2015).

International Organization for Standardization (ISO). 2005. *ISO/IEC 17025:2005: General requirements for the competence of testing and calibration laboratories*. Available at http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=39883 (last accessed on 17 September 2015).

International Accreditation New Zealand – Specific criteria for accreditation – Biological Testing, Fourth Edition May 2011. <http://www.ianz.govt.nz/services/accreditation-2/accreditation/laboratories/biological/> (last accessed on 17 September 2015).

- Krogh, P.** 2012. *File lifecycle: understanding workflow*. Philadelphia, PA, American Society of Media Photographers. Available at <http://dpbestflow.org/file-lifecycle/file-lifecycle-overview> (last accessed on 17 September 2015).
- Lo, P.L. & Blank, R.H.** 1989. A survey of armoured scale species (Hemiptera: Diaspididae) in kiwifruit orchards. *NZ Entomologist*, 12: 1–4.
- Standard Operating Procedures** (2014). Unpublished. New Zealand, Plant Health and Environment Laboratory, Ministry for Primary Industries.
- Thompson, M., Lyons, A., Kumarasinghe, L., Peck, D.R., Kong, G., Shattuck, S. & La Salle, J.** 2011. Remote microscopy: a success story in Australian and New Zealand plant biosecurity. *Australian Journal of Entomology*, 50: 1–6. DOI: 10.1111/j.1440-6055.2010.00803.x.
- Walker, K.** 2012. PaDIL user guide. Unpublished user guide. Museum Victoria, Australia.

Section 2 – Laboratory Workflow

Outline

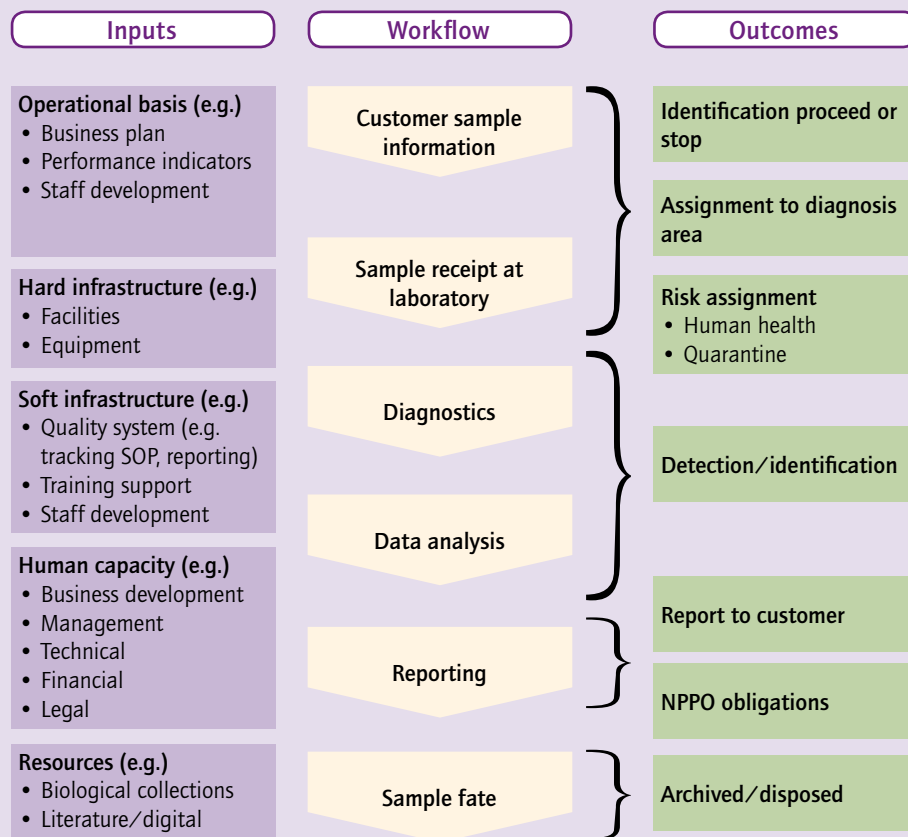
This section provides an overview of the key methods used to detect and identify plant pests. There is a wide range of methods available to the diagnostician, from visual microscopy through to DNA sequencing. General guidance will be given on the process of examining and sampling a specimen and the steps taken to make a diagnosis. Details of commonly used methods are described, along with some examples for different groups of pests.

Figure 14 sets out a basic schematic for the operation of a plant pest identification service, with workflow (centre), inputs (left) and outcomes

(right). This aims to provide a “big picture” view of the overarching activities, stages and decision-points that characterize a pest identification service. It is not a “prescription of practice” that is to be followed and is not set out as an exhaustive list of content; but is intended to guide independent thinking as to how the capability may be realized in context of your particular situation.

The schematic reflects the structure for the following sections and chapters, and shows how these areas work together to give a functional pest identification service.

Figure 14: Basic schematic for the operation of a plant pest identification service



4. Sample Management

Introduction

Samples may be received at the laboratory from a multitude of sources and with many different requests. It is important that these samples and the requests that accompany them are properly logged and are then passed through the laboratory and tracked until a final decision can be reached.

4.1 Sample reception

Mostly it may be expected that samples will be received via inspectors; however, and depending on the operation of the laboratory, samples may also be received from farmers, industry and the public. The extent to which information can be gathered on a sample will therefore be variable. This notwithstanding, the more background that can be obtained on the sample the better the likelihood of obtaining a quick and accurate diagnosis. It is therefore advised that plant diagnostic laboratories provide sample submission information sheets for customers to document pertinent information. These sheets should contain details such as:

- ◆ contact details of the person or organization submitting the sample
- ◆ sample details, e.g. type of plant, age of plants
- ◆ the problem, e.g. symptoms, percentage of plants affected
- ◆ growing history of the plant.

The sample-reception process should also set out the expectation of the customer, i.e. if there is a specific test, cost and time frame for reporting.

The extent to which the submission form may provide leading descriptions and descriptors on pest symptoms is open; however, a minimum level of description should be aimed for, such as whether the disorder is affecting leaves, shoots, fruits or roots, and whether causing necrosis, yellowing, dieback or wilt.

4.2 Sample registration

When a sample arrives at the laboratory for analysis, it should be accompanied by sample-submission paperwork (as outlined above; e.g. Figure 15). Details of the sample should then be captured on a registration system and the sample given a unique identification code or number. This will allow traceability of the sample and allow test results to be easily linked to the customer details to enable swift and accurate reporting.

Samples should be delivered to a central point where they can be opened and registered. Assess the sample paperwork for details of testing required or main symptoms stated by the customer to determine to which diagnostic team the sample should go. Check that you have all essential details, such as:

- ◆ name of sender – company and contact
- ◆ address and contact numbers/e-mail address
- ◆ customer's reference number(s)
- ◆ identity of host plant(s)/sample(s)
- ◆ what testing is required.

If there is no sample-submission form or the information is insufficient, contact the customer to obtain the information required.

When registering the sample, the following details should be captured:

- ◆ customer details (as above)
- ◆ whether the sample is part of a specific survey
- ◆ identity of host plant(s)/sample(s)
- ◆ plant variety
- ◆ sample type
- ◆ country of origin
- ◆ date received
- ◆ diagnosis team (department) to deal with the sample
- ◆ unique sample identification code or number.

Figure 15A: Page 1 of the standard, two-page sample submission form used by Fera







Your Details		Fera Reference: (For internal use only)	
Name:		Your Ref:	
Company:		Purchase order no:	
Address:		Name & Address for Invoice (if different)	
Postcode:		VAT no:	
Tel:		Fax:	
Mobile:		Email:	
Would you like to register for SamTrack?  (Our free and secure on-line sample tracking system, giving quick & easy access to your sample information)*			
The Sample - To help us with our diagnosis, please try and fill in as much information as possible.			
Plant genus, species and variety: (Or Seed/Plant material/Soil type)			
Propagation / planting method (e.g. seed, cutting etc.):		Age of plants or sowing date:	
Are the plants grown outside, under cover, or is this a stored product?			
Which pesticides / herbicides have been used and when?			
The Problem			
What symptoms have you observed? e.g. leaf spot, dieback, wilt			
What do you think the cause might be?	Bacteria  Fungi  Pest  Nematode  Virus 		
Distribution of symptoms & part of plant effected (roots, stems etc)?		% of plants affected	
When was the problem first seen?			
If you would like a specific test from our Price list please state test here:			
Other Information: e.g. cropping history and types of neighbouring plants, slope, temperature, humidity, irrigation, reporting.			
I hereby authorise Fera Plant Clinic to carry out testing on this sample and agree to Fera's Standard Terms and Conditions.			
Signed:		Date:	
PLEASE NOTE: Unsigned forms will not be processed.			
We may use your contact details to send you information about our services/offers/events; however your details will not be passed to any other parties outside of Fera. Please tick here if you wish to opt out of receiving this kind of information and only receive information related to your sample(s) <input type="checkbox"/>			
<small>Fera hereby excludes all liability for any claim, loss, demands or damages of any kind whatsoever (whether such claims, loss, demands or damages were foreseeable, known or otherwise) arising out of or in connection with the services and the preparation of any technical or scientific report, including without limitation, indirect or consequential loss or damage; loss of actual or anticipated profits (including loss of profits on contracts); loss of revenue; loss of business; loss of opportunity; loss of anticipated savings; loss of goodwill; loss of reputation; loss of damage to or corruption of data; loss of use of money or otherwise, and whether or not advised of the possibility of such claim, loss demand or damages and whether arising in tort (including negligence), contract or otherwise. This statement does not affect your statutory rights.</small>			

Figure 15B: Page 2 of the standard, two-page sample submission form used by Fera

Sending your sample for diagnosis

How to select your sample – The sample you send provides the key for diagnosis, so please consider the following:

- Try to send a sample that is representative of the problem - we need to see the full range of symptoms.
- For diseases, try to include the boundary between healthy and diseased tissue and where possible, include healthy material for comparison and mark as such.
- If symptoms on leaves or shoots show general discoloration or dieback, suggesting possible root damage, then please send us a whole plant (where possible) and include the roots and surrounding soil.
- If you suspect the problem is due to nematodes, please include separate soil samples from both the affected area and the edge of the affected area.

How to package your sample

Whole plants	Enclose moist root ball in a plastic bag sealed around the plant stem. Place the whole plant in a second plastic bag, inflate slightly and seal.
Leaves and shoots	
Virus diagnosis:	Place in a plastic bag, inflate slightly and seal.
Other/Unknown diagnosis:	Wrap sample in slightly moist absorbent paper, place in a plastic bag, inflate slightly and seal.
Fleshy items	For example: fruit, vegetables, tubers (except potatoes), bulbs, corms, etc. Wrap in dry, absorbent paper, if rotting/breaking down please wrap individually. Place in a plastic bag, inflate slightly and seal
Invertebrate pests	Samples of invertebrate pests (insects, spiders, mites etc.) should be put in a sealed plastic container.
Potato tubers	
Invertebrate diagnosis:	Wrap in dry, absorbent paper. Place in a plastic bag, inflate slightly and seal.
Other/Unknown diagnosis:	Wrap in dry, absorbent paper. Do not place in a plastic bag.
Soil samples	For nematode analysis. Place 500g of soil in a strong plastic bag and seal.
Seed samples	Ensure seeds are securely packaged.

All samples should be put in a strong cardboard box and packed securely with screwed up paper. Include your Sample Submission Form in a separate bag and seal the box

Send your sample to:

**Plant Clinic
Fera
Sand Hutton
York
YO41 1LZ
United Kingdom**

Ideally, samples should be dispatched by either express courier service or first-class post to arrive at Fera next day. If possible to avoid sending samples over the weekend or a bank holiday, however if unavoidable mark the outer packaging with 'Please refrigerate on arrival' if necessary.

If you have any queries, contact:

Fera Plant Clinic Helpline
Tel: 01904 462324
Fax: 01904 462147
E-mail: plantclinic@fera.gsi.gov.uk

* **SamTrack** is our free and secure on-line sample tracking system, giving quick & easy access to your sample information. You can register for SamTrack at <http://samtrack.fera.defra.gov.uk/>. Once you've registered you will receive a unique username and password along with instructions on how to use the system.

Using **SamTrack** gives **current information**, 24 hours a day. You can...

- Check when we have received your sample(s) and who is dealing with your sample(s).
- Check what pests and diseases have been identified, check your report and see a summary of all the samples you've sent to us.
- Access information outside normal working hours.

4.3 Sample examination

Once a sample has been received and the details captured, it is ready for examination. Initial checks may include:

- ◆ host details and sample numbers tally
- ◆ that the sample is fit for examination, i.e. not obviously excessively rotted
- ◆ whether there are flying or mobile insects that may need containment
- ◆ that the sample sent is relevant to the problem reported.

Having satisfied an initial check, the sample will progress to the diagnosis phase. For samples for which a specific test is stated (e.g. potato testing for viruses), passing the sample on to the appropriate diagnostician is straightforward. However, with samples where the request is to determine the aetiology of an unknown disorder, a preliminary investigation is required. The next sections set out some basic observations that can lead to a best approximation of causal pest taxa and course of diagnosis.

4.3.1 Roots, tubers, bulbs and corms

Symptoms – check for malformation, physical damage, galls or cysts, root congestion, rotting, etc. If rotting, ascertain if dry or wet, deep or superficial, major or minor, extending into crown.

Examination – initially examine the sample without disturbing any attached soil. Then gently remove soil using a blunt instrument or by shaking and re-examine. Finally, wash off any soil and check again. At each step, examine surfaces under a dissecting microscope and look for mycelium, fruiting bodies, sclerotia, runner hyphae, bootlaces, etc. Cut transverse and longitudinal sections through larger pieces of material (e.g. tubers) and look for any vascular discoloration or general decay.

Examination under the microscope – make a slide preparation and look for fungal structures; examine for bacterial streaming.

Tests – isolate from the leading edge, float, bait, incubate, inoculate, check soil (pH and electrical conductivity).

Review sample information sheet – cultural problems (e.g. waterlogging), storage issues (e.g. frost, overheating, oxygen starvation), ground compaction or hard pan, groundwater pollution (e.g. sewage leak), planting or potting-on date, onset of symptoms, host range affected, distribution (patchy or widespread), previous cropping history, nutritional analyses, application of chemicals (e.g. pesticides or growth regulators such as sprout suppressants), etc.

4.3.2 Stem base

Symptoms – check for lesions, cankers, galls, adventitious roots, vascular discoloration, evidence

of invertebrate pest damage (e.g. frass, webbing, slug or snail slime).

Examination – look for mycelium, fruiting bodies (e.g. sclerotia, pycnidia), cut transverse and longitudinal sections, look for vascular discoloration, graft incompatibility, etc.

Examination under the microscope – make slide preparations and look for fungal structures, vascular discoloration, examine for bacterial streaming.

Tests – isolate from the leading edge, incubate, inoculate and float.

Review sample information sheet – vertebrate pest damage (e.g. rabbits, deer, etc.), physical damage (e.g. grass strimming), weather damage (e.g. wind rock, frost, drought, rock salt application in winter), soil improvers and mulches, etc.

4.3.3 Stems and trunks

Symptoms – check for physical damage and wounds, lesions, cankers, galls, vascular discoloration, invertebrate pest damage (e.g. insect bore holes, webbing, frass), etc.

Examination – look for mycelium, fruiting bodies, toadstools, bracket fungi, cut transverse and longitudinal sections, etc.

Examination under the microscope – make slide preparations and look for fungal structures, vascular or heartwood discoloration, examine for bacterial streaming.

Tests – isolate from the leading edge, incubate, inoculate, float.

Review sample information sheet – vertebrate pest damage (e.g. squirrels, deer, etc.), invertebrate pest damage (e.g. wood boring insects), whether any mushrooms or toadstools have been seen, physical damage (e.g. lightning damage).

4.3.4 Leaves, flowers and fruits

Symptoms – examine upper and lower surfaces and, for larger specimens, cut transverse and longitudinal sections to determine extent of decay. Check for any discoloration or spots and ascertain distribution (e.g. old or new growth, interveinal or veinal, marginal, apical or distal, single, numerous,

coalesced), malformation and distortion (atypical shapes and colour, prolific hair production, epinasty, etc.). Record if spots are necrotic, chlorotic or water soaked. Check for physical damage and wounds, lesions, cankers, galls, invertebrate pest damage (e.g. insect bore holes, webbing, frass).

Examination – look for mycelium, fruiting bodies, mildews, rusts, slime moulds, etc., examine for bacterial streaming.

Examination under the microscope – make slide preparations and look for fungal structures.

Tests – isolate from the leading edge, incubate, inoculate, float.

Review sample information sheet – pesticide applications, weather damage (e.g. hail, frost, sun or wind scorch, windblown soil or sand).



5. Diagnosis

Introduction

This section provides an overview of the key methods used to detect and identify plant pests and diseases. There is a wide range of methods available to the diagnostician, from visual microscopy through to DNA sequencing. General guidance is given on the process of examining and sampling a specimen and the steps to be taken to make a diagnosis. Details of commonly used methods are given along with some examples for different groups of pests and diseases.

5.1 Diagnostic methods

This section outlines some of the diagnostic methods that can be used to identify pests and highlights some examples. A range of diagnostic methods is typically available for any given pest, therefore guidelines on identifying the most suitable method with consideration of infrastructure and human resource capacity are outlined. Critical here is a clear appreciation of the question to be answered, the level of confidence in the identification required, the time frame within which you are operating, the costs and other requirements of the technique and the number of samples that require the test (diagnosis). The choice of method is often a trade-off between different factors including cost, specificity, sensitivity and number of samples.

5.1.1 Conventional bioassay testing

These tests typically take longer and are more labour intensive than the other laboratory assays described below. Nevertheless, they can still be very important in the diagnosis; for example, host indexing of viruses and viroids is still often required to verify the result of a laboratory test. Bio-assays are usually carried out in insect-proof compartmented glasshouses and, in some climates, screen houses will be required for raising and holding the plants. Growth cabinets, able to provide controlled temperature, light and day length may be required for year-round work. Selection of test plants for routine diagnostic

use is dependent upon the type of plants and pests tested. The continued maintenance of reference material is a consideration.

5.1.2 Morphological identification

Determination of the morphology of a pest is a fundamental part of all plant pest diagnosis. The ability to characterize pests accurately and quickly is important for understanding the significance of the pest detected. Morphology is the comparison of the pest being identified with known references, which allows the interpretation of observed symptoms or features to arrive at the identification. Often this identification is followed up with confirmatory tests that support the identification or provide further details, such as specific species type. The extent to which morphological techniques are used depends on the discipline. Morphology can range from looking at sexual reproductive organs of insects, to looking at fungal structures by microscopy, to looking at size and colour of bacterial colonies.

5.1.3 Metabolite analysis (mainly bacterial identification)

The majority of bacterial pathogens can be isolated and grown on media and then subjected to analysis of metabolite composition and properties that provide taxonomic information. Traditionally, these methods have involved a sequence of tests, mainly substrate utilization tests, that followed a dichotomous key and, as a result, identification would take weeks rather than days to achieve. The methods also required a high level of technical competence for reproducible data to be generated. Alternative formats for substrate utilization assessments have subsequently been commercially developed, such as the Biolog system (<http://www.biolog.com>), which enables quicker and more reliable identification.

Another commercially available system for identification of bacteria is the MIDI system (<http://www.midi-inc.com>), which relies on the

identification and quantification of cell-wall fatty acids. As with Biolog, an identification can be achieved within 48 h of obtaining a pure culture. Both Biolog and the MIDI system are commercial products and come with extensive libraries of bacterial species. The level of taxonomic identification achieved by these systems is similar. As a general rule, both Biolog and the MIDI system enable reliable species-level identification for plant bacterial pathogens. More confident and lower taxonomic (i.e. pathovar) identification can be achieved in some species through the addition of specific metabolite profiles of samples to the internal databases of these systems.

5.1.4 Serological methods

Serological methods are based on a property of mammalian and avian immune systems. When foreign material (termed antigen) such as a micro-organism, protein or complex carbohydrate is injected into an animal, the animal's immune system responds by producing antibodies in its blood serum. These antibodies bind specifically with the antigen that triggered their production.

Many plant pathogens can be detected using serological or immunological methods. This technology is particularly important for the diagnosis and identification of plant viruses; the molecular simplicity of viruses allows a highly specific response. However, such methods have also been developed for bacteria and more complex organisms such as fungi.

Antibodies can be polyclonal or monoclonal. Polyclonal antibodies are so called because they consist of many antibodies each with a different specificity, which bind to several different epitopes (binding sites) of the antigen. Monoclonal antibodies contain multiple identical copies of just one antibody that binds to one specific epitope. Because of these properties, a generalized view of mono- and polyclonal antibodies is that monoclonal antibodies provide greater specificity than polyclonal antibodies. The merits of this difference then depend on the intended purpose and target pest and whether high specificity is needed. Immunoassays can visualize the antibody-antigen binding either directly or indirectly.

Detecting the quantity of antibody or antigen can be achieved by a variety of methods. One of the most common is to label either the antigen or antibody. The label may consist of an enzyme, colloidal gold (lateral flow assays), radioisotope, magnetic label or fluorescence. Other techniques include agglutination, nephelometry, turbidimetry and Western blot.

In laboratory-based immunoassays, most of these techniques have been superseded by ELISA (enzyme-linked immunosorbent assay). In ELISA, an unknown amount of antigen is fixed to a surface, such as a 96-well plate. This is followed by a blocking step to prevent non-specific antibody binding. A specific antibody is then attached to the surface in order to bind to the antigen. The antibody is linked to an enzyme and, in the final step, a substrate is added that the enzyme can convert to some detectable signal. For example, in fluorescence ELISA, when the sample is exposed to light of an appropriate wavelength, any antigen-antibody complexes will fluoresce. The amount of fluorescence can be used to determine the amount of antigen present in a sample. ELISA remains one of the most widely used methods for the routine detection of plant viruses, although nucleic acid amplification technologies are now being used more routinely. ELISA assays are highly sensitive, easily replicated, can quantify levels of the pathogen and can be automated. ELISA is a robust test that can be performed in almost any laboratory and requires minimum training.

Although it has many advantages, the drawbacks of ELISA include the fact that the test may not capture all strains of a pathogen and that ELISA kits have not been developed for many targets. The production of antibodies can also be resource intensive, for example, requiring the use of live animals, in contrast to PCR techniques where a wealth of sequence data is available and assays can be designed using a personal computer with Internet access. ELISA is also less sensitive than PCR.

5.1.5 Nucleic acid based detection methods

The science of nucleic acid based detection of plant pests has progressed rapidly in the past 20–30 years and many different methods populate

the literature. In very simple terms, the vast majority of these methods rely on knowledge of the nucleic acid sequence of an organism and inference of taxonomic specificity (e.g. family, genus, species, strain). The choice of method is strongly influenced by the outcome required, especially the level of specificity required and the volume of material for testing.

The nucleic acid sequence of any organism comprises regions conserved at various levels during evolution: high, moderate and low sequence conservation. Typically, genes that code for essential processes (such as rRNA genes) are highly conserved, whereas less critical genes may exhibit moderate conservation and non-coding regions low conservation. These differences in sequence have both provided supporting evidence for the current taxonomies of organisms and led to taxonomic revisions. These same sequences also provide the knowledge base for designing short nucleic acid sequences that are taxon specific (genus, species, etc.) and that can be used either as a probe in hybridization or as a primer in PCR-based methods (as outlined below). With the revolution in whole genome sequencing now in train, the level of sequence data available in databases such as GenBank is ever increasing, allowing highly intelligent diagnostics to be developed.

Introductions to some of the main and most recent nucleic acid methods as applied to pest diagnostics follow.

5.1.5.1 Hybridization-based detection

Hybridization is based on the principle of bringing nucleic acid sequences of the target (unknown) and test (known) organism together to allow annealing (hybridization) of homologous sequences if present and the production of a detectable signal. The procedure generally requires either the target or the test nucleic acid to be anchored to a physical matrix, such as a nitrocellulose membrane. The detection signal can be radiation or, more recently, some form of dye-based chemistry. Recent advances have seen major changes in the nature of the physical matrix and the miniaturization of the detection unit. The microarray exemplifies the current design of hybridization platforms where many tens of thousands of hybridization units can be contained

on the "footprint" of an Eppendorf. In these microarray systems, the sequence of each detection unit can be designed and thus microarrays can be tailored to detection as required. An example of this type of technology for plant viruses is the Defra BioChip project (<http://biochip.rvc.ac.uk/>) and, on a commercial basis, ClonDiag (<http://www.clondiag.com>). In both of these example systems, the microarrays are looking for identifications from a single sample.

In many cases the question to be answered requires looking at many samples for a specific pest of quarantine concern. In these circumstances a microarray is not suitable. A more appropriate method for high-throughput analysis of many samples is nucleic acid spot hybridization (NASH). With this method many samples (often a stem imprint) are spotted onto a matrix (nitrocellulose membrane) and then a test probe specific to the pest of concern is applied under conditions suitable for hybridization and development of a signal. The method and application is highly analogous to ELISA and microplates. Experience with NASH is required to interpret the signal and to distinguish the difference between a very weak positive signal and a mark due to discoloration caused by the sample.

5.1.5.2 Conventional and real-time polymerase chain reaction (PCR) based detection methods

The application of conventional and real-time PCR to pest diagnosis and characterization has been substantial in the context of plant pathology.

The polymerase chain reaction directs the exponential multiplication of template nucleic acid sequence by thermostable enzymes such as *Taq* DNA polymerase under cycles of heating and cooling that drive DNA denaturation and annealing, respectively. The directed nature of PCR is determined mainly by the nucleic acid sequence of short oligonucleotides, called primers, which work in pairs to "prime" the PCR reaction, and the annealing temperature. The primer sequence dictates the region of the template DNA that will be amplified. Where sequence information is known, primers can be designed to amplify target regions; otherwise they can be designed to amplify

randomly. PCR of RNA targets, such as viruses, requires a reverse transcription step to generate cDNA from the RNA template prior to DNA amplification. An overview of PCR is provided by NCBI. Polymerase chain reaction (PCR). Available at <http://www.ncbi.nlm.nih.gov/probe/docs/techpcr/> (last accessed on 17 September 2015).

The key outcome of PCR is the production of many copies of DNA of identical sequence to that of the template. Depending on the design of the primers, a single product or many amplification products of various sizes can result from the PCR. The amplified DNA can then be the subject of further processing (restriction analysis, sequencing, labelling) or visualized. Traditionally, PCR products have been visualized on an agarose gel. However, an evolution of conventional PCR is real-time PCR that allows real-time product analysis through the monitoring of a chemical reaction that parallels amplification. In real-time PCR, a probe labelled with a reporter dye attaches between the primers. When amplification occurs, the dye is activated resulting in reporter signal. The signal is measured without the need to open the PCR tube, largely reducing any chances of cross-contamination.

In most examples, a PCR-based diagnostic is based on the production of a single amplification product, seen on an agarose gel or by a fluorescence reading and recorded as a positive or negative outcome. In addition, more considered PCR diagnostics will include an internal control to check that the reaction has proceeded as expected, alongside the standard controls one would use to detect false positive and false negative results. With conventional PCR the internal control has to yield a fragment of a different size to the pest fragment, and with real-time PCR make use of a different dye chemistry. Some PCR methods also include quantification of the pathogen based on the intensity of the signal by comparison to the internal standard.

Other PCR-based diagnostic methods have made use of a fingerprint of amplification fragments that is characterized and known to be specific to an organism. A further and major application of PCR and diagnostics is to sequence the amplified fragment and then compare this sequence against

known sequences on GenBank or an in-house DNA sequence library using a tool such as BLAST.

Many studies have sought to compare PCR and ELISA. The experience of most laboratories has been that real-time PCR is ten times more sensitive than conventional PCR and a hundred times more sensitive than ELISA. However, PCR and real-time PCR both require specialist equipment and consumables; and while these costs are coming down they exceed those for ELISA and can be a constraint in terms of initial capital outlay and running costs.

5.1.5.3 Loop-mediated isothermal amplification (LAMP)

As mentioned above, one disadvantage of PCR is the cost of the specialized equipment required to perform accurately controlled thermal cycling and, in the case of real-time PCR, concurrent monitoring of fluorescence. Loop-mediated isothermal amplification (LAMP) is a method for the detection of specific nucleic acid sequences and has the potential to overcome many of the limitations of PCR-based methods. The ability of LAMP to amplify a target nucleic acid sequence under isothermal conditions removes the need for thermal cycling equipment, allowing testing to be carried out with minimal equipment (a water bath or heated block). Furthermore, simplified methods for the detection of amplification products facilitate the use of LAMP-based methods in the field or in less-well-resourced settings.

LAMP is an amplification method that uses two pairs of primers (internal and external primers) and a DNA polymerase with strand displacing activity to produce amplification products that contain loop regions to which further primers can bind, allowing amplification to continue without thermal cycling. Amplification is accelerated by the use of an additional set of primers (loop primers) that bind to those loops that are incorrectly oriented for the internal primers to bind. A high level of specificity results from the requirement for primers to bind to up to eight regions of the target sequence. LAMP has been used for the detection of a range of plant pathogens.

LAMP reactions generate a large amount of amplification product that can be detected

by conventional agarose gel electrophoresis, by using spectrophotometric equipment to measure turbidity, in real time by using intercalating fluorescent dyes, or by visual inspection of turbidity or colour changes. While detection methods based on visual inspection have the advantage of requiring no equipment, assessment of colour or turbidity with the unaided eye is potentially subjective. Equipment-free methods for unambiguous detection of LAMP products would increase the feasibility of using LAMP for detection of phytopathogens outside the laboratory. One such method is the use of lateral flow devices (LFDs) for the detection of labels incorporated into the amplification products.

Like PCR, LAMP can be used to detect RNA targets by incorporating a reverse transcription step to generate cDNA from the RNA template prior to amplification; reverse transcription and LAMP can be carried out in one tube, at a single temperature. The significant advantages of LAMP are therefore: (i) the ability to perform amplification reactions under isothermal conditions, obviating the need for thermal cycling equipment; (ii) the high specificity inherent in a mechanism that requires the recognition of six regions (or eight regions if loop primers are used) of the target sequence for amplification to occur; and (iii) an efficiency of amplification that generates a very large amount of product in less than 1 h, allowing the use of novel detection strategies.

5.1.5.4 Pyrosequencing and next generation sequencing

Approaches using PCR and LAMP rely on sequence knowledge to design appropriate primers and a basic appreciation of what the causal organism is. One limitation of the reliance on sequence knowledge is when trying to identify a new pest or to verify that material is clean of pests. In these situations one would need to use one or more primer sets of broad taxonomic specificity in the expectation that these would detect a pest if present. However, with this approach a negative result does not remove all uncertainty because an unknown pest may be genetically very distinct and the target sites of the primers may lack sufficient homology

for PCR to initiate. A new approach to diagnostics that has particular merit in identifying unknown pests and verifying that material is not infected is next generation sequencing, which uses universal and randomly targeting primers to massively amplify all nucleic acid within a sample. The short amplification products are then sequenced and sophisticated informatic software used to analyse the small sequences and join them into sequence lengths of potential taxonomic value. The identity of constructed sequences can then be examined by searching known sequence databases. This technology is highly specialized, would be limited to a few laboratories and justified only in exceptional cases.

5.1.6 Selection of an appropriate diagnostic method

Once a pathogen is required to be identified, consideration should be given to choosing an appropriate diagnostic method. Consideration should also be given to assessing the resources the laboratory presently has, or could have, for testing. Possessing specific items of equipment, for example, a real-time PCR machine, may be one of the initial considerations, but additional thought should be given to other, wider resource-related factors, including availability of staff and their relative competences, laboratory space and continued maintenance of capital equipment. Then specific requirements of the test should be considered, such as sensitivity, specificity and robustness of the assay relative to the data output of the assay (sequence data, viability data, qualitative and quantitative results). Finally, practical concerns, such as time taken to obtain a result and the cost of individual tests, should also be considered. With these factors in mind, literature searching can then begin for the range of diagnostic assays available. A useful start is an Internet search and then a bibliographic database search, usually with the type of method required and the target pathogen.

5.2 Verification of new methods

Once an appropriate diagnostic method has been selected, the laboratory will need to verify that it can perform the test competently. Under ISO

17025:2005 "the laboratory shall confirm that it can properly operate standard methods before introducing the tests or calibrations." This is different from validation, which is the "confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled" (Weigers 2003, 303). Method verification involves a number of experiments using positive and negative control material with the aim of ensuring that the laboratory can obtain a suitable limit of detection and replication of a test.

5.3 Training laboratory personnel

For successful and reliable diagnosis of plant pests, it is essential that staff have (i) adequate training, (ii) the opportunity to build experience and (iii) can demonstrate competence. A number of training sessions may be required until the trainer and trainee agree that the trainee is competent to undertake the task unsupervised; all training sessions should be documented. As a rule, it would be expected that diagnosticians must have a minimum of two years' experience in a diagnostic laboratory prior to attempting to complete a full diagnosis (due to the huge array of plants, pests, symptoms; experience is key to carrying out quality diagnosis).

All stages of the training must be recorded and shall cover:

- ◆ Stage 1 – Reading relevant instructions (e.g. SOPs)
- ◆ Stage 2 – Observing the task being performed by a trained member of staff
- ◆ Stage 3 – Carrying out the task under supervision
- ◆ Stage 4 – Assessment of competence to carry out the task unsupervised.

Wherever possible, evidence or experience used as part of the assessment of competence should be recorded. Competence is assessed using, where possible, at least one of the following:

- ◆ spiked recovery experiments
- ◆ repeat analysis of previously analysed samples
- ◆ analysis of reference or proficiency test materials
- ◆ comparison of results of trainer and trainee.

Criteria of acceptance are documented and are normally set at the quality control acceptance

limits for the method. The date of authorization to carry out a task unsupervised is recorded on the assessment form, along with confirmation by the line manager. Line managers should ensure that the evidence presented and documented is correct and appropriate.

Laboratories will often try to participate in proficiency testing schemes. Proficiency testing determines the performance of individual laboratories for specific tests or measurements and is used to monitor laboratories' continuing performance. This will also provide ongoing evidence of an individual's competence.

5.4 Methodologies for diagnosis

The aim of this section is to provide an introduction to the methodologies required to isolate and identify a suspected plant pest. It is by no means an exhaustive guide to the identification of plant pests.

5.4.1 Bacteriology

An introduction to plant pathogenic bacteria is provided by Vidaver, A.K. & Lambrecht, P.A. 2004. Bacteria as plant pathogens. *The Plant Health Instructor*. DOI: 10.1094/PHI-I-2004-0809-01. Available at <http://www.apsnet.org/edcenter/intropp/pathogengroups/pages/bacteria.aspx> (last accessed on 17 September 2015).

The work should be undertaken in a laboratory that has suitable containment in place to work with quarantine pests. The equipment required is as follows:

- ◆ dissecting trays
- ◆ scalpels, knives and secateurs
- ◆ 70 percent ethanol
- ◆ blue roll/tissue
- ◆ small flame source
- ◆ sterile disposable 90 mm Petri dishes
- ◆ 0.1 percent sterile peptone or 0.85 percent NaCl
- ◆ sterile disposable 1 µl and 10 µl inoculating loops
- ◆ sterile distilled water
- ◆ growth media
- ◆ incubators (28 °C & 25 °C ±1 °C and 21 °C ±2 °C)
- ◆ autoclave bags.

5.4.1.1 Diagnosis

Diagnosis of bacterial plant pathogens in symptomatic samples includes examination, isolation, identification and confirmation of causal organisms. The level of identification for an appropriate diagnosis can be at the genus, the species or the subspecific level.

Bacterial infections of plants are often seasonal. Infections are also influenced by climatic conditions and agricultural practice. A good background knowledge of these factors must be acquired over several growing seasons.

Descriptions of symptoms and methods of isolation and confirmation of the commonest bacterial plant diseases are found in *Methods for the diagnosis of bacterial diseases of plants* (Lelliot and Stead, 1991), which is an essential reference. Other useful and essential references are listed in section 5.5.1.3.

All unknown symptomatic samples should be treated as quarantine material and waste disposal must comply with local procedures.

The diagnostician should record a detailed description of the symptoms on the lab sheet, including diagrams where appropriate. Pictures can be taken before destructive processing of the samples.

Isolation

- ◆ With reference to the literature, a clinical decision is made as to which genera of bacteria are known to cause the symptom in that host.
- ◆ Excise a small amount of tissue from the leading edge of the symptom (where healthy plant tissue meets symptom) using sterile scalpels. If no leading edge is present, isolation can be attempted from another part of the plant where bacteria are likely to be prevalent.
- ◆ Macerate the excised material in a few drops of sterile (1 percent) peptone or saline (0.85 percent NaCl) using a sterile Petri dish as a clean surface and let stand for a few minutes before streaking onto media.
- ◆ The initial isolation is best undertaken on a low nutrient media to suppress rapid growth of saprophytes. A majority of bacteria can be successfully isolated on nutrient agar; however, more

selective, richer growth media may be recommended where specific genera are suspected.

- ◆ Label the base of the agar plate (not the lid) with the diagnostic reference number and date.
- ◆ Inoculate the growth media with the macerate by streaking out. Incubate the inoculated plates. Most genera of plant pathogenic bacteria are incubated at 25 °C ±1 °C for 48 h. However, if looking for certain specific bacteria, other temperatures may be required (e.g. 28 °C ±1 °C or 21 °C ±2 °C). Other slow-growing bacteria will also require longer incubation times.

Identification

- ◆ Examine the plates of growth media after appropriate incubation (48–72 h), for typical bacterial colonies of presumptive plant pathogens.
- ◆ As a guidance, the most probable candidate colonies will likely be the most numerous and not necessarily the first visible colonies that appear at 24 h.
- ◆ If no presumptive bacterial pathogens are isolated, re-evaluate the symptom description and make a clinical decision on whether the symptom is due to infection by organisms other than bacteria or has a physiological cause.
- ◆ When bacterial infection is not indicated, a final report of "No primary bacterial plant pathogens isolated from the sample provided" is recorded.
- ◆ Purify presumptive pathogens within mixed populations by subculturing onto appropriate fresh media and reincubate at a suitable temperature.
- ◆ If colonies are still mixed, using a 1 µl inoculation loop take a small amount of a mixed colony and resuspend in 5 ml of distilled water and, using a 10 µl loop, streak onto fresh media.
- ◆ With a pure culture, plate out onto a richer medium, such as KB, as this will support more prolific growth and may maintain the viability of the culture better.
- ◆ Generally, for short-term storage (1–3 weeks) grown cultures are stored at 5 °C, or at room temperature on agar. For long-term storage, glycerol stocks should be prepared at –20 °C or –80 °C on ceramic beads.

- ◆ When well-isolated colonies of presumptive pathogens have been achieved it is appropriate to progress to the identification stage.
- ◆ Before proceeding with specialized testing (such as fatty acid profiling or PCR) it can be useful to conduct Gram testing (e.g. KOH test), assess colony morphology and pigmentation, anaerobic growth test, spore determination and oxidase test to assist with differentiation of some of the common genera.

Confirmation (Koch's postulates)

- ◆ Full confirmation is the demonstration of pathogenicity of the bacterium on the required host, causing symptoms consistent with those initially described, followed by reisolation and repeat identification from that host.
- ◆ For confirmation – inoculate a purified fresh culture of the identified bacterial culture into healthy growing plants of the appropriate host species and grow-on alongside uninoculated controls.
- ◆ Grow on the inoculated plants and observe for symptom development.
- ◆ Reisolate from the newly developed symptoms.
- ◆ Confirmation is complete when the identified bacteria is recovered from the host and identified.
- ◆ Full identification is necessary with a suspected quarantine-listed organism or for a new or unusual finding that merits a peer-reviewed science publication.

5.4.2 Mycology

An introduction to plant pathogenic fungi is provided in the *Diagnostic manual for plant diseases in Vietnam*. Section 7. *Fungal taxonomy and plant pathogens*. Available at http://aci.gov.au/files/node/8613/MN129_part4.pdf (last accessed on 17 September 2015).

The work should be undertaken in a laboratory that has suitable containment in place to work with quarantine pathogens.

The equipment required is as follows:

- ◆ dissection tray
- ◆ scalpels and single-edge razor blades.
- ◆ mounted needles or disposable hypodermic needles

- ◆ forceps
- ◆ glass microscope slides
- ◆ glass cover-slips no. 2 (18×18, 22×22 and 18×50 mm or other as appropriate)
- ◆ clear self-adhesive tape (Sellotape)
- ◆ mounting medium: lactoglycerol
- ◆ stains: trypan/cotton blue in lactoglycerol
- ◆ Petri dishes containing potato dextrose agar (PDA) or other appropriate medium
- ◆ boiling tubes
- ◆ small flame source
- ◆ chemicals for boiling roots: sodium hydroxide or potassium hydroxide (KOH)
- ◆ anti-bumping granules, if available.

5.4.2.1 Diagnosis

It is important to accurately describe symptoms prior to examination (which is frequently invasive and destructive) and then to prepare material for examination under a compound microscope for identification of any fungal structures. Pure cultures may also be prepared to aid identification.

Initial examination

Check the customer information sheet for details about the host, country of origin, percentage level of infection and comments.

Examine sample material with naked eye and note any damage to specimen, such as:

- ◆ apparently dried and dead tissues
- ◆ discoloration of foliage
 - yellowing (chlorosis)
 - browning (necrosis)
 - coloured deposits
- ◆ spots or holes on leaves
 - symmetrical
 - irregularly shaped
 - associated with chlorotic or discoloured haloes
- ◆ malformation or stunting of leaves or shoots
- ◆ stem browning or canker formation
- ◆ stunted, contorted, blackened or rotted root system
- ◆ firmness of tubers, bulbs or fruits
- ◆ sticky exudates, gummosis.

Record the extent and position of any such damage outlined above and then place the sample under the dissecting microscope and select the

lowest magnification. Slowly examine healthy tissue so that the appearance of non-infected tissue (especially leaves) can be recognized. Then examine diseased material for any fungal structures, recording their position, occurrence, shape and relationship to the diseased areas. Decide whether the fungal structures are consistently associated with symptoms.

Having detected a fungal structure, increase magnification (up to about $\times 50$) to determine its morphology (mycelium, conidiophore, sporodochium, pycnidium, acervulus, apothecium, cleistothecium, perithecium, etc.), pigmentation (hyaline – colourless, brightly coloured or dematiaceous – darkly coloured) and position of structure on host (superficial, erumpent, partially erumpent, raised, sunken, emergent from stomata, lenticel, etc.). After examining host surfaces it may be necessary to cut into the sample (especially for tubers, bulbs, roots, stems and fruits) to determine extent of penetration or damage.

If an area of interest is located, then proceed to examination of the material using a compound light microscope.

Fruiting body present

Place a small drop of lactoglycerol (with or without stain) on the surface of a clean glass microscope slide. Keeping the sample material under the dissecting microscope, carefully pick off a specimen of several suspect fungal fruiting bodies with the tip of a fine, alcohol-flamed scalpel blade or mounted needle or new sterile hypodermic needle. Examine the slide under the dissecting microscope to confirm that structures were successfully removed and are correctly oriented for examination (excessive amounts of host material can also be teased away at this stage and discarded). Gently lower a suitably sized glass cover-slip onto the slide and then press down lightly to expel any air bubbles. If necessary, holding the slide by the end, gently warm the slide over the flame of a spirit burner till any air pockets start to expand, remove from flame and place on the bench top to cool. This removes any further pockets of air and also assists in clearing of tissues by the mountant. Transfer the slide to a compound light microscope.

Aerial mycelium, chemical deposits or unknown structures

Cut off a small piece of clear adhesive tape such as Sellotape (up to 30 mm long) and place adhesive side down onto sample material, then gently press the tape down. Specimen should have adhered to tape. Lift off the tape and then place the desired portion (adhesive side down) onto a small drop of lactoglycerol on a glass microscope slide. Press down gently to remove air bubbles and then warm very carefully to avoid boiling, which may dissolve the adhesive. Allow slide to cool and transfer it to the compound microscope.

No structures observed but fungi suspected

Remove a thin transverse or longitudinal section from the host (leaves, stems, roots, tubers and bulbs) with a scalpel or single-edged razor blade and place into a drop of trypan/cotton blue in lactoglycerol on a glass microscope slide. (Trypan/cotton blue is used because it assists in differentiating host from fungal material; the latter usually stains darker blue.) Lower a cover-slip gently and warm the slide as above. Transfer the slide to compound microscope.

Fine young roots may be examined whole; however, any older, tougher roots will need boiling to soften them to allow stain to enter and stain any fungal spores contained within. Prepare such roots by boiling as below.

Boiling roots for observation

- ◆ Select suspect area of roots, avoiding if possible excessively rotted or disintegrating strands. Cut off 3–6 cm lengths of an appropriate amount of root in relation to sample size.
- ◆ Put roots into a large boiling tube, fill the tube with no more than an inch of water and add 4–6 anti-bumping granules (if available). The granules are not essential but they help to minimize the risk of the hot contents splashing out of the tube.
- ◆ Add 2–3 pellets of either sodium hydroxide or KOH. (Avoid handling pellets with bare fingers.)
- ◆ Use a test-tube holder to hold the boiling tube over a medium flame on a Bunsen burner or gas flame (a spirit burner is not hot enough, so do

not use) and boil the roots for several minutes to soften and clarify them, then remove from heat.

- ◆ Place the boiling tube in a tube rack until cool enough to remove specimen for observation.
- ◆ Once cool, place specimen in an empty Petri dish to aid root selection.
- ◆ Using forceps place some of the boiled root specimen onto a slide and add 1–2 drops of trypan/cotton blue in lactoglycerol.
- ◆ Place a large, rectangular (22×64 mm) cover-slip over the roots.
- ◆ Gently tap the cover-slip to flatten the roots for examination.

Examination of mounted material with a compound microscope

- ◆ If necessary, set up the compound microscope for optimum resolution and clarity.
- ◆ Select a low power objective (e.g. ×10) and locate the specimen.
- ◆ Scan the specimen at low power and ascertain if structures are fungal.
- ◆ Ascertain if spores are present and determine how they are being formed, e.g. from mycelium, within a fruiting body.
- ◆ If a fruiting body is located, record its external morphology and dimensions and then attempt to expel its contents by gently pressing down on the cover-slip with a blunt mounted needle. This can be done with extreme care on the microscope stage, while observing at ×10 or remove slide from microscope and place on bench and tap very gently with a pencil.
- ◆ Re-examine under high power and describe morphology of spores and spore-producing structures.
- ◆ It may be necessary to cut a thin section of a fruiting body if details of spore attachment are not clear. This can be done with either a sharp single-edged razor blade or a scalpel.
- ◆ Compare morphology and initial tentative diagnosis of sample to reference information.
- ◆ A final diagnosis may be possible at this stage; also make use of suggested selected reference sources to aid diagnosis.
- ◆ If fungal specimen cannot be identified with

confidence (for whatever reason) then proceed to culture the suspect fungus to make the identification easier.

Isolating fungi from plant material

Initial isolation can be carried out on tap water agar. In addition, the vast majority of commonly occurring plant pathogens and spoilage fungi will grow very well on PDA. This can be prepared from a commercial formulation. For the first isolation, the media may be supplemented with antibiotics such as penicillin and streptomycin to counteract any bacteria. Incubation temperature may be selected on the basis of what is known about the fungus; most commonly occurring plant pathogens grow quite well at 17–22 °C.

- ◆ Flame sterilize a scalpel, or use a new single-edged razor blade to cut a small piece (2–3 mm max.) of tissue from the leading edge of a stem lesion, internal necrosis or leaf spot.
- ◆ If not looking for *Phytophthora*, place part of the sample in 10 percent bleach for 2–5 min. Alternatively, soak the material in 50 percent ethanol for 10 s. (This method can be used even if *Phytophthora* is suspected.) Rinse with sterile distilled water and dry between paper towels.
- ◆ Label the base of the agar plate with the sample reference number and date. If taking tissue from more than one type of lesion, label the plates accordingly. Place the tissue onto the agar in each plate, making sure the pieces are evenly distributed.
- ◆ Incubate for 5–7 days, observing every couple of days to follow culture progress and to see whether the culture is pure. At the early stage of incubation, it can be replated onto fresh medium if there is any unwanted organism also growing with it.
- ◆ If necessary, refer to suggested selected reference sources for aid with any further specialist culturing techniques that may be required and for subsequent identification.

Plating out fungal spores or fruiting bodies

Take a suitably sized sample of inoculum. It is prudent to leave a little bit of original fungus material on the sample in case the first culture is

not successful. Place the inoculum of the fungus in the centre of a Petri dish of PDA medium. Incubate and check for fungal growth every couple of days until the colony produces spores. To prevent the agar from drying out, the Petri dishes may be sealed with Parafilm or kept in a loosely secured bag.

5.4.3 Virology

An introduction to plant pathogenic viruses is provided by Gergerich, R.C. & Dolja, V.V. 2006. Introduction to plant viruses, the invisible foe. *The plant health instructor*. DOI: 10.1094/PHI-I-2006-0414-01. Available at <http://www.apsnet.org/edcenter/intropp/PathogenGroups/Pages/PlantViruses.aspx> (last accessed on 21 September 2015).

The work should be undertaken in a laboratory that has suitable containment in place to work with quarantine pests.

5.4.3.1 Diagnosis

Examine sample material with the naked eye and note any symptoms, such as:

- ◆ abnormal colour
- ◆ dwarfing or stunting
- ◆ discoloration of the leaves, e.g. chlorosis, mosaic, mottle, ringspots, vein yellowing or vein necrosis
- ◆ malformation of the leaves, e.g. distortion, narrowing or epinasty
- ◆ discoloration on stems, fruit and roots
- ◆ other symptoms such as wilting or defoliation.

Symptoms and background information such as host species, along with the experience of the diagnostician, will provide an indication of the potential causal agent. However, further diagnostic tests will be required to confirm the presence of a specific virus. Due to their nature, viruses are generally diagnosed using inoculation onto indicator plants, serological and nucleic acid-based tests. Electron microscopy can also be used, but this requires access to costly specialized equipment and trained staff.

Inoculation onto an indicator host

Viruses can only be spread by means of a wound. In plants, mechanical inoculation is a method

used for their detection and diagnosis. However, it is important to note that not all viruses are sap transmissible.

Healthy herbaceous indicator plants are inoculated with suspect sample inoculum and any symptoms that may be produced in those indicators are recorded after a period of time, which may take days or weeks. Depending on the reactions produced on different indicator plants, a diagnostician may be able to reach a specific diagnosis. There are a number of variations in the mechanical inoculation technique depending on the particular virus suspected or the host on which it appears. As a result, a variety of different indicator plants and extraction buffers are used.

Equipment required:

- ◆ clean pestle and mortar
- ◆ grinding buffers, e.g. phosphate buffer pH 7.0⁴
- ◆ pot labels
- ◆ pencil
- ◆ tray
- ◆ healthy indicator species
- ◆ sample material
- ◆ Celite (diatomaceous earth)
- ◆ microspatula
- ◆ wash bottle filled with tap water
- ◆ bucket filled with a bleach solution
- ◆ lab test sheet
- ◆ cotton bud
- ◆ 1–5 ml variable volume pipette
- ◆ pipette tips
- ◆ grinding bags.

Indicator plants will normally include *Chenopodium amaranticolor*, *Chenopodium quinoa*, *Nicotiana occidentalis* P1, *Nicotiana benthamiana* and *Nicotiana clevelandii*. Others may be added depending on the host being tested and the virus suspected. Choose plants that have at least six true leaves (except cucumber which should have one true leaf and French bean which should have cotyledons only). Place the indicator plants in the dark for 12–24 h prior to inoculation to increase their susceptibility. Choose two to four leaves of

⁴ Note: additives to the buffer such as polyvinylpyrrolidone (PVP) and sodium sulphite are important to help overcome inhibitors in the plant material to be used as the inoculum.

good size on the indicator plant. Mark the leaves to be inoculated by making a hole in the tip of each of the chosen leaves using a clean pipette tip.

Inoculum is prepared by grinding the plant material in buffer (and an abrasive powder such as Celite). The inoculum is then rubbed gently onto the marked leaves of the indicator plants and rinsed off with water 3–5 min later. A negative (inoculation buffer only) and a positive (a virus that is moderately transmissible and produces clear symptoms on the indicator plants) control should be included in each batch of inoculations. Note that after each inoculation the bench and equipment should be disinfected with bleach. The inoculated plants should then be placed into a greenhouse at a temperature of 15–18 °C and incubated for 4–28 days. The plants should be examined twice a week for symptoms and a record of findings maintained.

The symptoms should be evaluated using the following criteria:

- ◆ host
- ◆ indicator plants used
- ◆ reactions of those indicator plants, both local and systemic lesions
- ◆ time taken to produce reactions.

Testing for viruses using ELISA

ELISA is an immunological technique for the diagnosis of specific plant viruses developed by Clark and Adams (1977). It has become one of the principal methods of plant virus diagnosis as it enables large numbers of samples to be processed at once, is very specific and is quantitative.

With double-antibody sandwich (DAS) ELISA, virus particles are trapped and sandwiched between the immunoglobulin G (IgG) fraction of specific antiserum and IgG that has been enzyme labelled (conjugated) with alkaline phosphatase. The presence of the virus is confirmed by the addition of a substrate to alkaline phosphatase and the development of a yellow colour due to substrate breakdown products. The absorbance of light is measured at 405 nm on a colorimeter.

Equipment required

- ◆ ELISA plate
- ◆ variable-volume pipettes to cover the ranges:
 - 1–5 µl
 - 5–50 µl
 - 50–200 µl
 - 200–1 000 µl
 - 1–5 ml
- ◆ variable-volume eight-channel multipipette: 50–250 µl
- ◆ disposable pipette tips of appropriate sizes
- ◆ sap extractor or grinding apparatus
- ◆ colorimetric plate reader, e.g. Thermo Scientific Multiskan FC plate reader
- ◆ bovine serum albumin
- ◆ dried skimmed milk powder
- ◆ 4-nitrophenyl phosphate disodium salt hexahydrate
- ◆ phosphate-buffered saline with Tween (PBST)
- ◆ Tween-20
- ◆ antibodies and conjugates
- ◆ buffers
- ◆ plate plans
- ◆ waterproof marker pen
- ◆ cling film
- ◆ paper towel
- ◆ wash bottle
- ◆ grinding bags.

A plate plan should be produced to map where samples and controls are to be placed on the 96-well plate. This should be based on the following testing criteria.

- ◆ Each sample must be tested in duplicate. Therefore columns of the plate must always be allocated to each virus in pairs (e.g. columns 1+2 ArMV, columns 3+4+5+6 CMV, columns 7+8 TBRV).
- ◆ For each virus test there must be a minimum of one healthy and one infected control (also run in duplicate).

On the plate plan paperwork, assign samples and controls to wells of the plate, working vertically. Write which column(s) will be for which virus test at the top. Use a standard plate plan where possible (which already has healthy control positions assigned).

Note: Indirect triple-antibody sandwich (TAS) ELISA tests should be on a separate plate plan as more incubation steps are required (unless performing it as a cocktail, during which the monoclonal antibody and anti-species conjugate are combined together and added to the plate at the same time).

Double-antibody sandwich (DAS) ELISA procedure

- ◆ Coating plate:
 - Label the ELISA plate with an ELISA plate number sticker and write this on the plate plan paperwork. Write on the plate itself which virus test is in each column according to the plate plan.
 - Mix the IgG antibody specific for the virus being tested with coating buffer in a plastic weigh boat. Add 100 µl per well. Cover with plastic film. Initial and date paperwork, and fill in which company the antiserum was from.
 - Incubate at 37 °C for 2–4 h or overnight.
- ◆ Sample preparation:
 - Place samples in grinding bags and grind. Add 5 ml of extraction buffer (2 percent w/v PVP in PBST) prior to grinding or immediately after. This volume can be adjusted between 2 and 10 ml for small to large seeds. *Note:* For healthy controls use the same host as the sample being tested if possible.
 - Wash ELISA plate with PBST; either three times by hand or use a plate washer. Blot dry on several layers of paper towel.
 - Following the plate plan, load the plate with samples, healthy controls and infected controls. Add 100 µl extract per well. Cover with cling film.
 - Incubate overnight in the refrigerator at 4 °C.
- ◆ Addition of enzyme conjugate:
 - Wash ELISA plate with PBST once by hand to remove plant or seed material. Then either use a plate washer or wash a further three times with PBST by hand. Ensure that no coloured residue remains. If necessary wash again. Blot dry.
- Mix the alkaline phosphatase conjugate antibody that is specific for the virus being tested with conjugate buffer (0.2 percent w/v BSA in PBST) in a plastic weigh boat. Add 100 µl per well. Cover with cling film. Ensure the use of conjugate antibody from the same company detailed in the coating section of the paperwork. Initial the conjugate section of the paperwork once added.
- Incubate for 2–4 h at 37 °C.
- ◆ Addition of substrate:
 - Wash ELISA plate with PBST and blot dry as before.
 - Make solution of 0.1 percent w/v 4-nitro-phenyl phosphate disodium salt hexahydrate (substrate powder) in substrate buffer, in a plastic weigh boat. Make 1 ml per column of the plate (*note:* 10 ml is enough for a full plate). Add 100 µl per well. Keep solution in the dark when not in use as it is light sensitive. Initial the paperwork and detail the time the substrate solution was added.
 - Keep the plate in the dark at room temperature and check periodically for colour development. Typically, this would be a maximum of 1 h and probably less in warmer climes (e.g. non-air-conditioned laboratories in tropical countries). It is important to monitor the blank control as this should remain without colour, but will discolour with time. For some ELISA reactions an optimal incubation is reached that differentiates the positive tests.
- ◆ Analysis of results: There are a number of plate scanners with associated analysis software available. The following provides an example of one system.
 - Read the absorbance according to the plate scanner instructions.
 - Check that the infected controls are above the positive threshold and ideally are above the 0.5 absorbance value; if the reading is too low then leave to develop for longer. Take a hard-copy printout and attach it to the plate plan. If printing, then highlight on the printout the values that are above the positive threshold.

Detection of viruses using PCR

PCR is described in section 5.1.5.2. Before PCR can be used to detect a plant pest, the nucleic acid needs to be extracted from the test material. Several methods are available to extract nucleic acids for analysis. The choice of methods in any laboratory depends on the major sample source and the nature of the test you are carrying out. Regardless of the method, the extraction process will involve three main steps; lysis, removal of inhibitors and recovery of the nucleic acid.

Crude lysis may involve physical methods such as grinding, freeze–thawing or sonication, or chemical methods using detergents, enzymes, chaotropic agents or just boiling of cells in a buffer. Nucleic acid extraction methods may rely on the different solubility of nucleic acids and proteins in phenol and water, as in phenol/chloroform extraction methods, or the ability of nucleic acids to bind to silica, as in silica/guanidinium isothiocyanate extraction methods. Concentration (recovery) of extracted nucleic acid can be accomplished by precipitation with ethanol or using silica beads.

Commercial kits containing all the necessary reagents are available, based on spin columns or magnetic beads. In the absence of a kit, the following protocol, commonly termed CTAB due to use of Cetyl trimethylammonium bromide, is adapted from Chang, S., Puryear, J. & Cairney, J. 1993. A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter*, 11: 113–116. It can be used for extracting RNA from most types of plant material.

RNA extraction protocol – plant leaf

For each sample, carry out an extraction from the plant or plants to be tested and also from a known healthy plant (if possible from the same species) as a negative control. All centrifugation steps are carried out at 13 000 *g* in a microcentrifuge, unless stated otherwise.

Equipment required:

- ◆ grinding tool and grinding bags
- ◆ 2 ml microcentrifuge tubes (4 per sample)
- ◆ pipettes (P1000 and P200)
- ◆ pipette tips
- ◆ water bath or other incubator preheated to 65 °C
- ◆ centrifuge (with appropriate rotor for spinning microcentrifuge tubes up to 13 000 *g*)
- ◆ fridge
- ◆ fume hood
- ◆ reagents:
 - CTAB grinding buffer (at least 1 ml per sample)
 - chloroform: isoamyl alcohol (IAA) 24:1
 - 4 M lithium chloride (LiCl)
 - isopropanol
 - 70 percent v/v ethanol
 - Tris-EDTA buffer containing 1 percent SDS
 - Nuclease-free sterile water.

Procedure:

- ◆ Place tissue, 100–200 mg, in 10×15 cm (or similar) grinding bag and freeze in liquid nitrogen before grinding into a fine powder using a small hand roller. *Note:* check that you haven't created a hole in your grinding bag, if so, cover with sticky tape.
- ◆ Grind until thawing begins and tissue forms a "smooth paste". Add 1–2 ml (i.e. 10 volumes) of grinding buffer and mix thoroughly using roller.
- ◆ Decant 0.7 ml of ground sap into a 1.5 ml microfuge tube and incubate sap at 65 °C for 10–15 min.
- ◆ After incubation, add 700 µl of chloroform:IAA (24:1) and mix to emulsion by inverting the tube.
- ◆ Centrifuge at maximum speed in a microfuge for 10 min at room temperature.
- ◆ Optional step for "difficult tissue": Carefully remove upper (aqueous) layer and transfer to a fresh tube. Add an equal volume of chloroform:IAA, mix and spin as before.
- ◆ Remove aqueous layer, taking extra care not to disturb interphase. Add an equal volume of 4 M LiCl, mix well and incubate at 4 °C overnight or 1 h at room temperature.
- ◆ Spin for 20–30 min at maximum speed at 4 °C to pellet the RNA.
- ◆ Resuspend the pellet in 200 µl of TE buffer containing 1 percent SDS. Add 100 µl of 5 M NaCl and 300 µl of ice-cold isopropanol. Mix well and incubate at –20 °C for 20–30 min.

- ◆ Centrifuge for 10 min at maximum speed at 4 °C to pellet nucleic acid. Decant off salt and ethanol supernatant.
- ◆ Wash pellet by adding 500 µl 70 percent ethanol and spinning for 3–4 min at 4 °C.
- ◆ Decant off the ethanol and dry the pellet to remove residual ethanol. *Note:* Do not dry completely as the pellet will become difficult to resuspend.
- ◆ Resuspend pellet in 50 µl of nuclease-free sterile water.

PCR workflow and controls

Ideally different areas of the lab (or different labs) will be dedicated to the different parts of the PCR process, to ensure freedom from contamination. The parts to separate are (i) extraction, (ii) PCR set-up, (iii) DNA spiking and (iv) post-PCR.

Controls for contamination should be included in each stage of the process. Thus, for each set of extractions a known healthy control should be included (ideally of the same species or a closely related species; a blank buffer control can also be used). This control will be tested alongside the diagnostic samples and will identify any contamination during the extraction process.

Water controls of several kinds should be included in the process as follows.

- ◆ Tubes capped following the addition of master mix (pre-capped) – this indicates how clean the reagents being used are.
- ◆ Tubes left open during DNA spiking but closed afterwards (post-capped) – this highlights any cross-contamination during set-up (especially when using plates).
- ◆ Finally, tubes where water is added at the end of the process to indicate cross-contamination from sample to sample or associated with the pipette during set-up.

Conventional RT-PCR protocol

PCR reactions should be set up on ice, or with the stock reagents sitting on ice. Typical reaction conditions are either 25 or 50 µl; for diagnostic use 25 µl is ample to allow the gel electrophoresis to be repeated, while for development work and sequencing 50 µl might be more suitable. Reverse

transcription PCR (RT-PCR) can be performed in one-tube (with a single reaction) or two-tube (separate RT step) format. In a two-tube format, cDNA is added from the reverse transcription reaction into the PCR reaction, while in the one-tube format an RT step is performed immediately prior to PCR cycling. The latter is preferable for diagnostic use since it is simpler and contamination is less likely as the tube is only opened post-PCR, optimization of one-step RT-PCR usually involves reducing the amount of M-MLV reverse transcriptase included in the reaction mix.

Setting up your PCR reactions

Equipment required:

- ◆ pipettes (P10 and P200)
- ◆ pipette tips
- ◆ 96-well PCR plates
- ◆ plate seals or caps
- ◆ PCR set-up cabinet
- ◆ reagents:
 - Mastermix
 - samples.

Plan how many reactions you will need and which wells you will use for each sample using a plate plan. Calculate what volumes you will need to add to make up your RT-PCR Mastermix (see Table 1). Remember that ideally you should test your samples in duplicate.

Aliquot out 24 µl Mastermix per well and then add 1 µl sample or control to each of the relevant wells. Place the plate in the PCR machine and run the plate on an RT-PCR programme.

5.4.4 Nematology

5.4.4.1 Introduction to plant nematology

There are numerous reference works that detail the biology and pathogenicity of both plant-parasitic and free-living nematodes. The introductions provided by Decraemer and Hunt (2006) and Hockland *et al.* (2006) are summarized, in part, below.

Nematodes are pseudocoelomate, unsegmented worm-like animals, commonly described as filiform or thread-like. Nematodes are the most numerous Metazoa on earth. They are either free-living or parasites of plants and

Table 1. Example calculation of one step RT-PCR Mastermix

Reagent	Volume for one reaction	Volume required
Total number of reactions		11
Sterile, nuclease-free water	34.75 µl	382.25
10× reaction buffer	5 µl	55
25 mM MgCl ₂	3 µl	33
Primer 1 10 µM	2 µl	22
Primer 2 10 µM	2 µl	22
dNTP mix (10 mM each)	1 µl	11
MMLV (diluted 1/50 in SDW)	1 µl	11
<i>Taq</i> polymerase (5 u/µl)	0.25 µl	2.75
RNA	1 µl	

animals. Although they occur in almost every habitat (Cobb, 1915), they are essentially aquatic animals. Nematodes depend on moisture for their locomotion and active life. Therefore soil moisture, relative humidity and other environmental factors directly affect nematode survival. However, many nematodes can survive in an anhydrobiotic state.

It has been estimated that a single acre of soil from arable land may contain as many as 3 000 000 000 nematodes. In order to constrain this limiting factor in agricultural production, it is vital to accurately identify nematode pests and to understand their biology. Many plant-parasitic nematodes cause economic damage to a wide range of crops. However, their presence is not always apparent to growers and symptoms are often attributed to nutritional disorders or other causes. In the past, the difficulty associated with detecting nematodes and the lack of information about their biology and the damage they cause has contributed to the increased risk of their inadvertent movement in trade unnoticed with their associated hosts or in soil residues.

Many nematodes that may be of phytosanitary importance are intercepted in international trade by plant health inspectors at points of entry. Often these are unknown species that have the potential to become significant pests if allowed to enter and establish. Nematode pests that were previously unknown or are not well known

may be subject to emergency quarantine actions to avoid possible introduction and spread before the risks are better understood. Measures should be modified as appropriate based on experience and the acquisition of new information. Cannon *et al.* (1999) describe how the United Kingdom adopted a systematic protocol to determine appropriate measures and include examples of nematode pests intercepted in the United Kingdom on Chinese penjing (dwarfed trees); other examples include certain species of root-knot nematodes (*Meloidogyne* spp.) intercepted by member states of the European Union on imported rooted cuttings.

Nematodes display a wide range of feeding habits or trophisms. Some species of nematodes are microphagous or microbotrophic, feeding on small micro-organisms, while others are saprophagous, feeding on dead and decaying organic matter. Many species of nematodes are phytophagous, obtaining nourishment directly from plants, while others are omnivorous or predatory. Parasitism of invertebrates or vertebrates is also common. There are three main types of plant parasitism: ectoparasitic, endoparasitic and semi-endoparasitic.

Ectoparasitic: The nematode remains in the soil and does not enter the plant tissues. It feeds by using its stylet to puncture plant cells – the longer the stylet the deeper it can feed within the plant tissues. The majority of ectoparasitic species remain

motile whereas others, e.g. *Cacopaurus*, become permanently attached to the root by the deeply embedded stylet.

Endoparasitic: In this type of parasitism the entire nematode penetrates the root tissue. Migratory endoparasites, such as *Pratylenchus* and *Radopholus*, retain their mobility and have no fixed feeding site within the plant tissue, whereas the more advanced sedentary endoparasites have a fixed feeding site and induce a sophisticated trophic system of nurse cells or syncytia. Establishment of a specialized feeding site enhances the flow of nutrients from the host, thereby allowing the females to become sedentary and obese in form and highly fecund. Sedentary endoparasites also have a migratory phase before the feeding site is established. In root-knot and cyst nematodes it is only the J2 and adult male that are migratory, but in *Nacobbus*, for example, all juvenile stages, the male and the immature vermiform female are migratory, only the mature female being sedentary.

Semi-endoparasitic: Only the anterior part of the nematode penetrates the root, with the posterior part remaining in the soil.

5.4.4.2 Laboratory requirements

Laboratory facilities for diagnosing nematode pests require suitable containment in place to work with quarantine organisms. Detailed descriptions of extraction equipment can be found in the EPPO (European and Mediterranean Plant Protection Organization) Standard on nematode extraction (EPPO, 2013a).

5.4.4.3 Diagnosis

Nematode identification underpins all aspects of research, advisory work, implementation of quarantine legislation and selection of control strategies. Classical taxonomy is now complemented by molecular diagnostics.

Standard protocols for the extraction of regulated nematode species are provided by the IPPC and by each regional plant protection organization (RPPO). Diagnostic protocols for all quarantine and regulated species for the European region are provided by EPPO (<http://archives.eppo.int/EPPOstandards/diagnostics.htm>). These

documents provide typical host symptoms of nematode feeding and parasitism.

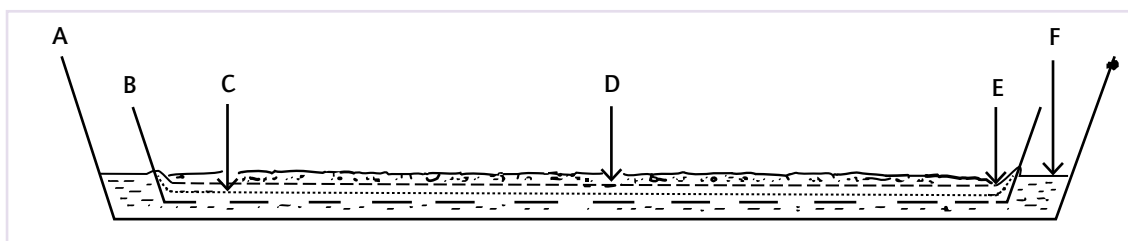
Extraction from substrate

There are many methods for extracting nematodes from substrate, including passive, dissection and flotation techniques. Vermiform nematodes can be extracted from plant tissues, seed, soil or growing medium using such techniques as the Baermann funnel (Figure 16), modified Baermann-tray method (Hooper and Evans, 1993; Figure 17), an adapted sugar-flotation method (Coolen and D'Herde, 1972) or the misting technique (Hooper *et al.*, 2005). There are also various methods described for isolating cyst nematodes from substrate, for example, the Fenwick can (Fenwick, 1940) or Wye Washer (Winfield *et al.*, 1987). Detailed descriptions of extraction procedures can be found in the EPPO Standard on nematode extraction (EPPO, 2013a).

Plant material suspected to be infested with plant-parasitic nematodes should be processed as soon as possible to avoid further deterioration and infection by secondary pathogens. Soil and growing medium should be stored at around 5 °C

Figure 16: Modified Baermann funnel for extracting nematodes from plant material or soil



Figure 17: Modified tray method for extracting nematodes

A, photographic dish; B, plastic coated wire basket; C, coarse mesh plastic supporting gauze; D, filter (single-ply tissue, milk filter or cotton/nylon cloth); E, thin layer of soil; F, sufficient water to wet but not flood the subsample.

out of direct sunlight, until processed. To ensure good recovery, it is recommended that soil should be handled gently to avoid damaging nematode specimens.

The Baermann funnel method is a simple technique for extraction of active nematodes from soil, seed and plant material.

It comprises a funnel with a piece of rubber tubing attached to the stem and closed by a spring or screw clip (the recommended slope of funnel is approximately 30°). The funnel is placed in a support and almost filled with tap water. A plastic sieve or wire basket with a large enough aperture size to allow nematodes to actively pass through is placed just inside the rim of the funnel. Soil or plant tissue is cut into small pieces and placed either directly on the mesh or onto a single-ply tissue supported by the mesh; the water level is then adjusted so that the substrate is only just submerged. Active nematodes pass through the mesh and sink to the bottom of the funnel stem. Alternatively, funnels made of plastic or stainless steel, or using silicone tubes can be used. However, regarding the latter, diffusion of oxygen into water is lower than for polyethylene (Stoller, 1957), which could slowly lead to asphyxiation of nematodes. Depending on the substrate, most (50–80 percent) of the motile nematodes present will be recovered within 24 h; however, samples can be left on the funnel for up to 72 h to increase recovery rate. For longer extraction periods, regular tapping and adding of fresh water increases nematode motility and therefore recovery rate. Efficacy of extraction can also be improved by adding 1–3 percent H_2O_2 for oxygen supply (Tarjan, 1967, 1972). Following

the extraction period, a small quantity of water containing the nematodes is run off and observed under a stereomicroscope (Flegg and Hooper, 1970).

The Seinhorst mistifier technique differs from the Baermann funnel method in that any plant sap and toxic decomposition products are washed away. In this method a Baermann funnel or Oostenbrink dish is placed in a mist or fog of water to avoid the depletion of oxygen. The mist is produced by nozzles spraying water at a certain pressure over the plant material or by nozzles spraying water upwards so that droplets fall softly back onto the plant material. Active nematodes leave the plant tissue and are washed into the funnel or dish where they sediment. The nematodes are collected every 24–48 h in a glass beaker by opening the screw clip on the funnel stem or by collecting the specimens on a 20–25 μm sieve. Extraction can be continued for up to four weeks. This technique is described by Hooper (1986).

Motile and immotile nematodes can be extracted from plant material by the Coolen and D'Herde (1972) method. The plant material is washed, cut into pieces of about 0.5 cm, and 5 g portions are macerated in 50 ml of tap water in a domestic blender at the lowest mixing speed for 2 min. The suspension of nematodes and tissue fragments is washed through a 750 μm sieve placed on top of a 45 μm sieve. The residue on the 45 μm sieve is collected and poured into two 50 ml centrifuge tubes. About 1 ml kaolin is added to each tube, the mixture is thoroughly stirred and then it is centrifuged at 3 000 rpm for 5 min. The supernatant is decanted and sucrose solution

(1.13 g/cm³) is added to the tubes. The mixture is thoroughly stirred and centrifuged at 1 750 rpm for 4 min. The supernatant is washed through a 45 µm sieve, the residue is collected and the nematodes are studied under a stereo microscope. As an alternative to sucrose, ZnSO₄, MgSO₄ or colloidal silica can be used.

Preliminary identification

Definitions of terminology used in the following sections can be found in EPPO (2013b).

Differentiating plant-parasitic nematodes from other trophic groups requires full training from a specialist. A dissecting stereomicroscope with a magnification of at least 40× is required to observe morphological characters and prepare nematode specimens for study. The three basic types of feeding apparatus and corresponding pharyngeal regions found in plant-parasitic nematodes, are presented in Figures 18 and 19, respectively.

Preparation of nematode specimens

Morphological observation should be carried out on as many adult specimens as possible. There

are numerous published methods for fixing and processing nematode specimens for study, most recently summarized in Manzanilla-López and Marbán-Mendoza (2012). Nematodes processed to an anhydrous state and mounted in glycerol are recommended for examination as important taxonomic features can be obscured if specimens are not cleared sufficiently.

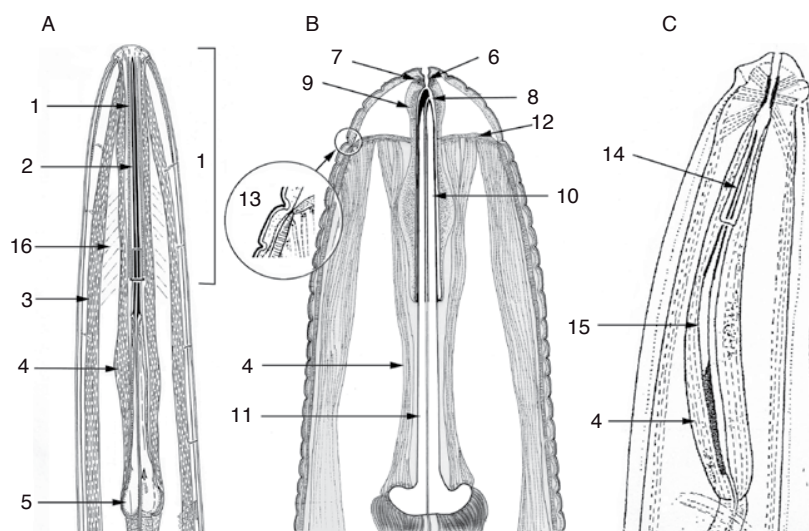
Temporary microscope slide preparations can be made quickly for instant examination, but such slides may remain usable for several weeks only.

If possible, permanent slides should be prepared for future reference and deposited in nematode reference collections. Methods of preparing permanent slide mounts of nematodes have been described in detail elsewhere (Seinhorst, 1962; Hooper, 1986). The slow evaporation method as described by Hooper (1986) is outlined below.

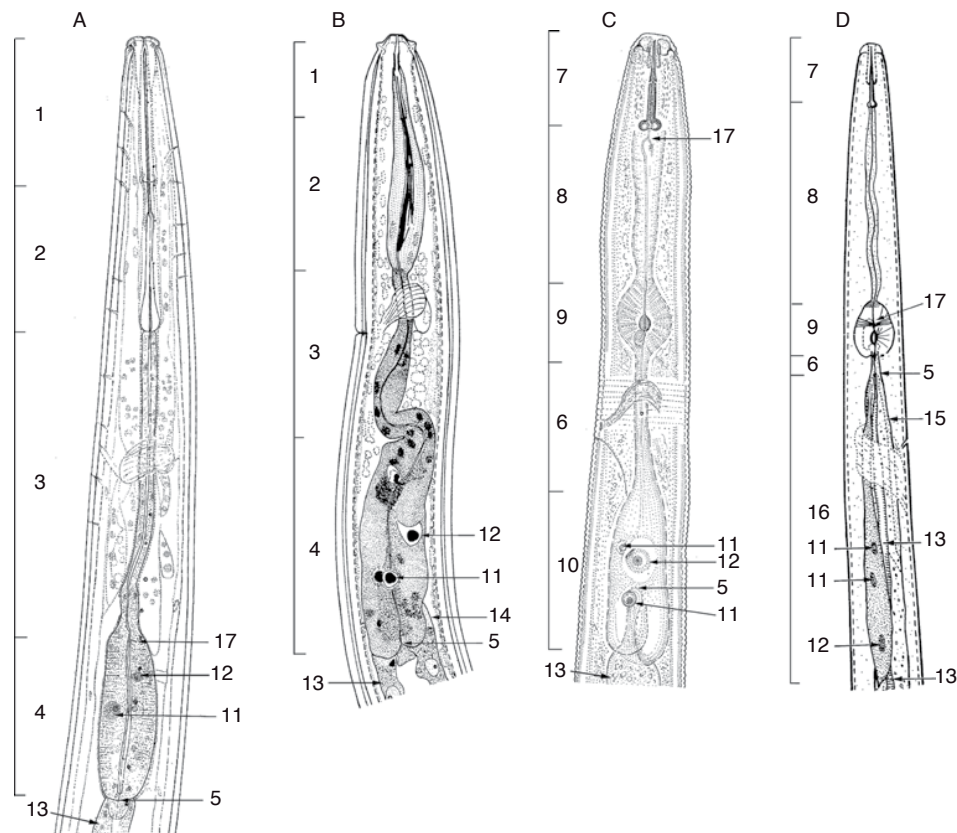
Temporary preparations

A small drop of water is placed on a glass cavity slide, enough to sufficiently fill the well. The nematode specimens are transferred to the water and heated to 65 °C. It is vital that the heating

Figure 18: Stoma region and types of feeding apparatus in plant-parasitic nematodes



A, Odontostyle and odontophore (Longidoridae). B, Stomatostylet with detail of body cuticle (inset) at base of cephalic framework (Tylenchomorpha). C, Onchiostyle (Trichodoridae). 1, cheilostome; 2, odontostyle; 3, somatic muscles; 4, stylet protractor muscles; 5, odontophore with flanges; 6, prestoma; 7, thickening of cuticle around prestoma; 8, stylet opening; 9, stoma; 10, stylet conus; 11, stylet shaft and knobs; 12, basal cephalic framework; 13, body cuticle in detail, showing disappearance of median and striated basal zone in head region; 14 and 15, onchiostyle with onchium (14) and onchiophore (15); 16, dilators buccae. Reproduced from Decraemer and Hunt (2006) with permission.

Figure 19: Digestive system of plant-parasitic nematode taxa

A, *Paraxiphidorus* (Longidoridae). B, *Paratrichodorus* (Trichodoridae). C, *Pratylenchoides* (Pratylenchidae). D, *Aphelenchoides* (Aphelenchoididae). 1, cheilostome; 2, pharyngostome; 3, narrow anterior region of pharynx; 4, pharyngeal bulb; 5, pharyngeal-intestinal junction; 6, isthmus; 7, stomatostylet; 8, procorpus; 9, metacarpus; 10, post-corpus; 11, ventrosublateral pharyngeal gland nuclei; 12, dorsal pharyngeal gland nucleus; 13, intestine; 14, intestine dorsally overlapping pharynx; 15, pharyngeal-intestinal junction valve cell; 16, pharyngeal gland lobe; 17, dorsal pharyngeal gland orifice. Reproduced from Decraemer and Hunt (2006) with permission.

should be just sufficient to kill the nematodes, as prolonged heating will result in distortion and deterioration of the specimens. In practice, 10–15 s on a hotplate will be sufficient time for most species, but it is recommended to check the slide at intervals to monitor progress and remove from the heat only when movement of all the nematodes has ceased.

A glass slide, free of dust is selected and put on the side of the microscope stage. A small drop of single strength TAF fixative (7 ml formalin (40 percent formaldehyde), 2 ml triethanolamine, 91 ml distilled water) or another appropriate fixative is put in the centre of the slide and an appropriate amount of paraffin wax shavings is positioned around the drop (the wax will help support the cover-slip and seal it to the slide).

The nematodes are transferred from the cavity slide to the TAF so that they are positioned beneath the meniscus in the centre of the drop and not overlapping one another. The number of specimens able to fit on a slide will vary according to the size of the nematodes.

An appropriately sized cover-slip is carefully cleaned with lens tissue. It is gently lowered onto the wax shavings so that contact is made with the drop of TAF. The slide is placed on a hotplate and observed until the wax has just melted, the air that may be lodged under the cover-slip is removed by gently tapping the slide. The slide is then removed from the heat and examined.

There should be a clear area of TAF containing the nematodes in the centre and a complete ring of wax to seal the slide.

Should the seal be broken or the nematodes become embedded in the wax, the slide is heated again, the cover-slip carefully removed, the nematodes recovered and remounted on a new slide. If the wax has spread beyond the cover-slip, it is cleared away with a fine blade.

The cover-slip is sealed with a ring of clear nail varnish. When the varnish has dried, the specimens are ready for study.

Permanent preparations

A small drop of water is placed on a glass cavity slide, enough to sufficiently fill the well. The nematode specimens are transferred to the water and heated to 65 °C. It is vital that the heating should be just sufficient to kill the nematodes, as prolonged heating will result in distortion and deterioration of the specimens. In practice, 10–15 s on a hotplate will be sufficient time for most species, but it is recommended to check the slide at intervals to monitor progress and remove from the heat only when movement of all the nematodes has ceased.

The nematodes are transferred to an embryo dish or suitable watch glass half full of single strength TAF (7 ml formalin (40 percent formaldehyde), 2 ml triethanolamine, 91 ml distilled water). It is covered and left to fix for a minimum of one week.

The specimens are transferred to a watch glass containing a 3 percent glycerol solution with a trace amount of TAF. The nematodes should be submerged. A cover-slip is placed over the watch glass and left overnight.

The cover-slip is moved slightly so that a small gap is produced to allow evaporation, and the watch glass is left in an incubator (approximately 40 °C) until all water has evaporated (this may take at least one week). At the same time, a small beaker of glycerol is placed in the incubator to ensure it becomes anhydrous.

Using a syringe or dropper, a small drop of the anhydrous glycerol is dispensed onto the centre of a glass slide and the nematodes are transferred to this, arranging them centrally.

Three cover-slip supports are carefully selected, such as glass beads, of similar diameter

to that of the nematodes, and placed at intervals in the margin of the glycerol drop, so that they form an even support.

Small amounts of paraffin wax shavings are placed at regular intervals around the circumference of the glycerol drop.

A cover-slip is heated on a heating block for a few seconds. The cover-slip is cleaned with lens tissue and gently lowered on to the wax, so that contact is just made between cover-slip and glycerol.

The slide is placed on the heating block and, as soon as the wax has melted and any air bubbles have been expelled by the settling cover-slip, the slide is removed from the heat and the wax allowed to reset.

When the wax is completely hard, any excess wax is removed from around the cover-slip with a scalpel.

The cover-slip is sealed with a ring of sealant such as Glyceel or clear nail varnish. The slide is labelled with an indelible marker, or with a slide label affixed to it. This includes classification, date of slide preparation, host, locality, sample number (if applicable) and method of preservation used.

Identification to species

Classification of a nematode population is difficult and requires an understanding of nematode morphology, phylogeny and taxonomy. If a quarantine-listed organism or a new finding is suspected, confirmation should be carried out by a specialist.

For identification using light microscopy, a magnification of 400× to 1000× (oil immersion lens) and differential phase contrast (DIC) is recommended.

Standard protocols for the morphological identification and molecular confirmation of regulated nematode species are provided by the IPPC and each RPPO.

5.4.5 Entomology

An introduction to insect identification is provided by the Royal Entomological Society: http://www.royensoc.co.uk/insect_info/introduction.htm

Work should be undertaken in a secure laboratory that has containment measures in place

that will prevent the escape of pests. The basic equipment required is as follows.

Large equipment:

- ◆ binocular dissecting microscope (up to 160× magnification) for general low-power examination of specimens and sample screening
- ◆ cold light source with fibre optic light guides
- ◆ research microscope (up to 1000× magnification with inbuilt light source) for the examination of slide-mounted specimens
- ◆ heating block or boiling plate (range up to 120 °C)
- ◆ slide oven for slide preparation
- ◆ laboratory incubator to rear specimens
- ◆ domestic refrigerator for sample storage
- ◆ domestic freezer for sample disposal.

Small equipment:

- ◆ white enamelled sample trays
- ◆ disposable plastic petri dishes of various sizes
- ◆ embryo dishes and watch glasses
- ◆ microscope slides (standard and cavity)
- ◆ cover glasses (13 mm and 18 mm)
- ◆ steel ruler (20 cm – 1/10th mm divisions)
- ◆ entomological pins
- ◆ dissection equipment including: seekers, scalpels and blades, forceps (various, including entomological forceps), fine paint brushes, minute pins or similar and Pasteur pipettes
- ◆ spirit burner.

Basic reagents:

- ◆ demineralized water
- ◆ ethanol (diluted with demineralized water to 60 and 70 percent solutions)
- ◆ methanol
- ◆ KOH 10 percent solution
- ◆ white spirit (mineral spirit)
- ◆ glacial acetic acid
- ◆ clove oil
- ◆ Heinz slide mounting media
- ◆ Canada balsam and solvent
- ◆ acid fuchsin tissue stain.

Sundry equipment:

- ◆ replacement microscope bulbs and fuses
- ◆ microscope immersion oil
- ◆ lens tissues
- ◆ compressed air in spray canister
- ◆ scalpel blade changer
- ◆ sharps disposal bin
- ◆ reagent bottles and rack
- ◆ waste reagent bottle
- ◆ Pyrex boiling tubes, tube holder and rack
- ◆ cork tile
- ◆ laboratory tissue (roll)
- ◆ disposable gloves
- ◆ indelible marker pens (course and fine)
- ◆ clear plastic screw-top sample tubes
- ◆ Eppendorf tubes (0.5 ml and 1.0 ml)
- ◆ rearing boxes
- ◆ clear polythene bags
- ◆ autoclave bags.

5.4.5.1 Diagnosis

Traditional insect identification is based primarily on the microscopic examination of morphological characters that may require specimens to be specially prepared for examination, the use of keys (usually dichotomous) and by comparison with either a single or preferably a range of verified voucher specimens. This approach remains the most cost-effective and accessible means of delivering insect diagnosis. Most, but not all, keys are based on the characteristics of the adult life stage, but in plant health diagnostics it is the immature stages that are most frequently encountered often moving in trade (i.e. eggs, larvae or pupae); in such instances it is often necessary to rear these through to an identifiable stage. In addition, other threads of evidence can also aid or expedite identification, for example, host association, geographical origin and pest distribution data.

About one million insect species have thus far been described, which accounts for 80–90 percent or all known animal life forms, but there are still many more that have yet to be formally described and it has been estimated that the final total may be between 6 million and 10 million species. Of the known extant insect species, taxonomists

have thus far divided them into approximately 23 different orders. With such a vast array of taxa it is not within the capability or experience of one person to deliver fully comprehensive diagnostic services. The majority of the plant pest species do however belong to just five of these orders, namely, the Coleoptera (beetles), Diptera (flies), Hemiptera (bugs), Lepidoptera (moths and butterflies) and Thysanoptera (thrips). A good general plant health entomologist should be able to broadly recognize these orders and the key pest species within them, but beyond that diagnosis may require the identification skills of a specialist.

One tried and tested method for delivering entomology diagnosis services operates on a "triage" system, whereby general entomologists prioritize newly arrived samples identifying the majority of routine samples. This involves scanning samples such as plant material or sticky traps for invertebrates of concern and, where necessary, preparing invertebrates for examination themselves or by the senior diagnosticians (e.g. clearing specimens and subsequently mounting them on microscope slides). When they have taken the diagnosis as far as they can, then more senior or specialized diagnosticians are called in as required. Once a diagnosis has been completed it should only be signed-off by a designated entomologist. For example, under ISO 17025 accreditation only those entomologists that have been formally trained and tested to make the identifications (and with this clearly indicated in their training records) may sign for an identification.

For general entomologists and specialists alike, expertise and experience is paramount in order to deliver an effective and accurate service. Separation between species can be subtle and confirmation of diagnosis can only be reliably done by an experienced diagnostician. Such skills can only be developed over time through training, mentoring and by collaborating and networking with other experts and specialists.

5.5 Additional information

The Internet provides access to a vast array of information to underpin diagnosis and development of expertise. This section provides information on

key reference materials for plant pests as well as centres of excellence and expert databases.

5.5.1 Manuals and websites

Key information sources include American Phytopathological Society (APS), CAB abstracts, EPPO, as well as more general Internet searches (e.g. Google). Recent reviews or books dealing with pests of particular crops are especially useful and can be relatively easily located using Internet searches of key words (e.g. viruses, cassava).

Useful starting points are the CABI Crop Protection Compendium and the CABI Forestry Compendium. Searching by host in these compendia can generate a crude list of pests associated with the host, which can then be augmented by searches on the EPPO PQR system (database on quarantine pests). It is useful to include all known synonyms of the pest even if these have not been used for some time.

In some cases, it may be necessary to consider all organisms associated with a particular host and, therefore, consideration must be given to whether it is a genuine pest, secondary pathogen or just an incidental record.

Names of organisms change as more taxonomic information becomes available and so it is important to check the currently approved names of the organisms as well as all synonyms and obsolete names.

One useful general resource is the Catalogue of Life website (<http://catalogueoflife.org>) and (<http://www.species2000.org/>), which provide the currently accepted names for a wide range of organisms. Approved fungal names and synonyms are available on the Indexfungorum website (<http://www.indexfungorum.org>), while approved bacterial and phytoplasma names are available on the ISPP website (<http://www.isppweb.org>) and in Firrao *et al.* (2004) <http://taxonomicon.taxonomy.nl/TaxonTree.aspx?id=1007020>, respectively.

Key sources for each pest group are listed below.

5.5.1.1 Pathogens – general

Notes in the journal *Plant Disease*:

<http://www.apsnet.org/publications/plantdisease/Pages/default.aspx>

Plantwise Knowledge Bank:

<http://www.plantwise.org/KnowledgeBank/home.aspx>

International Society for Infectious Diseases, Program for Monitoring Emerging Diseases (ProMED), About ProMED-mail. Available at <http://www.promedmail.org> (last accessed on 16 September 2015).

BSPP *New Disease Reports*: <http://www.ndrs.org.uk/>
CABI Crop Protection Compendium. Available at <http://www.cabi.org/cpc/> (last accessed on 16 September 2015).

Molecular Plant Pathology:

<http://onlinelibrary.wiley.com/journal/10.1111/%28ISSN%291364-3703>

EPPO (European and Mediterranean Plant Protection Organization), EPPO activities on plant quarantine. Available at <http://www.eppo.int/QUARANTINE/quarantine.htm> (last accessed on 16 September 2015).

5.5.1.2 Viruses and viroids

National Center for Biotechnology Information (NCBI) ICTVdB:

<http://www.ncbi.nlm.nih.gov/genomes/Genomes-Group.cgi?taxid=10239&opt=Virus>

Descriptions of plant viruses (DPV):

<http://www.dpvweb.net/>

Viroids:

<http://www.ncbi.nlm.nih.gov/genomes/Genomes-Group.cgi?taxid=12884&opt=Viroid>

5.5.1.3 Bacteria

Lelliot, R.A. & Stead, D.E. 1991. *Methods for the diagnosis of bacterial diseases of plants*. Oxford, Wiley. 224 pp.

CABI & EPPO. 1997. *Quarantine pests for Europe*, 2nd edn. Wallingford, UK, CAB International and Paris, European and Mediterranean Plant Protection Organization. 1425 pp.

Weller, S.A., Aspin, A. & Stead, D.E. 2008. Classification and identification of plant-associated bacteria by fatty acid profiling. *EPPO Bulletin*, 06/2008; 30(3–4): 375–380. DOI: 10.1111/j.1365-2338.2000.tb00914.x.

Snowdon, A.L. 2010a. *A colour atlas of post-harvest diseases and disorders of fruits and vegetables*. Vol. 1. *General introduction and fruits*. Boca Raton, CRC Press. 320 pp.

Snowdon, A.L. 2010b. *A colour atlas of post-harvest diseases and disorders of fruits and vegetables*. Vol. 2. *Vegetables*. Boca Raton, CRC Press. 416 pp.

Janse, J.D. 2010. *Phytobacteriology: principles and practice*. Wallingford, UK, CAB International. 368 pp.

Bradbury, J.F. 1985. *Guide to plant pathogenic bacteria*. Wallingford, UK, CAB International. 332 pp.

The International Society of Plant Pathology (ISPP): <http://www.isppweb.org/>

Schaad, N.W., Jones, J.B. & Chun, W., eds. 1988. *Laboratory guide for identification of plant pathogenic bacteria*, 2nd edn. St Paul, MN, USA, APS Press, American Phytopathological Society. 164 pp.

5.5.1.4 Phytoplasmas

Old names as well as names under the revised nomenclature: Firrao *et al.* (2004) <http://taxonomy.con.taxonomy.nl/TaxonTree.aspx?id=1007020>

5.5.1.5 Fungi

Index fungorum:

<http://www.indexfungorum.org/>

Kirk, P.M., Cannon, P.F., Minter, D.W. & Stalpers, J.A., eds. 2008. *Dictionary of the fungi*, 10th edn. Clayton, Victoria, Australia, CSIRO Publishing. 640 pp.

Barnet, H.L. & Hunter, B.B. 1972. *Illustrated genera of imperfect fungi*, 3rd edn. Minneapolis, MN, Burgess. *IMI descriptions of fungi and bacteria*. Wallingford, UK, CAB International. <http://www.cabi.org/dfb/> (last accessed on 16 September 2015).

Ellis, M.B. & Ellis, J.P. 1997. *Microfungi on land plants: an identification handbook*. Slough, UK, Richmond Publishing. 868 pp.

Snowdon, A.L. 2010a. *A colour atlas of post-harvest diseases and disorders of fruits and vegetables*. Vol. 1. *General introduction and fruits*. Boca Raton, CRC Press. 320 pp.

Snowdon, A.L. 2010b. *A colour atlas of post-harvest diseases and disorders of fruits and vegetables*. Vol. 2. *Vegetables*. Boca Raton, CRC Press. 416 pp.

Leslie, J.F. & Summerell, B.A. 2006. *The Fusarium laboratory manual*. Oxford, Blackwell. 388 pp.

Erwin, D.C. & Ribeiro, O.K. 1996. *Phytophthora diseases worldwide*. St Paul, MN, APS Press, American Phytopathological Society. 592 pp.

Lane, C.R., Beales, P. & Hughes, K.J.D., eds. 2012. *Fungal plant pathogens*. Wallingford, UK, CAB International. 328 pp.

5.5.1.6 Arthropods

CABI Arthropod Name Index (1996) on CD-ROM. Gives information on synonyms and links to old *Review of Applied Entomology* volumes (including pre-1973).

Hill, D.S. 1987. *Agricultural insect pests of temperate regions*. Cambridge, UK: Cambridge University Press. 672 pp.

Hill, D.S. & Waller, J.M. 1982. *Pests and diseases of tropical pests: principles and methods of control*. London, Longman. 175 pp.

5.5.1.7 Insects

Carroll, L.E., White, I.M., Freidberg, A., Norrbom, A.L., Dallwitz, M.J. & Thompson, F.C. Pest fruit flies of the world. Available at http://delta-intkey.com/ffl/www/_wintro.htm (last accessed on 16 September 2015).

Carroll, L.E., Norrbom, A.L., Dallwitz, M.J. & Thompson, F.C. Pest fruit flies of the world – larvae. Available at http://delta-intkey.com/ffl/www/_wintro.htm (last accessed on 16 September 2015).

Natural History Museum. Lepindex – the global Lepidoptera names index: <http://www.nhm.ac.uk/research-curation/projects/lepindex/>
ScaleNet: <http://www.sel.barc.usda.gov/scalenet/query.htm>

5.5.1.8 Nematodes

Luc, M., Sikora, R.A. & Bridge, J., eds. 2005. *Plant parasitic nematodes in subtropical and tropical agriculture*, 2nd edn. Wallingford, UK, CAB International. 896 pp.

Perry, R.N. & Moens, M., eds. 2013. *Plant nematology*, 2nd edn. Wallingford, UK, CAB International. 568 pp.

Siddiqi, M.R. 2000. *Tylenchida parasites of plants and insects*, 2nd edn. Wallingford, UK, CAB International. 864 pp.

5.5.2 Databases of accredited labs and expertise

http://www.eppo.int/DATABASES/diagnostics/diag_quest.htm

5.6 Bibliography

Barnet, H.L. & Hunter, B.B. 1972. *Illustrated genera of imperfect fungi*, 3rd edn. Minneapolis, MN, Burgess.

Boonham, N., Glover, R., Tomlinson, J. & Mumford, R. 2008. Exploiting generic platform technologies for the detection and identification of plant pathogens. *European Journal of Plant Pathology*, 121: 355–363. DOI: 10.1007/978-1-4020-8780-6_15.

Boonham, N., Tomlinson, J. & Mumford, R. 2007. Microarrays for rapid identification of plant viruses. *Annual Review of Phytopathology*, 45: 307–328. DOI: 10.1146/annurev.phyto.45.062806.094349.

Bradbury, J.F. 1985. *Guide to plant pathogenic bacteria*. Wallingford, UK, CAB International. 332 pp.

CABI & EPPO. 1997. *Quarantine pests for Europe*, 2nd edn. Wallingford, UK, CAB International and Paris, European and Mediterranean Plant Protection Organization. 1425 pp.

Cannon, R.J.C., Pemberton, A.W. & Bartlett, P.W. 1999. Appropriate measures for the eradication of unlisted pests. *Bulletin OEPP / EPPO Bulletin*, 29: 29–36.

Coolen, W.A. & D'Herde, C.J. 1972. *A method for the quantitative extraction of nematodes from plant tissue*. Ghent, Belgium, Ministry of Agriculture, State Agricultural Research Centre. 77 pp.

Decraemer, W. & Hunt, D.J. 2006. Structure & classification. In R.N. Perry & M. Moens, eds. *Plant nematology*, 1st edn, pp. 3–32. Wallingford, UK, CAB International.

Ellis, M.B. & Ellis, J.P. 1997. *Microfungi on land plants: an identification handbook*. Slough, UK, Richmond Publishing. 868 pp.

- EPPO. 2013a. Diagnostic: PM 7/119 (1) Nematode extraction. *EPPO Bulletin*, 43: 471–495.
- EPPO. 2013b. *Diagnostic protocols for regulated pests: pictorial glossary of morphological terms in nematology*. EPPO Technical Document No. 1056 (rev. 4). Paris, European and Mediterranean Plant Protection Organization. Available at http://www.eppo.int/QUARANTINE/diag_activities/EPPO_TD_1056_Glossary.pdf (last accessed 16 September 2015).
- Erwin, D.C. & Ribeiro, O.K. 1996., *Phytophthora diseases worldwide*. St Paul, MN, APS Press, American Phytopathological Society. 592 pp.
- Fenwick, D.W. 1940. Methods for the recovery and counting of cysts of *Heterodera schachtii* from soil. *Journal of Helminthology*, 18: 155–172.
- Flegg, J.J.M. & Hooper, D.J. 1970. Extraction of free-living stages from soil. In J.F. Southey, ed. *Laboratory methods for work with plant and soil nematodes*, pp. 5–22. Technical Bulletin 2. London, Ministry of Agriculture, Fisheries and Food.
- Hockland, S., Inserra, R.N., Millar, L. & Lehman, P.S. 2006. International plant health – putting legislation into practice. In R.N. Perry & M. Moens, eds. *Plant nematology*, 1st edn, pp. 327–345. Wallingford, UK, CAB International.
- Hooper, D.J. 1986. Extraction of nematodes from plant tissue. In J.F. Southey, ed. *Laboratory methods for work with plant and soil nematodes*. Reference Book 402, 6th edn, pp. 51–58. London, Ministry of Agriculture, Fisheries and Food.
- Hooper, D.J. & Evans, K. 1993. Extraction, identification and control of plant parasitic nematodes. In K. Evans, D.M. Trudgill & J.M. Webster, eds. *Plant parasitic nematodes in temperate agriculture*, pp. 1–60. Wallingford, UK, CAB International.
- Hooper, D.J., Hallmann, J. & Subbotin, S.A. 2005. Methods for extraction, processing and detection of plant and soil nematodes. In M. Luc, R.A. Sikora & J. Bridge, eds. *Plant parasitic nematodes in subtropical and tropical agriculture*, 2nd edn, pp. 53–86. Wallingford, UK, CAB International.
- IMI descriptions of fungi and bacteria*. Wallingford, UK, CAB International. <http://www.cabi.org/dfb/> (last accessed on 16 September 2015).
- Janse, J.D. 2010. *Phytobacteriology: principles and practice*. Wallingford, UK, CAB International. 368 pp.
- Lelliot, R.A. & Stead, D.E. 1991. *Methods for the diagnosis of bacterial diseases of plants*. Oxford, Wiley. 224 pp.
- Leslie, J.F. & Summerell, B.A. 2006. *The Fusarium laboratory manual*. Oxford, Blackwell. 388 pp.
- Luc, M., Sikora, R.A. & Bridge, J., eds. 2005. *Plant parasitic nematodes in subtropical and tropical agriculture*, 2nd edn. Wallingford, UK, CAB International. 896 pp.
- Manzanilla-López, R.H. & Marbán-Mendoza, N., eds. 2012. *Practical plant nematology*. Mexico City, Biblioteca Básica de Agricultura, Grupo Mundi-Prensa. 883 pp.
- Monger, W.A., Alicai, T., Ndunguru, J., Kinyua, Z.M., Potts, M., Reeder, R.H., Miano, D.W., Adams, I.P., Boonham, N., Glover, R.H. & Smith, J. 2010. The complete genome sequence of the Tanzanian strain of *Cassava brown streak virus* and comparison with the Ugandan strain sequence. *Archives of Virology*, 155: 429–433. DOI:10.1007/s00705-009-0581-8.
- Seinhorst, J.W. 1962. On the killing, fixing and transferring to glycerin of nematodes. *Nematologica*, 8: 29–32.
- Siddiqi, M.R. 2000. *Tylenchida parasites of plants and insects*, 2nd edn. Wallingford, UK, CAB International. 864 pp.
- Smith, J.J., Waage, J., Woodhall, J.W., Bishop, S.J. & Spence, N.J. 2008. The challenge of providing plant pest diagnostic services for Africa. *European Journal of Plant Pathology*, 121: 365–375.
- Snowdon, A.L. 2010a. *A colour atlas of post-harvest diseases and disorders of fruits and vegetables*. Vol. 1. *General introduction and fruits*. Boca Raton, CRC Press. 320 pp.
- Snowdon, A.L. 2010b. *A colour atlas of post-harvest diseases and disorders of fruits and vegetables*. Vol. 2. *Vegetables*. Boca Raton, CRC Press. 416 pp.
- Tarjan, A.C. 1967. Influence of temperature and hydrogen peroxide on the extraction of burrowing nematodes from citrus roots. *Plant Disease Reporter*, 51: 1024–1028.
- Tarjan, A.C. 1972. Observations on extracting citrus nematode, *Tylenchulus semipenetrans*, from citrus roots. *Plant Disease Reporter*, 56: 186–188.

Tomlinson, J. & Boonham, N. 2008. Potential of LAMP for detection of plant pathogens. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, 3: 1–7.

Weller, S.A., Aspin, A. & Stead, D.E. 2008. Classification and identification of plant-associated bacteria by fatty acid profiling. *EPPO Bulletin*, 06/2008; 30(3–4): 375–380. DOI: 10.1111/j.1365-2338.2000.tb00914.x.

Weigers, A.L. 2003. Valid methods: the quality assurance of test method development, validation, approval, and transfer for veterinary testing laboratories. *J. Vet. Diagn. Invest.*, 15(4): 303–310.

Winfield, A.L., Enfield, M.A. & Foremann, J.H. 1987. A column elutriator for extracting cyst nematodes and other small invertebrates from soil samples. *Annals of Applied Biology*, 111: 223–231. DOI: 10.1111/j.1744-7348.1987.tb01449.x.

6. Imaging Specimens

Introduction

Scientific imaging, or scientific photography, is a method that is widely used in surveillance work for the purpose of identification and documentation. It involves making photographs or drawings of specimens, in the most accurate and unbiased manner possible. This chapter provides an introduction to some of the reasons for creating images of specimens and some of the processes and techniques involved. For a more detailed treatment, the reader is referred to the [Phytosanitary Resources website](#).

6.1 Why take images for diagnostics?

6.1.1 Documentation

One of the most common reasons for taking photographs of specimens is to document them, either for future reference or as a backup to a physical collection. An accurate, unbiased representation of a specimen can be very useful insurance against colours fading, damage, degradation or loss.

6.1.2 Reference material

Electronic images can be shared online in an image library or by e-mail, and can be used as reference material in the same way the original specimen might be. Images of specimens that have been positively identified can be used for comparison purposes to identify further specimens. Sharing images online allows a larger audience to have access to specimens than was previously possible. This also exposes the information to intense scrutiny by a larger volume of discerning "reviewers", often resulting in improvements to accuracy.

6.1.3 Identification

In cases where the specimen has not been positively identified, images of the appropriate diagnostic

features can be sent for identification to an expert in lieu of the original specimen. The speed and ease with which images can be sent to experts, as well as the reduced risk of the specimen being lost or damaged in transit, makes imaging an important weapon in a surveillance laboratory's arsenal.

6.2 What is a scientific image?

The main aim of taking scientific photographs and illustrations is to accurately represent the sample with as much detail as possible. For a diagnostic photograph the aim is to have:

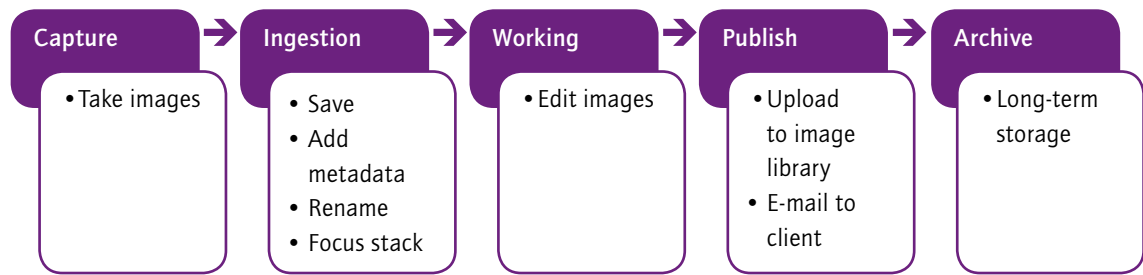
- ◆ entire subject matter in focus (by using focus stacking techniques)
- ◆ high level of subject detail (high-resolution images)
- ◆ high level of colour accuracy (by using grey card, colour cards)
- ◆ high level of measurement accuracy (by including a scale bar)
- ◆ standardized views (dorsal, ventral, lateral)
- ◆ uniform lighting
- ◆ correct exposure
- ◆ seamless monotone background, preferably colourless.

6.3 Workflow

Imaging specimens can be complex and time-consuming. If intending to undertake imaging of many specimens, establish a workflow that is functional and efficient. An example of a workflow that it may be useful to adopt is shown in Figure 20.

As noted above, there is more detailed information about the optical imaging of pinned, wet- and slide-mounted specimens, especially of arthropods, available online.

Figure 20: An example of an imaging workflow



7. Remote Diagnostics for Plant Pests

Introduction

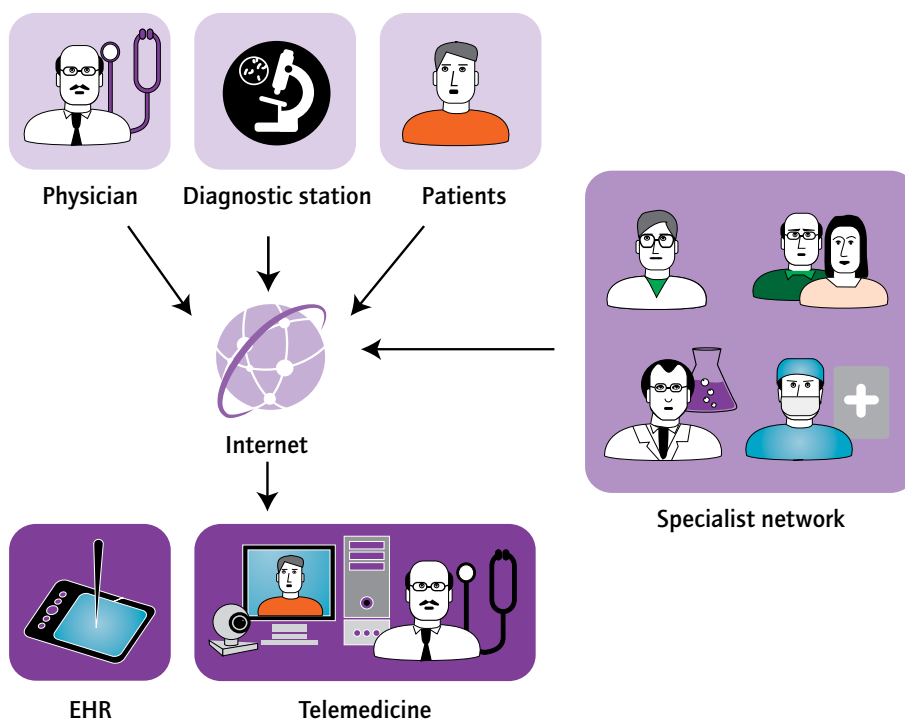
As global trade and the movement of people increase, countries are faced with greater risks from pest incursions that may reduce the quality and quantity of agricultural production and result in restrictions to market access. At the same time there has been a decline in taxonomic expertise and a concentration of specialists in city centres, far from where pests are found and where the services of expert taxonomists are needed most. As these factors conspire to potentially elevate the risk of pest incursions, we need to find ways of providing pest identification processes that are faster, cheaper and better. Mobile technologies and the Internet are providing new solutions to similar problems across the globe and may well be applied to these pest issues as well, but it will require adaptation, a shift to new processes and a new way of doing diagnostics.

7.1 What is remote diagnostics and why do we need it?

For some years now, physicians have been using communication technologies to create the concept of telemedicine (Figure 21), where patient consultations are conducted from a distance. Telemedicine has been particularly useful for people living in isolated communities or remote regions, and where access to specialists or specialist opinion is limited.

The constraints of distance and expertise are the same for plant pest diagnostics, where we are facing a global decline in both taxonomic expertise and in the numbers of pest diagnosticians generally. Moreover, our specialists tend to be concentrated in major city centres, whereas most pest species are intercepted in rural and remote areas, or at some distance from the nearest specialist. At the same time, globalization has led to an increase in

Figure 21: Telemedicine uses the Internet to share patient information in real time



international trade and the movement of people. With these increases in movement of goods and people comes the increased likelihood of pest movement. The demand for diagnostic services is increasing as specialist human resources are in decline and so we are forced to find solutions to this problem that are fast, cheap and effective.

Remote diagnostics for plant pests is like telemedicine in that it uses the Internet and other communication technologies to share images of a pest or symptoms caused by pests with specialists in different locations. A range of digital communication tools and devices can be used to share pest images and information. The process may occur in real time or not and use software to capture and store the information that is shared during the process, creating a permanent digital record of the pest and its identification. The following sections describe some of the systems used to perform remote pest identifications.

7.1.1 Basic principles

A typical laboratory set-up for remote diagnostics would include a microscope (dissection or compound), a video camera attached to the microscope and either a computer or Internet server with a graphical interface. Images captured from the video camera can be shared by connecting either the computer or the server to the Internet and assigning a static IP address to the location. Directly connecting the camera to the Internet enables

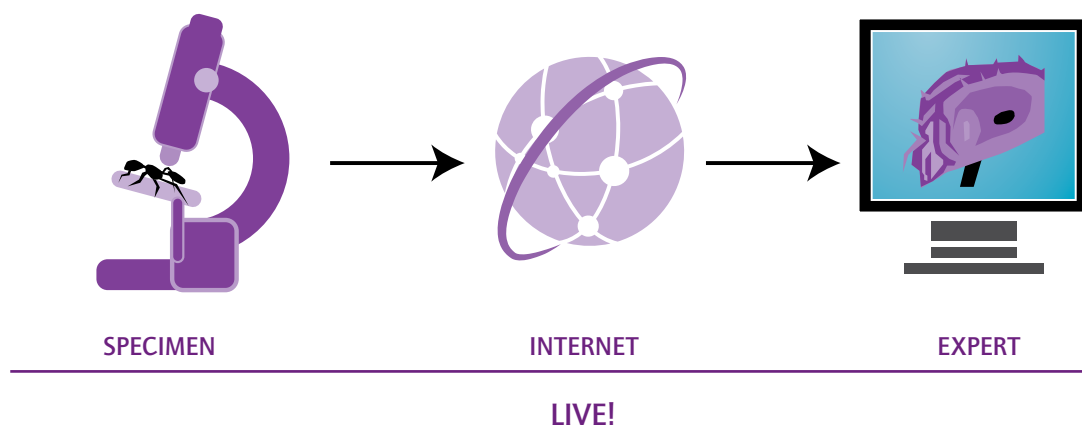
people in other locations to view the images from the camera in real time on their computer by using their web browser to locate the IP address of the microscope camera (Figure 22). The person sharing the image (camera location) can communicate with the person viewing the image (specialist) by using any of a variety of communication tools.

Remote microscopy has been found to be effective in real-world settings. Trials conducted by the Australian Quarantine and Inspection Service (AQIS) and the Ministry of Agriculture and Forestry Biosecurity New Zealand that tested the efficacy of remote microscopy in plant quarantine settings showed that in Australia, a diagnosis to a level at which realistic biosecurity decisions could be made occurred on 77 percent of occasions (Thompson *et al.*, 2011).

Several different types of system are available that can facilitate Internet sharing of live images. To capture images from the microscope to a computer requires special software to capture and share the image over the Internet. This software usually includes image editing and archiving and would require the allocation of a static IP address to the computer. Examples of this system include Olympus NetCam software and the Leica Network LAS module.

Alternatively, Nikon offers the DS-L series camera control unit with graphical interface, which captures the microscope image and offers it directly to the Internet without any computer

Figure 22: A specialist can access a pest image over the Internet from the Nikon DS-L2 hardware connected to a microscope camera



device or additional software. The DS-L has its own unique operating system through which the user can select their own IP settings. It also has its own image-editing suite and tools for annotating and highlighting the live image, and current models have a touch screen for easy operation.

In both types of systems, image sharing can either be restricted to a local area network (LAN) or be made available to a wide area network (WAN), such as the Internet. Each type of network will require certain security protocols that will be determined by the organization, and sharing within and between these networks will require compliance with the relevant security protocols. For example, organizations often create layers of firewalls to limit the flow of data within their network as a precaution against the spread of malicious software ("viruses") that may infect the network. They also create firewalls that are designed to protect the network from external attack and therefore limit the flow of certain kinds of access and data entering their network. Networks may occasionally limit the data that can leave the LAN.

In order to share images from a microscope located within a LAN, the images need to be captured by a device that has an IP address that can be accessed over the Internet. In order to directly contact the IP address of the device,

people external to the LAN have to pass through the organizational firewall. Without any security protocol, this would give external users direct access to the internal network. Organizations that wish to participate in an external remote microscope network would need to set certain firewall security protocols to protect their systems (Figure 23). The type of security required will depend on what type of hardware system is used to capture images from the microscope camera. Computer-based systems like Olympus Netcam and Leica Network LAS that use computers for image capture are more susceptible to security breaches because computers have common operating systems (Windows, Linux, Apple/Mac OS) and hard drive storage that is often connected to the LAN. The Nikon system is less susceptible because it does not have data storage capacity and uses a very restricted Nikon operating system; as a result it is not susceptible to attack and cannot store viruses.

7.1.2 Nikon digital sight hardware

The Nikon digital imaging system consists of a digital microscope camera that is matched to a dedicated stand-alone camera control unit (Figure 24).

The camera control unit has its own screen and operating system so no additional computer is required. It has full network capability and can be connected to a LAN or to the Internet. Web browser

Figure 23: Firewall protection for LAN Networks

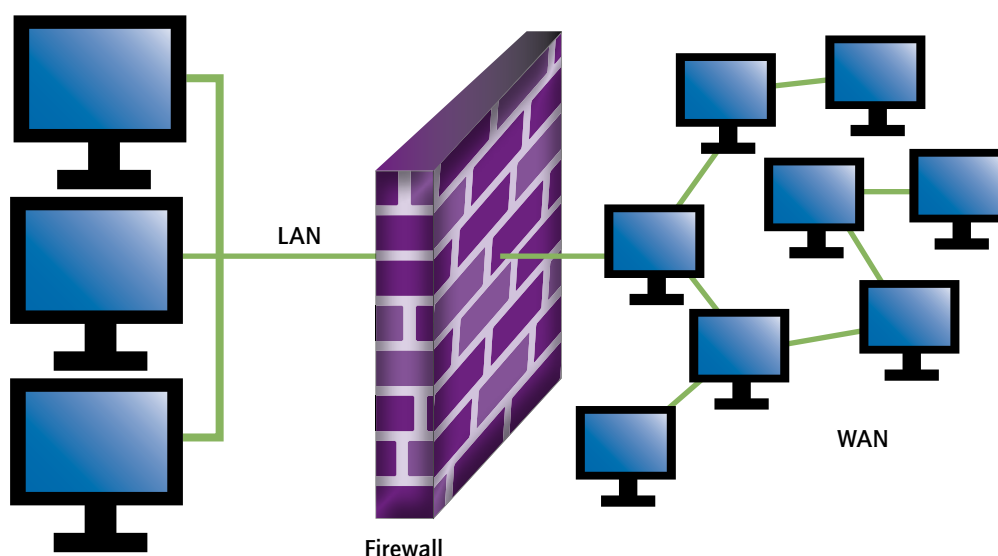
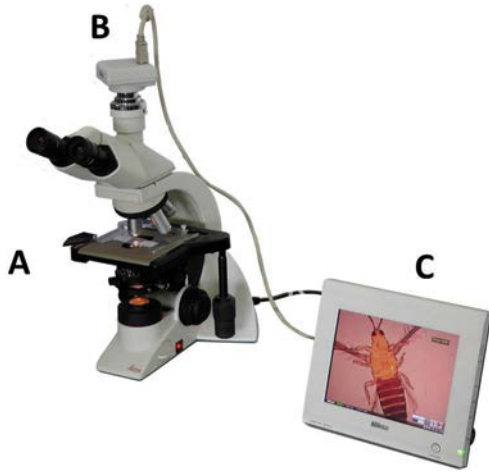


Figure 24: Fi-1 camera and DS-L2 console connected to A, microscope; B, Nikon digital camera (Fi-1); C, Nikon Camera Control Unit (DS-L2) that connects to the Internet



support is available for live image viewing and connection via standard Internet communication and file transfer protocols (HTTP, telnet, FTP) and is DHCP compatible for the allocation of IP addresses. The unit can also be connected to a computer or a

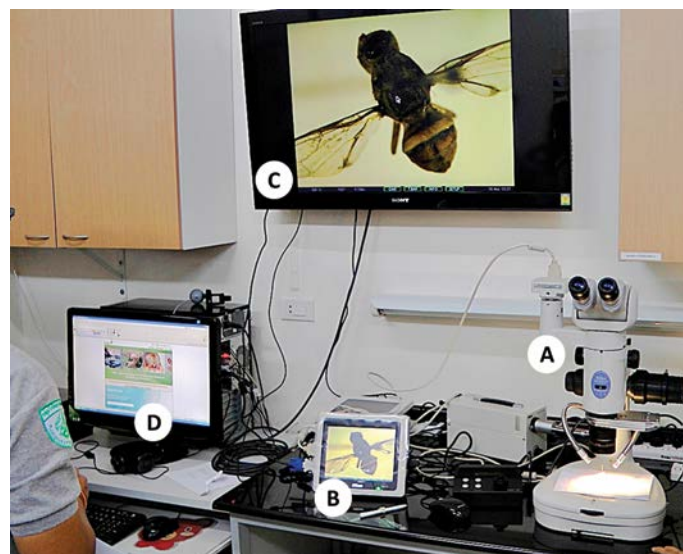
large external monitor and can capture images to a USB memory stick. A comprehensive set of menus is available for imaging and for measuring and annotating images. An example set-up of a unit in Thailand is shown in Figure 25.

7.1.2.1 Internet access and configuration with Nikon DS-L

The Nikon DS-L series consoles contain a web server which supports a website that provides access to the camera. A fixed IP address is required to connect the camera to the Internet. The DS-L server is usually located inside the firewall of a network, so anyone who wishes to access the camera images must pass through any organizational firewalls to access the server.

At face value this may seem like a security risk because it requires opening a port in the firewall to allow access to the DS-L console, but, as mentioned above, there is limited ability for outsiders to access the LAN via the console. If an unauthorized person did connect to the DS-L console, they would only be able to see the microscope camera image – since this equipment is only connected when there is a need to identify a pest remotely, there is little opportunity to hack the system.

Figure 25: Nikon hardware set-up for remote microscopy at the Plant Quarantine Laboratories in Bangkok, Thailand



A, Nikon digital camera; B, DS-L2 Camera Control Unit; C, Extended monitor for the camera control unit and D, PC used to access taxonomic information on the Internet, communicate with remote users and access the shared camera image via the Internet or the LAN.

Although the console does not pose a high security risk to the LAN, extra measures can be put in place to isolate the console from the organizational network. Some strategies that can be used to eliminate the threat completely include using a reverse proxy or using a DMZ to isolate the DS-L console from the network (Figure 26).

7.2 Methods of communication for real-time remote microscopy

7.2.1 Standard voice telephone

While sharing a specimen image with a specialist, the parties involved must communicate with each other to discuss the specimen and to relay instructions. For example, the specialist may need to see key features of the specimen in order to determine an identification and therefore needs to instruct the non-specialist how to manipulate the specimen under the microscope during this process. Since the specialist can see the specimen in real time, the communication should be relatively fluid.

A simple telephone call is often adequate for most remote identifications and, whether fixed or mobile, the phone is perhaps the most common communication tool available. In any case, communications tools may be limited in many locations and other options may not be available.

7.2.2 Video conferencing system

Video conferencing may add to the interactions between those seeking identifications and those providing them. For example, in conferencing conditions, multiple specialists in different locations could simultaneously participate in a remote identification. Internet services such as Skype, Google Hangouts, Apple FaceTime and others are readily available and easily accessible to a global audience (Figures 27 and 28). Such applications have recording and chat facilities and can keep a record of the discussion that accompanies each remote identification.

7.2.3 Web-enabled "whiteboard" software

"Electronic whiteboards" can be used to increase information sharing and in teaching. They allow real-time annotation of an image with all parties being able to contribute to the discussion, independent of their location. At any time during the discussion, the image and comments can be saved for future reference. Whiteboard systems range in features from simple to elaborate. Microsoft Messenger's Whiteboard program, which comes free with Windows, is simple, can be learned quickly and meets the basic needs.

Figure 26: IT security options for setting up a microscope camera within a LAN to provide external Internet access

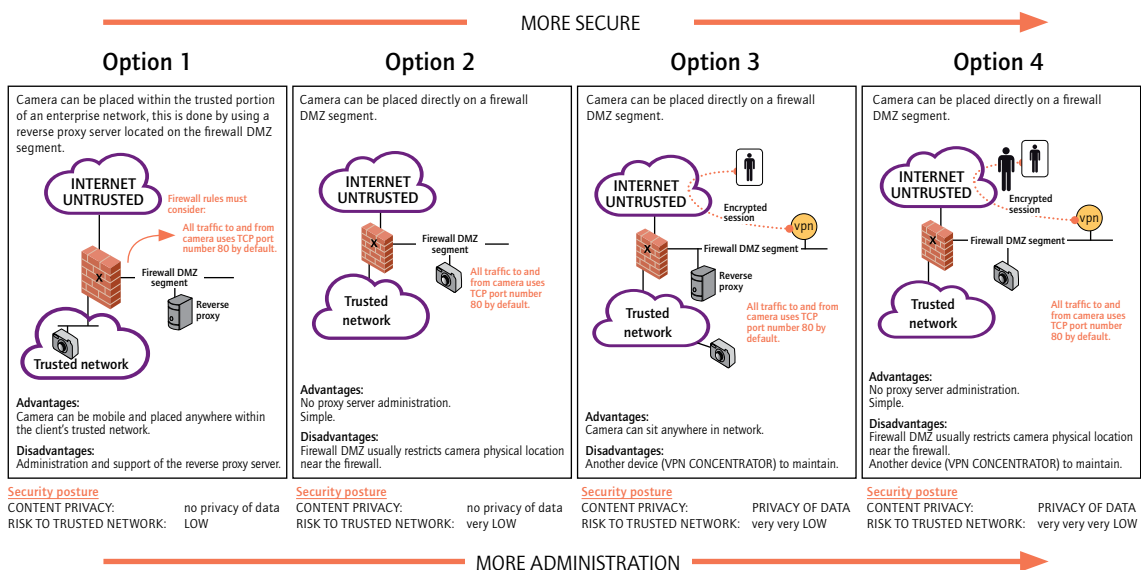


Figure 27: A non-specialist in Thailand uses a Skype video call to discuss a specimen with specialists in Vietnam



The specimen image is being shared with Vietnam over the Internet via the Nikon DS-L2.

7.2.4 Other methods for capturing and sharing live images

Web-enabled imaging systems such as those discussed above are relatively expensive and, although these produce the best quality images and provide a suite of additional features for image manipulation, there are simpler and cheaper ways of sharing live images. Providing that live (video stream) images can be captured on a PC, any program that allows desktop sharing can be used to share the images with other PCs in different locations via the Internet. These programs include Windows Remote Desktop, Skype, Ustream, Google Hangouts, Web-conferencing and other programs that are freely available. One drawback of this method is that the image quality may be compromised to the point that detail essential to making an identification is lost. This is because many of these types of programs apply their own image-processing conditions that can considerably reduce quality. In contrast, systems that allow direct access to the specimen image lose little or no quality.

In many instances, the loss of quality may not be too great a hindrance to making an identification. It all depends on the nature of the specimen and the level of detail that is needed. Where resources are limited, this approach can be considered as an option. Inexpensive image capture equipment such as USB microscopes can be easily used in this situation (Figure 29).

Figure 28: Specimens under the USB microscope may be shared with an expert via video software. In this case the conversation between the laboratory and the expert is over the telephone



Alternatively, Skype may be used for video and voice.

Note, however, that organizations may place security restrictions on the use of web-sharing tools similar to those described above.

7.3 Remote diagnostic processes for education and training

The ability to share live microscope images with Internet users across multiple locations presents an ideal opportunity for remotely training people. By sharing microscope images over the Internet, diagnostic specialists and taxonomists can demonstrate how to identify pest species or distinguish symptoms or features that are characteristic of a particular pest. Additional communication devices can be used to provide a video-conferencing environment where there can be discussion, whiteboards, chat, documentation, and image, video and audio capture. These provide a lasting record of the training that can be accessed for future reference by the participant. The result is interactive learning that is cheap and effective, where participants get to improve their skills by accessing a specialist from the comfort of their own office.

7.3.1 Field-based remote diagnostics

In many instances, pests or pest problems can be identified from images. The development of handheld computers, USB microscopes, phone cameras, wireless broadband and extensive mobile

Figure 29: Left: A USB microscope captures images to a PC at a border inspection point and can be shared with remote specialists via web collaboration tools such as Skype. Centre: A 20–200× magnification USB microscope. Right: A Border Inspection Officer examines pests and captures images using a USB microscope



network services now make it possible to capture highly magnified, high-resolution images from almost anywhere, and to share them over the Internet or via phone networks. These technologies allow the process of pest diagnosis to be streamlined so that images of a pest or symptoms can be shared with specialists directly from the field using mobile devices. Identification to species level is not always required in order to make a management decision. Instead, more timely application of pest control strategies may be more important than delaying for a specimen-based identification. Not all diagnostics can be done in this way, but a significant proportion of pest problems could be solved quickly and efficiently if field staff could share and communicate with specialists via mobile devices.

7.3.1.1 Hardware for mobile devices

A range of macro lenses and wireless microscopes is now available for mobile devices providing magnification from $\times 4$ to $\times 200$ in the field (see, e.g. <http://www.photojojo.com> and <http://www.chinavasion.com>). These devices are cheap and can be mobilized to a broad group of users in the field where high-quality images of pests can be captured and shared with networks of people who can collectively comment or offer an identification. These devices, together with the phenomenon of crowdsourcing information and social networking can greatly extend individuals' and groups'

capability to identify pests in a way that is fast and effective (Figure 30). At the field level, this means that pest management decisions can be made more rapidly and, where large networks are involved in the process, pest intelligence is widely disseminated. In addition, this collective approach to problem solving means that the more experienced members of a network share their knowledge with less experienced members, thereby raising the skills level of all individuals in the group.

7.3.2 Virtual networks for remote diagnostics

Crowdsourcing has become a powerful phenomenon for sharing and building knowledge in the digital space. Numerous websites are designed to target and connect people in many different ways and can provide a wide range of choice for users engaging in crowdsourcing activities. Social networking and sharing websites can provide the means for people to share information globally with known and unknown people or allow users to connect with specific individuals or private groups.

Sermo is an online community of physicians with more than 200 000 members who can choose to share clinical information in order to assist diagnoses and to build on the body of medical knowledge. Similar online networks could be used to share pest information for the purposes of identifying pests and recommending

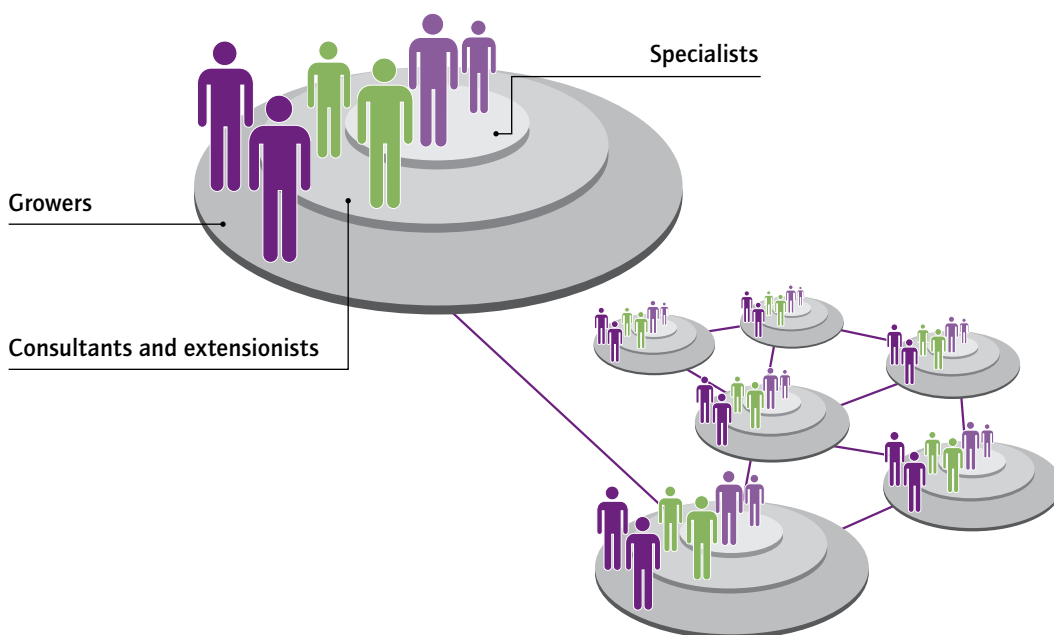
Figure 30: Centre: Various simple attachments enable mobile phones to capture highly magnified images of pests, which can be shared and discussed with communities via cloud networks. These communities can include local or regional NPPOs. Side images taken with the Hot Electronics wireless microscope and mobile phone. Left side images of papaya brown spot. Right side images show range of magnifications achieved with the wireless microscope



management strategies (Figure 31). Existing websites and software products can provide the basis of such networks where farmers, consultants, extension agronomists and specialists can share diagnostic information and make collective

decisions for managing pests. Virtual networks can be local, starting with existing peer-to-peer relationships and expanding to regional, national and even international networks and drawing on an increasing pool of expertise.

Figure 31: A proposed network of growers, consultants, extensionists and specialists



7.3.2.1 Software for mobile devices

An explosion in mobile phone ownership and wider service coverage has paved the way for mass communication and information sharing. The effectiveness of mobile phones for remote diagnostics can be improved by using a software application that not only facilitates the communication process, but also saves and stores data. E-mail is often used to share pest information; however, simply sharing pest images via e-mail services limits the interaction to a select group and does not allow the information to be saved, stored and shared with a wider group. Moreover, because e-mail interactions are dispersed and lack connection, data cannot easily be aggregated for future reference. It is therefore desirable to use websites or software that can share and store pest information in a network environment (Figure 32). Pest incidence information that is stored and aggregated can provide valuable information about the spatial and temporal incidence and distribution of pests. This information can then be used for pest management decisions, pest alerts, area freedom declarations, developing research and development (R&D) strategies and for compiling pest lists.

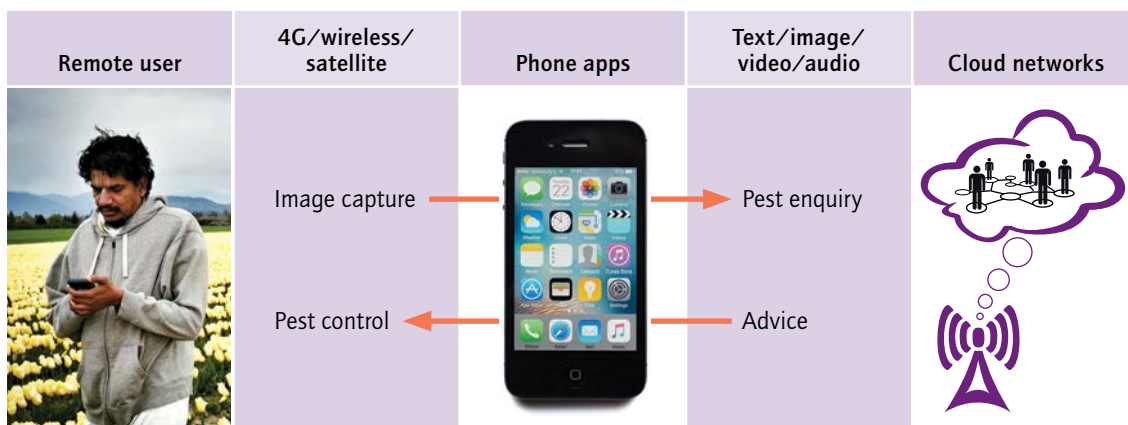
Although there are numerous mobile applications for engaging in social media, there are few such sites designed specifically for biologists and that can save and organize data. Software

tends to be either social-media oriented or data-management oriented, but not both. Consequently, several applications would have to be used in order to achieve both group sharing and data storage. However, Pestpoint (<http://www.Pestpoint.org.au>) is a software application that allows users to create private virtual networks for sharing, and that will capture and store pest information. It operates from both the web and mobile platforms.

7.4 Conclusions

Not all pest identifications can be done remotely. Some are simply too difficult to determine remotely and will require that a specimen be examined. The identification of insects from images will certainly be easier than the identification of plant diseases. Nevertheless, we should at least try to solve every pest problem in this way, if it means that some pests can be controlled faster. Creating a system for remote diagnostics that includes networks and data capture produces multiple benefits for the whole plant health system, in building knowledge by capturing and storing pest information over space and time, improving capability as less-experienced people learn from interactions with more experienced people through their networks and better intelligence about pests from observing patterns in their distribution and incidence.

Figure 32: Remote field diagnostics where pest images or video are captured on a mobile device and shared over the Internet with a network of people who may be able to offer an identification, management advice or both





8. Reference Collections

Introduction

A reference collection for phytosanitary purposes may contain any of the following types of specimens:

- ◆ fungi
- ◆ arthropods
- ◆ gastropods
- ◆ nematodes
- ◆ invasive plants
- ◆ parasitic plants
- ◆ plant parts affected by pathogens and pests.

A reference collection is essential for phytosanitary purposes as it:

- ◆ underpins quarantine and response decisions
- ◆ allows verification of the pathogen and pest records of a country to resolve trade disputes
- ◆ allows pathogen and pest records to be revisited as diagnostic methods are continuously improving and the taxonomy of organisms is dynamic; during taxonomic revision, one species can be revised and split into a number of different species or combined with other species.

A good reference collection should have:

- ◆ specimens that are well preserved, maintain clear diagnostic characters and allow analysis (e.g. molecular testing) in the future
- ◆ effective measures to prevent infestation of pests and moisture damage on specimens
- ◆ labels that contain collection details
- ◆ database that can retrieve data efficiently.

8.1 General requirements

8.1.1 Building

The structural requirements of the building for a reference collection are:

- ◆ a dedicated room with minimum vibration: may consider earthquake-resistant structure, no thoroughfare and no unaccompanied visitors
- ◆ the room should have enough space for storing reference specimens with work space for curator

- ◆ a solid floor and walls without gaps in joinery to avoid infestation of pests
- ◆ large work benches for sample processing, microscopic examination and culture maintenance
- ◆ a storage system that can be in the form of boxes, drawers, cabinets, shelves or cupboards (*note*: some mobile filing systems are more expensive but can save a lot of space)
- ◆ phone and network points placed in practical positions
- ◆ no water tap within the collection to avoid moisture damage.

Live specimens should not be handled in the same room as the reference collections to minimize disturbance to the collection and avoid infestation of pests such as mites, booklice and beetles.

8.1.2 Controlled environment

Reference specimens are fragile and vulnerable to pests, moulds and fungi. The following points will help prevent damage to the collection.

- ◆ Air conditioners and dehumidifiers are essential in regions with warm and wet climates. The running cost of these appliances can be significant; hence, it is advisable to incorporate the cost into the financial budget.
- ◆ It is recommended that temperature be maintained at 18–19 °C and relative humidity below 50 percent to prevent moisture damage, fungal growth and breeding of booklice and beetles that can damage specimens.
- ◆ Specimens keep longer in the dark. There should be no windows or skylights in the room and lights should be turned off when the room is not in use.
- ◆ To control pests, it is recommended to use airtight containers for specimens, use pest deterrents (e.g. camphor blocks, naphthalene sticky traps, tangle trap barriers), fumigate or apply insecticide if required, seal gaps in

joinery; freeze all incoming or returning dry herbarium specimens at -20°C for 48 h and on a regular basis (every 1–2 years).

- ◆ Separate reference collection from work space that is used for handling live plant material.

8.1.3 Safety and security

Reference collections hold valuable resources that are important for quarantine and response decisions, and can facilitate trade. Therefore, the collections should be kept at a reasonable level of security. In addition, safety measures should be implemented to protect staff working in the collections.

Some of the safety and security measures required are:

- ◆ a dedicated room, separating the collections from office space and other areas
- ◆ a lockable room or room in a lockable, restricted access building
- ◆ an alarm system for after-hours protection
- ◆ a telephone to allow emergency calls
- ◆ fire wise:
 - fire blanket, fire extinguisher, inert gas
 - do not use open flames
 - do not leave electric appliances, particularly heaters, on unattended
 - maintain electrical wiring
- ◆ consideration of natural disasters, e.g. earthquakes, tsunami, flooding, storms and wild fire
- ◆ no eating in the reference collection room
- ◆ no unaccompanied visitors
- ◆ wear covered shoes
- ◆ tie back long hair
- ◆ access for trained personnel only
- ◆ chemical and equipment should be handled by trained personnel.

Some of the chemicals and reagents used in the preparation of reference specimens are harmful. The following measures should be considered.

- ◆ Material Safety Data sheets (MSDS) and any provided product literature should be checked to have a clear understanding of any dangers associated with the chemical or reagent or kit contents (e.g. storage and handling requirements). Hard copies of MSDS for each chemical held in the laboratory should be easily accessible.

- ◆ Quantities of hazardous substances in the laboratory should be kept to a minimum, commensurate with needs and shelf life. Appropriate signage should be displayed when storing these substances.
- ◆ Staff should check that the work area is safe by locating fire extinguishers, nearest first-aid kit and any safety equipment that may be required.
- ◆ Appropriate personal protective clothing and equipment should be used as recommended in the MSDS, e.g. laboratory coat, gloves, safety glasses, safety goggles, face shields, masks.
- ◆ Legal requirements of the country for holding chemicals and reagents should be checked.
- ◆ Chemicals and reagents should be disposed of appropriately; consult your local authorities.

8.1.4 Containment requirement

Some countries require keeping herbarium specimens from intercepted material of significant biosecurity risk in a containment facility. Consult your local authorities to confirm which level of containment is required.

Plant material infected with exotic pests or diseases and any contaminated consumables (e.g. wipes, plastic bags, gloves) should be disposed of appropriately. Consult your local authorities.

8.1.5 Storage system

Specimens should be stored in alphabetical order of the pathogen or host, or by the inventory number.

A database should be created to allow efficient data entry for new records and information, and data retrieval with search and data filtering functions. A database can range from simple Excel spreadsheet to programs specially designed for the herbaria. Keeping records in a log book only is not recommended as it is difficult to update and retrieve data.

8.1.6 Routine maintenance

It is important to have dedicated staff appointed to look after and oversee the care and control of reference collections.

The reference collection should be routinely maintained by:

- ◆ regularly monitoring temperature, humidity, dehumidifier water bucket, security and fire alarms, pest control measures that may be in place
- ◆ checking permanent mounts are not drying and labels are not fading
- ◆ updating database with name changes and adding new information available.

If the sealed container of a dried herbarium specimen has been opened, it is necessary to repeat the drying process before sealing and returning the specimen to the collection.

If preserved infected plant tissues have been given away or exchanged, it is necessary to reinoculate the pathogens on herbaceous plants (wherever possible) or grafted specimens and preserve some new infected plant tissue to replenish the stock.

Some pathogens may lose their infectivity over time; it may be necessary to test the inoculated or grafted plants by serology or molecular techniques to check the transmission was successful (e.g. in case of latent transmission).

8.1.7 Improvement

Reference collections can improve their value by:

- ◆ exchanging specimens with other reference collections to obtain reference specimens
- ◆ inviting experts to re-examine specimens
- ◆ generating DNA barcode from specimens
- ◆ storing nucleic acid extractions in deep freezers (−80 °C is recommended)
- ◆ setting up a virtual reference collection.

Note: Digitized collection details, DNA sequence data and images of specimens in a data management system allow effective data retrieval, analysis and reporting, which in turn can facilitate prompt biosecurity decisions.

8.2 Entomology reference collection

8.2.1 Reference specimens (voucher and physical)

Entomological reference collections include all terrestrial invertebrates, as well as nematodes, worms, snails and slugs.

8.2.1.1 Criteria for selection of specimens to be retained (what to keep)

Space restrictions are the overall limiting factor that determine how many specimens it is possible to store; not all specimens can be retained for inclusion in the collection. As a guide, allow room for approximately ten specimens of the same species to be kept, though this may vary depending on specimen size and importance.

Entomology specimen retention criteria:

- ◆ local fauna, native or endemic species, common local crop pests, intercepted pests and newly established species
- ◆ male and female specimens of each species, where possible
- ◆ distribution: specimens of each species from different countries
- ◆ locality: specimens of each species from different localities or regions within the home country
- ◆ variation: intraspecific variations in colour, pattern, markings, size, etc.
- ◆ new species to the collection
- ◆ specimen is in better condition than those already in the collection
- ◆ collected from new host (a new host is when the insect was reared out of or on that host, not just resting on it)
- ◆ kept for survey reasons.

8.2.2 Equipment and supplies

8.2.2.1 Processing equipment

- ◆ microscope
- ◆ drying oven
- ◆ hotplate or microwave oven
- ◆ label baking oven
- ◆ freezer
- ◆ fridge
- ◆ magnifying glass or magnifying lamp.

8.2.2.2 Consumables and other items

- ◆ forceps, fine and ultra-fine, stainless
- ◆ insect mounting pins (stainless steel only) from entomological supplier (sizes 0, 3, 5)
- ◆ micropins for double mounts

- ◆ pinning block (Figure 33)
- ◆ micropins bent and made for lifting mites and small insects (Figure 33)
- ◆ cover-slips (16 mm diameter circular; 13 mm circular)
- ◆ microscope slides (25 mm × 75 mm × 1 mm)
- ◆ insect mounting points, cards (Figure 33)
- ◆ spreading boards (various sizes)
- ◆ closed cell polythene foam boards
- ◆ genitalia vials (microvials)
- ◆ label paper, pens or pencil (for ethanol-proof writing)
- ◆ specimen labels
- ◆ insect mounting (carding) glue
- ◆ scissors
- ◆ soda glass vials various sizes (Figure 33)
 - e.g. 50 × 12 mm
 - e.g. 75 × 25 mm
- ◆ staining blocks (watch glasses) with glass covers (Figure 33).

8.2.2.3 Chemicals and solutions

List of laboratory chemicals required for making media and preparing specimens:

- ◆ Canada balsam (as this is thinned with xylene, Euparal may be preferable)
- ◆ camphor
- ◆ chloral hydrate
- ◆ clove oil
- ◆ distilled water
- ◆ entomological carding glue
- ◆ ethanol (96 percent) also diluted to 70 percent (*note*: because pure ethanol is often difficult to obtain, some collectors use isopropanol (isopropyl alcohol))
- ◆ ethyl acetate
- ◆ Euparal
- ◆ acid fuchsin powder
- ◆ glacial acetic acid
- ◆ glycerol, glycerine
- ◆ gum arabic
- ◆ Histoclear (an orange essence used as a thinning agent for Euparal)
- ◆ household bleach (5 percent sodium hypochlorite solution)

Figure 33: Small equipment routinely needed for mounting.



- ◆ hydrochloric acid (10 percent)
- ◆ lactic acid (85 percent)
- ◆ phenol
- ◆ KOH (10 percent).

Some chemicals used in the preparation of insects for identification are extremely toxic or flammable. Utmost care must be taken when using them, preferably in a fume hood, on a downdraft station or, at the minimum, in a very well ventilated area. It is also important to wear personal protective equipment, such as gloves and lab coat.

Some chemicals must not be stored together and require special cabinets as a safety requirement (see Table 2).

8.2.2.4 Media

This section gives a list of media and their recipes for different insect orders.

Essig's/Wilkey's fluid

85 percent lactic acid	100 ml
Phenol ¹ (saturated aqueous solution)	10 ml
Glacial acetic acid	20 ml
Distilled water	5 ml

¹ *Safety warning*: Volatiles given off by Phenol are carcinogenic, so it must be used in a fume hood.

Table 2: Chemicals and their hazard ratings

Chemical	Hazard rating
Canada balsam	Flammable, serious eye irritation, skin irritation, toxic to aquatic life
Chloral hydrate	Skin and eye irritant
Clove oil	Toxic, skin irritant
Ethyl acetate	Highly flammable, serious eye irritation, damage to organs by skin contact
Euparal	Causes skin irritation, may cause allergy or asthma symptoms or breathing difficulties if inhaled, may cause an allergic skin reaction, may cause respiratory irritation, causes serious eye damage
Acid fuchsin powder	Hazard status is estimated as irritating to skin and eyes. Do not inhale or ingest
Glacial acetic acid	Flammable liquid, toxic, systemic toxicity, metal corrosive, skin corrosive, eye corrosive, aquatic ecotoxin, vertebrate ecotoxin
Glycerol	May cause skin, eye and respiratory tract irritation. May affect kidneys
Gum arabic	Respiratory and contact sensitizer
Household bleach (5 percent sodium hypochlorite solution)	See manufacturer's instructions
Hydrochloric acid	Fatal if swallowed, corrosive to metals, severe skin and eye burns, aquatic and vertebrate ecotoxin
Phenol	Acutely toxic, metal, skin and eye corrosive, aquatic and vertebrate ecotoxin
Lactic acid	Toxic, skin and eye corrosive, vertebrate ecotoxin
Potassium hydroxide (KOH)	Acutely toxic, metal, skin and eye corrosive, aquatic and vertebrae ecotoxin

Hoyer's mounting medium

Distilled water	50 ml
Chloral hydrate	200 g
Gum arabic (clear crystals)	30 g
Glycerine	20 g

Dissolve gum arabic in distilled water at room temperature.

Add chloral hydrate and leave for a day or two until dissolved.

Add glycerine and filter through glass wool.

Staining solution

Acid fuchsin	0.5 g 10 percent
Hydrochloric acid	25 ml
Distilled water	300 ml

8.2.3 Specimen processing for preservation

This section includes an outline of best methods of storage for each type of organism (e.g. ethanol, slide preparation dry-mounted or a combination). For detailed information on preserving insects, see CSIRO. *Simple methods of preserving insects and their allies*. Commonwealth Scientific and Industrial Research Organisation. Available at <http://www.ento.csiro.au/education/preserving.html> (last accessed on 17 September 2015).

8.2.3.1 Killing

After the specimen is captured or located it can be killed using the following methods

Soft bodied and immature specimens that will lose colour should be killed in just-boiled water and left for 1–5 min before placing in 70–80 percent ethanol. This helps prevent them from turning dark; larvae of Coleoptera, Lepidoptera and

Hymenoptera are fixed in this way. Larger insects take longer and may require several changes of boiling water.

Sclerotized (hard-bodied) insects can be directly killed in 70–90 percent ethanol. Scaly insects such as Lepidoptera, Trichoptera and Culicidae should not be killed using liquid agents, but use freezing or dry killing jars.

Parasitic Hymenoptera are best killed and preserved in 96 percent ethanol. This high concentration prevents the membranous wings from becoming twisted and folded, hairs from matting and soft body parts from shrivelling.

Most specimens can be killed by placing in the freezer for a minimum of 30 min; larger insects will take longer to kill. This technique is used to immobilize active insects before killing with other methods.

Small insects for slide mounting are killed directly in clearing and staining fluids.

Insect specimens are also killed in ethyl acetate vapour (flammable and irritant chemical) in a killing jar.

Snails and slugs are killed by drowning in oxygen-depleted (boiled) water in a jar with tight-fitting lid, remove as much air as possible and leave for 24 h. Use narcotized water (tobacco-soaked) and do not boil the specimen as this makes it go hard.

Earthworms (Annelida: Oligochaeta) can be killed in 70 percent ethanol.

8.2.3.2 Dry mounting

The following references may be useful.

Walker, A.K. & Crosby, T.K. 1988. *The preparation and curation of insects*, new revised edn. New Zealand DSIR Information Series 163. Wellington, DSIR Science Information Publishing Centre. 92 pp. Available at <http://www.landcareresearch.co.nz/resources/collections/nzac/specimen-preparation-and-curation> (last accessed on 17 September 2015).

Schauff, M.E., ed. 2001. *Collecting and preserving insects and mites: techniques and tools*. Washington, DC, Systematic Entomology Laboratory. Available at <http://www.ars.usda.gov/News/docs.htm?docid=10141> (last accessed on 17 September 2015).

Hunter, G. 2006. *Curation of insect specimens*. Conserve O Gram No. 11/8. Washington, DC, National Park Service. Available at <http://www.nps.gov/museum/publications/consveogram/11-08.pdf> (last accessed on 17 September 2015).

8.2.3.3 Pinning

- ◆ Bees, wasps, flies, alate termites, moths and butterflies should all be pinned through the thorax. Bees, wasps and flies should all be pinned slightly to the right of the centre to avoid damaging delicate diagnostic characters.
- ◆ Bugs (Hemiptera) should be pinned through the scutellum.
- ◆ Grasshoppers (Orthoptera) should be pinned through the pronotum.
- ◆ Beetles (Coleoptera) should be pinned through the right elytron (Figure 34).

Pins must be stainless steel, otherwise they will corrode and ruin the specimen. Pins should be inserted into the body using appropriate pin size. Number 3 pins suitable for direct pinning of large insects should be used whenever possible. Number 5 pins are used for very large insects and number 0 pins for narrow-bodied insects. Anything smaller than that should be mounted on a micro pin (minuten pin) (see section 8.2.3.4 Double mounting).

Note: Numbers 00 and 000 pins are too springy and will risk damage to the specimen when handling.

Figure 34: Dorsal and lateral view of pinned beetle



8.2.3.4 Double mounting

Small insects pinned with micro pins should be double mounted on 3 mm square by 10–12 mm long Nu Poly Strips or plastazote strips on a number 3 insect pin (Figure 35).

8.2.3.5 Card pinning

All small (<5 mm), hard-bodied insects should be glued to triangular card points (Figure 36).

Beetles (Coleoptera) should be mounted so that the ventral side of the body is visible.

Flies, wasps and other insects in which wings are extended above the body should be mounted on their right side, preferably with wings oriented vertically.

Specimens should be orientated with the head on the right side of the card point. Only a minimum amount of glue is to be used.

Card points are attached to a number 3 insect pin.

8.2.3.6 Cardboard rectangles

Very small insects, especially elongate soft-bodied beetles, minute Hymenoptera and Coleoptera, may be glued to rectangular cards (Figure 37). This method is best used when there is more than one specimen from the same series, as each specimen can be glued at a different angle to aid future identification.

Puparia, pupal cases from specimen rearing are also mounted this way. Cards are attached to a number 3 insect pin.

Note: Mounting specimen on cardboard rectangles is not a preferred method.

Figure 35: Lateral view of double-mounted insect



Figure 36: Dorsal and lateral view of card-mounted insect

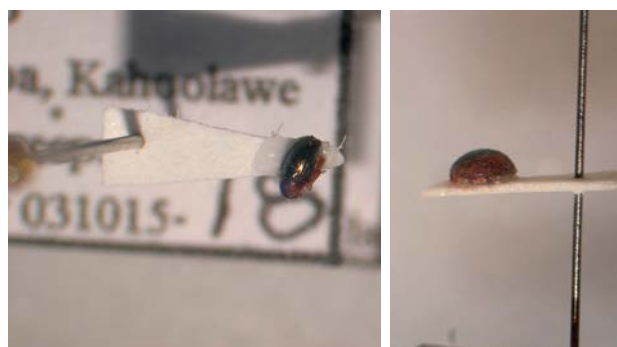


Figure 37: Insect on card rectangle



8.2.3.7 Spreading and setting

Butterfly and moth wings are spread to show the colour pattern (Figure 38). The rear margins of the forewings are set at right angles to the body. The hind wings should then be far enough forward so that there is no large gap between the front and hind wings. There should be no wrinkles in the hind wings. The wings are entirely covered with non-waxed paper, lightweight airmail paper or tracing paper.

An online tutorial is available: Science Learning, 2009. *Mounting moths*. Available at <http://www.sciencelearn.org.nz/Contexts/Hidden-Taonga/Sci-Media/Video/Mounting-moths> (last accessed on 17 September 2015).

8.2.3.8 Ethanol preserving

The following soft-bodied terrestrial or aquatic invertebrates, once killed or fixed should be stored in 70–75 percent ethanol:

- ◆ larval forms of most insect orders
- ◆ termites (Isoptera) – all stages
- ◆ silverfish (Thysanura)
- ◆ snails and slugs (except empty snail shells)
- ◆ worms, centipedes, millipedes, amphipods.

Specimens for molecular work should be placed in 96 percent ethanol immediately and the ethanol must be changed after several days to dehydrate the specimens for storage.

8.2.3.9 Slide mounting

Slide mounting should be used for the insect groups listed below. Methods of preparation for slide mounting vary for each group. Also see slide-making methods outlined in Dooley, J. 2002. *Specimen preparation*. San Francisco, CA, PPQ. Available at http://keys.lucidcentral.org/keys/v3/Dones_Lourdes/other_files/slide_prep_training.pdf (last accessed on 17 September 2015).

- ◆ Mites (Acari) (can be slide mounted directly into Hoyer's and cleared on a hotplate)
- ◆ Nematodes
- ◆ Thrips (Thysanoptera)
- ◆ Mealy bugs (Pseudococcidae)
- ◆ Scale insects (Coccoidea)
- ◆ Aphids (Aphidoidea)
- ◆ Other orders, larval skins as required
- ◆ Whitefly (Homoptera: Aleyrodidae)
- ◆ Dissected parts of insects – this technique requires specialized micro tools (Figure 39) and a certain amount of skill. The tools are relatively easy to make with micro pins heated over a flame and gently bent into required position with very fine forceps, and then inserted and glued into small bamboo skewers or wooden tooth picks.

Note: Mounting on slides is an excellent way to preserve and study small soft-bodied insects and genitalia.

Figure 38: Dorsal and lateral view of moth with wings spread



Subsequent reidentification labels

These must be added to the specimen without removing the original determination.

Note: Following reidentification, any filed records relating to the specimen may need to be changed.

Positioning of labels on pinned specimens

The locality label should be placed approximately 5 mm from the pinned specimen. Examples of labels are given below.

For pinned specimens (Figure 40A), the pinhole should be about in the middle of the label, avoiding any words. This method offers maximum protection to the brittle appendages of the specimen.

For directly pinned specimens, the label is centred under the specimen with the long axis of the label coinciding with the long axis of the specimen.

For card- and double-mounted specimens on pins (Figure 40B), insert the pin through the centre of the right side of the label, with long axis of the

label oriented in the same direction as the card point. The pinhole goes to the right of the words. This is less likely to interfere with writing while still offering some protection for the specimen.

Leave enough room between each label so the data can be read without having to slide or tilt the label. Tilting labels can make them loose, can swing on the pins and risk damaging neighbouring specimens.

When labels become loose, remove the label and close pinhole with a thumb nail, then repin by making a new pinhole.

Positioning of labels for specimens stored in vials

This includes labels for specimens stored in glass or plastic vials, dry or in ethanol. These labels are larger than pinned specimen labels (up to 15 mm × 40 mm), the locality and identification parts are on the same label, sitting along the length of the vial. Use only one label, which is to be placed inside the vial (Figure 41).

Locality data goes on the left side, identification data on the right.

Figure 40A: Directly pinned insect showing position of labels

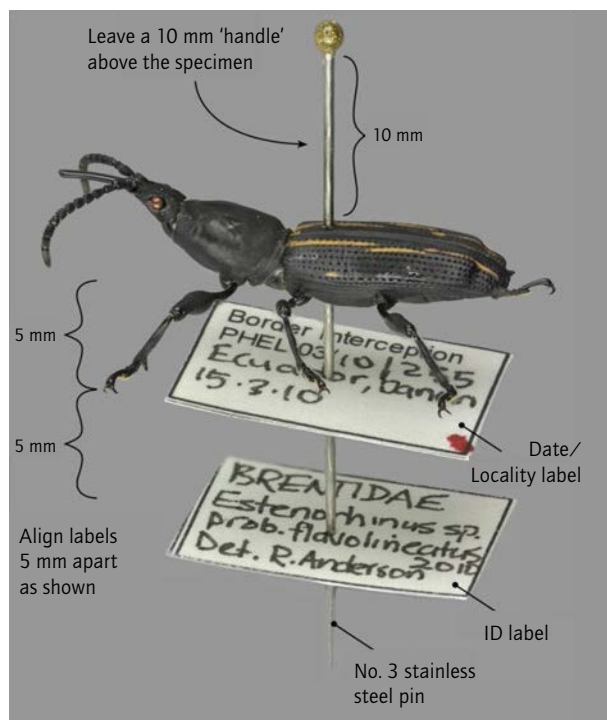


Figure 40B: Card-mounted specimen showing position of labels and pin

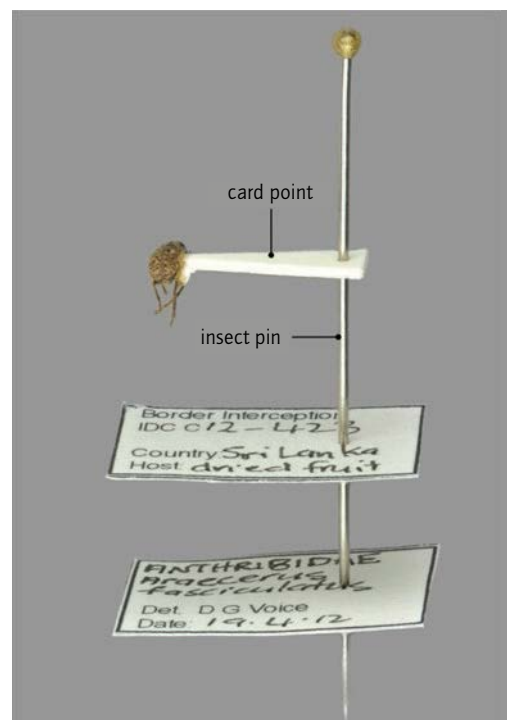
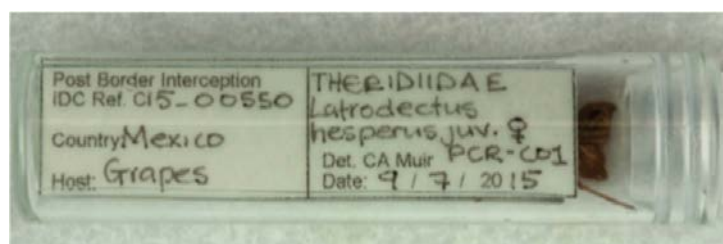


Figure 41: Example of a label for specimens stored in a vial



Roll the label around a pencil, words outermost, to curl it slightly, before placing it in the vial. This allows the label to stay against the glass so the words don't become obscured by floating specimens.

If it is necessary to insert a second label: to ensure both labels can be read, insert a plain piece of paper the width of the vial to separate them with the words of both labels facing outwards.

If specimens are stored in 96 percent ethanol for molecular work, this must be indicated on the label.

Positioning of labels for slide-mounted specimens

The locality label is placed on the right side when the specimen is facing head downwards.

The identification label is placed on the left side of the slide (Figure 42).

8.2.5 Relaxing dried specimens

Relaxing is not required for freshly killed specimens. Some specimens, however, die in transit to the lab and become dry and brittle; these need to be relaxed to stop appendages breaking off during pinning.

Simple relaxing procedure

- ◆ Cover the bottom of the relaxing chamber (air-tight container or desiccator) with warm water to about 1 cm, place a grille above the water and cover with filter paper or tissue.
- ◆ Add a few drops of clove oil or a disinfectant to prevent mould growth.
- ◆ Place specimens on filter paper in a shallow dish, such as a Petri dish or plastic lid.
- ◆ Close the relaxing chamber and leave overnight. Relaxing times differ for different insects and sizes.

Alternatively, for quick results place the relaxing chamber in the oven at 40 °C, the specimens should be relaxed enough to handle in a few hours.

Ensure that labels in the relaxing chamber are not written in water-soluble ink.

8.2.6 Drying specimens

Specimens for dry storage, including snail shells, must be dried in suitable containers in the oven at 40 °C, or air-dried in a secure container at room temperature, before being stored in the collections. No dried specimens from the collection should

Figure 42: Labelling of a slide-mounted specimen



be left out in the laboratory overnight unless in a covered container.

Drying ovens in entomology laboratories are kept at an optimum temperature of 40 °C for drying entomological specimens. Generally, specimens may remain in the drying oven until they are placed in permanent storage.

Pinned specimens can be placed in unit trays in the oven or in store boxes for drying. The length of time for drying depends on the size and colour of a specimen, from a few hours or overnight (e.g. small Diptera) to one week (e.g. large black Coleoptera).

Slide-mounted material should be dried in the entomology oven for at least 4 weeks. Different media and different amounts of media may take longer to dry completely. Slide-mounted specimens are placed in aluminium slide trays in the oven for drying.

8.2.7 Dissections

Dissection is a skilled task and requires practice. Any part of the insect's body can be dissected and slide mounted for study in Hoyer's. However, once a three-dimensional appendage is placed under a cover-slip distortion can occur, so placing in a staining well with sand and 95 percent ethanol enables examination at different angles. The appendages need to be stored in 80 percent ethanol with 5 percent glycerol added. Very small appendages can be slide mounted on a cavity slide.

8.2.7.1 Genitalia

- ◆ Remove abdomen and elytra in 70 percent ethanol; if insect is freshly killed placing in ethanol is not necessary, heat in 10 percent KOH for up to 1 h.
- ◆ Carefully remove genitalia together with last modified abdominal segment in distilled water.
- ◆ Transfer to 90 percent ethanol or, after making observations, mount into Hoyer's medium straight from distilled water – be aware that distortion may occur.

Alternatively, temporarily slide-mount in glycerine for examination, or examine in water in a watch glass.

Genitalia and macerated parts are best preserved in glycerine in a microvial pinned with the specimen (on the same pin).

Lepidoptera genitalia dissection and preparation references

- Hayden, J.E., Lee, S., Passoa, S.C., Young, J., Landry, J.-F., Nazari, V., Mally, R., Somma, L.A. & Ahlmark, K.M.** 2013. Digital identification of microlepidoptera on *Solanaceae*. Fort Collins, CO, USDA-APHIS-PPQ Identification Technology Program (ITP). Available at <http://idtools.org/id/leps/micro/> (last accessed on 17 September 2015).
- Hoare, R.J.B.** 2000. A new genus of primitive Nepticulidae (Lepidoptera) from eastern Australia, with a revised diagnosis of nepticulid subfamilies. *Zoological Journal of the Linnean Society*, 128(3): 289–317. (p. 292).

8.2.7.2 Diptera larvae and other insect parts

- ◆ Place the larva in water in a dissecting dish and cut the cuticle with fine dissecting scissors along one side, starting close to the anterior end, passing below the lateral spiracle and continuing almost to the posterior end.
- ◆ Place the larva in Essig's/Wilkey's solution and heat gently for 1 h. When the larva is well macerated, remove the body contents.
- ◆ Almost separate the posterior spiracular area from the remainder of the skin and pull the cephalopharyngeal skeleton a short way out of the body. Place the skin in 95 percent ethanol.
- ◆ Slide mount, in Hoyer's solution, with the skin opened outward so that the cephalopharyngeal skeleton, with the mouth hooks, lies away from the skin and the posterior spiracular area lies with both spiracles upward.

For some other orders, the head of the specimen is mounted carefully on slides so that mouthparts can be examined.

Other parts of the insect body, such as antennae, legs and palps, may be slide mounted straight into Hoyer's solution.

8.2.7.3 *Stuffing of Orthoptera, Cicadidae and Phasmatodea*

Large grasshoppers, large Hemiptera (e.g. Cicadidae) and other orthopteran insects may need to be dissected for permanent dry storage. Using dissecting scissors, a cut is made underneath the abdomen, the gut contents are removed with forceps, the cavity swabbed out with ethanol-soaked cotton wool then clean cotton wool is inserted to fill out the space. The cut edges can be brought together and glued in place before the specimen is oven dried.

Phasmatodea may need to have the gut contents removed and a nylon bristle inserted from the end of the abdomen to keep the abdomen rigid before being oven dried.

8.2.7.4 *Lepidoptera wing preparation procedure*

- ◆ Carefully cut wings at basal attachment of specimen.
- ◆ Bleach Lepidoptera wings by immersing in ordinary household or laundry bleach (5 percent sodium hypochlorite solution) for 1–3 min. Wetting them first with ethanol will activate the bleach. *Note:* the bleaching process should be watched carefully under the microscope as wings may be damaged if left in the chemical too long.
- ◆ As soon as the veins become visible, remove from the bleach and wash in plain water.
- ◆ Remove Lepidoptera scales in the water by brushing the wings carefully with a fine brush.
- ◆ De-scaled wing may then be stained with acid fuchsin solution, if desired.
- ◆ Wash in 95 percent ethanol and slide mount in glycerine or Hoyer's solution.
- ◆ To make permanent mount, place wings in clove oil for 5–10 min before mounting in Euparal or Canada balsam.
- ◆ Position the wing as desired, turning it over if necessary and making sure that its basal part is well stretched out and all the veins are visible.

8.2.8 *Slide mounting procedure for small arthropods*

Slide preparation of all specimens for the reference collection needs to follow the permanent mounting procedure. A temporary mounting procedure is

also provided below, which may be used for quick identification and short-term storage, and allows reprocessing for making permanent slides.

8.2.8.1 *Procedure for Collembola, aphids and immature Hemiptera (plant bugs)*

- ◆ Place specimens in Essig's/Wilkey's solution in a staining well.
- ◆ Cover with glass lid and label with a unique identification number.
- ◆ Heat until clear (30 min to 1 h) on a hotplate or under a light bulb in the fume hood.
- ◆ If required, add 3 or 4 drops of acid fuchsin staining solution.
- ◆ Tease out body contents and repeat, if necessary, using new Essig's/Wilkey's solution.

For permanent mounts

- ◆ Transfer to 25 percent ethanol for 5–10 min.
- ◆ 50 percent ethanol for 5–10 min.
- ◆ 75 percent ethanol for 5–10 min.
- ◆ 100 percent ethanol for 5–10 min.
- ◆ Clove oil for 5–10 min.
- ◆ Finally, mount directly into Canada balsam.

For temporary mounts

Mount directly into Hoyer's solution mounting medium (Collembola can also be cleared in lactic acid in a 40 °C oven).

8.2.8.2 *Thrips (Thysanoptera)*

Permanent mounting procedure

- ◆ Place in 10 percent KOH or in Essig's/Wilkey's for a variable period, depending on the colour of the specimen:
 - black or dark brown forms – several hours
 - pale forms – 2 h.
- ◆ Transfer to water (at least 1h, can be left for several days).
- ◆ Transfer to 30 percent ethanol (1 h, can be left for 2–3 days).
- ◆ Transfer to 70 percent ethanol (1 h, can be left for 2–3 days).
- ◆ Transfer to 95 percent ethanol (1 h, can be left for 2–3 days).
- ◆ Transfer to absolute ethanol (1 h, can be left for 2–3 days).

- ◆ Transfer to clove oil (at least 1 h, can be left for 2–3 days).
- ◆ Slide mount in Canada balsam, usually one thrips per slide, with the head of the specimen facing downwards.

Temporary mounting procedure

- ◆ Place thrips in Essig's/Wilkey's in watch-glass well for a variable period, depending on the colour of the specimen.
- ◆ Cover with glass lid, label and place under lamp for clearing.
- ◆ Upon clearing (approx. 1 h depending on the colour of the specimen), tease out internal parts; wash the specimen in 70–96 percent ethanol.
- ◆ Slide mount in Hoyer's mounting medium, usually one thrips per slide, dorsal side up, head towards operator.
- ◆ Label the slide with the head of the specimen facing downwards.

Reference: Thrips Wiki. 2013. Collecting and preparing thrips for study. http://thrips.info/wiki/Collecting_and_preparing_thrips_for_study (last accessed on 22 September 2015).

8.2.8.3 Temporary slide-mounting procedure for armoured and soft scales (Hemiptera: Diaspididae and Coccidae)

The aim of the preparation is to eliminate both the external secretion covering the body and the internal organs, without damaging the external diagnostic structures of the body.

- ◆ Remove specimens from the vials and plant substrate under binocular microscope and place in a staining well containing Essig's/Wilkey's solution.
- ◆ Gently remove scales from the cover (tests), puncture venter mid-thorax and add a few drops of acid fuchsin to stain.
- ◆ Cover with glass lid, label and place under lamp for clearing. Specimens may be pumped and teased lightly during this step to aid in the clearing process. Fresh specimens are usually cleared adequately in 1–2 h.
- ◆ Remove to fresh Essig's/Wilkey's solution, tease out internal parts and then wash the specimen in 75 percent ethanol.

- ◆ Slide mount ventral side up with the pygidium facing upwards in Hoyer's mounting medium.
- ◆ Label the slide.

8.2.8.4 Temporary slide-mounting procedure for mealybugs (Hemiptera: Pseudococcidae) and giant scales (Hemiptera: Monophlebidae)

The aim of this preparation is to eliminate both the external secretion covering the body and the internal organs, without damaging the external diagnostic structures of the body.

- ◆ Remove specimens from the plant substrate or vial under binocular microscope and place in a staining well containing Essig's/Wilkey's solution.
- ◆ Make a small incision, with a fine needle, on the dorsal side of the mealybug or scale between the hind coxae.
- ◆ Place a few drops of chloroform to remove body waxes and a few drops of acid fuchsin to stain.
- ◆ Cover staining well with glass lid, label and place under lamp for clearing. Specimens may be pumped and teased lightly during this step to aid in the clearing process. Fresh specimens are usually cleared adequately in 1–2 h.
- ◆ Remove to fresh Essig's/Wilkey's solution, tease out internal parts and then wash the specimen in 75 percent ethanol.
- ◆ Slide mount ventral side up with the head facing downwards in Hoyer's mounting medium.
- ◆ Label the slide.

8.2.8.5 Permanent mounting procedure using microwave for mealybugs and scale insects (Hemiptera: Coccoidea)

- ◆ Remove scales or mealybugs from plant material into a dry staining well. At this stage observations can be made – parasitism, whether live or dead, counts, tentative identification, etc.
- ◆ Drip 95 percent ethanol onto mealybugs to penetrate wax.
- ◆ Puncture venter mid-thorax – a minuten pin in a holder is ideal for this.
- ◆ Transfer insects to Essig's/Wilkey's solution fluid with one or two drops of acid fuchsin solution added; use 1 ml of fluid in a 5 ml vial.

Sit the lid loosely on top – do not screw it down. The lid must have 3–4 ventilation holes as the contents will boil up the vial (a sealed vial might explode).

- ◆ Place in microwave oven for 1 min. *Note:* Times and power level will depend on the power (wattage) of the microwave used. Phenolic compounds will boil off, so use a fume cabinet.
- ◆ As soon as the vial is cold enough to handle, pour fluid into a staining well. Insects should have cleared – if not, gently pump the insect to aid clearing, then return to vial and microwave a further minute.
- ◆ Transfer insects to 95 percent ethanol. Use flat-nosed forceps to gently dorsoventrally flatten them while expelling remaining contents. Insects could now be mounted in Hoyer's medium.
- ◆ Transfer immediately to clove oil in a staining well. Cover the well and microwave for 1 min. *Note:* Times and power level will depend on the power (wattage) of the microwave used. The oil doesn't boil, so a vial is not used. The oil dissolves traces of fat and wax which otherwise cloud Canada balsam mounts. In some insects, e.g. very fatty mealybugs or monophlebids, it may be necessary to repeat this step in fresh clove oil if the clove oil has a thickened cloudy appearance after microwaving.
- ◆ Transfer directly to Canada balsam slide mounts.

Reference: The slide-mounting procedure is modified from **Lo, P.L. & Blank, R.H.** 1989. A survey of armoured scale species (Hemiptera: Diaspididae) in kiwifruit orchards. *New Zealand Entomologist*, 12: 1–4.

8.2.8.6 Mites (Acari)

Well-sclerotized mites should be cleared in lactic acid prior to slide mounting. Avoid excessive clearing in lactic acid, as this can over-clear the mites and make them difficult to manipulate.

To pick mites from plant substrate, dampen a needle with lactic acid, Hoyer's or use a fine artist's paint brush.

- ◆ Place them in a staining well or cavity slide with lactic acid and heat under lamp, on a hotplate or in an oven at 70 °C.

- ◆ Slide mount cleared specimen in Hoyer's.

Lightly sclerotized and non-sclerotized mites can be slide mounted directly into Hoyer's. Place slide on a hotplate for clearing at 70 °C. Specimens are usually cleared for identification within the hour.

Reference: **Dooley, J.** 2002. Specimen preparation. San Francisco, CA: PPA. Available at http://keys.lucidcentral.org/keys/v3/whitefly/PDF_PwP%20ETC/Slide%20Prep%20Training.pdf (last accessed on 22 September 2015).

8.2.8.7 Immature whiteflies and psyllids (Hemiptera, Psylloidea: Aleyrodidae) (puparia)

- ◆ Collect specimens in 75 percent ethanol.
- ◆ Move specimens to 10 percent KOH and clear for several hours *at room temperature*. (*Note:* never heat whitefly specimens in KOH because this affects chitin and diminishes its ability to hold stain.) Do not place black specimens in KOH, as it wrinkles their skin.
- ◆ Soak in 75 percent ethanol (incisions are not necessary), then in bleach solution of 50:50 hydrogen peroxide and ammonium hydroxide.
- ◆ Make sure the specimens sink into the solution. Monitor every 15 min.
- ◆ As soon as the specimens turn uniformly brown, transfer to 75 percent ethanol to wash and neutralize KOH.
- ◆ Transfer to Essig's/Wilkey's solution with acid fuchsin stain.
- ◆ Transfer to clove oil. *Never* try to pump or tease out body contents of nymphs as setae are very fine and easily damaged. Leave for 5–10 min until clear.
- ◆ Place a tiny drop on the slide, spread out to cover cover-slip area.
- ◆ Align specimens; let the Canada balsam become a bit tacky.
- ◆ Place the cover-slip gently over the balsam and specimens.
- ◆ If balsam doesn't cover the cover-slip area, you can run in more.

8.2.8.8 Adult whitefly

Temporary mounting procedure

- ◆ Place the specimens into 70 percent ethanol.

- ◆ Place specimen onto slide with Hoyer's mounting medium. The specimens should be positioned on the slide dorsum up or in lateral aspect. The lateral aspect is more difficult to obtain but the necessary structures are more easily discerned.
- ◆ Heat slide at 40–60 °C for several hours.

Permanent mounting procedure

- ◆ Place specimens in 70 percent ethanol (incisions are not necessary and should not be made).
- ◆ Use a spatula wide enough to support most of the body parts and wings when making transfers from one reagent to another.
- ◆ Move specimens into 10 percent KOH and clear for several hours at room temperature. Overnight is most desirable unless a rapid determination is required. *Note: Never heat specimens in KOH!*
- ◆ Transfer to ethanol to wash and neutralize KOH.
- ◆ Transfer to Essig's/Wilkey's solution with stain (legs down, dorsum up; it is not necessary to submerge the whole specimen, otherwise the wings will almost certainly become entangled with the rest of the body); heat at 50 °C (do *not* try teasing out body contents as whitefly become very sticky in heated Essig's/Wilkey's solution).
- ◆ Transfer to clove oil and tease out internal body contents with a very fine bent needle (00 or 0 insect pin); return to Essig's/Wilkey's solution (stained or unstained) if necessary and heat. Retransfer to clove oil if the specimens have been reheated in Essig's/Wilkey's solution.
- ◆ Mount specimens on a slide with a propped cover-slip (props should be vinyl props or short lengths of monofilament fishing leader of about 0.25 mm diameter).

Note: It is important that the specimens be placed in very thin, runny Canada balsam, diluted with xylene so that it drips or runs, otherwise the antennae and often the legs will collapse and be worthless for identification purposes. Thick balsam cannot penetrate the small openings in these structures fast enough. Thicker balsam can be added before the cover-slip is applied and is probably a necessity in propped slides anyway as

the xylene will evaporate, leaving an inadequate amount of balsam to cover the specimens.

- ◆ Specimens should be positioned on the slide dorsum up or in lateral aspect. The lateral aspect is more difficult to obtain but the necessary structures are more easily discerned.

Reference: This procedure has been taken from a provisional key to adult whiteflies of California by R.J. Gill (1989, unpublished).

8.2.9 References for preparation and mounting techniques

The authors recommend the following literature regarding preparation and mounting techniques. These references stipulate methods for preparing various groups.

General

Naumann, D.I., ed. 1991. *The insects of Australia: a textbook for students and research workers* (Parts 1 & 2). Melbourne, Melbourne University Press. 1029 pp.

Walker, A.K. & Crosby, T.K. 1988. *The preparation and curation of insects*, new revised edn. New Zealand DSIR Information Series 163. Wellington, DSIR Science Information Publishing Centre. 92 pp. Available at <http://www.landcareresearch.co.nz/resources/collections/nzac/specimen-preparation-and-curation> (last accessed on 17 September 2015).

Stehr, F.W., ed. 1987. *Immature insects*. Dubuque, IA, Kendall/Hunt Publishing. 754 pp. (Vol. 1, pp. 9–17).

Acari

Krantz, G.W. & Walter, D.E. 2009. *A manual of acarology*, 3rd edn. Lubbock, TX, Texas Tech University Press. 816 pp. (pp. 83–96).

Jeppson, L.R., Keifer, H.H. & Baker, E.W. 1975. *Mites injurious to economic plants*. Berkeley, CA, University of California Press. 614 pp. (pp. 385–392).

Araneae

Ubick, D., Paquin, P., Cushing, P.E. & Roth, V., eds. 2005. *The spider genera of North America: an identification manual*. The American Arachnological Society. (pp. 8–9).

Coleoptera

Booth, R.G., Cox, M.L. & Madge, R.B. 1990. *IIE guides to insects of importance to man*. Vol. 3. *Coleoptera*. Oxford, Oxford University Press. 392 pp. (Adults, pp. 10–12; larvae, pp. 205–208).

Diptera

Ferrar, P. 1987. *A guide to the breeding habits and immature stages of Diptera Cyclorrhapha*. Part 1. *Text*; Part 2. *Figures*. Leiden, E.J. Brill; Copenhagen, Scandinavian Science Press. 907 pp. (pp. 10–12).

White, I. & Elson-Harris, M.M. 1992. *Fruit flies of economic significance: their identification and bionomics*. Wallingford, UK, CAB International. 600 pp. (pp. 24–29).

Drew, R.A.I., Hooper, G.H.S. & Bateman, M.A. 1978. *Economic fruit flies of south Pacific region*. Queensland, Department of Primary Industries; Canberra, Department of Health. 137 pp. (pp. 2–4).

Foote, R.H., Blanc, F.L. & Norrbom, A.L. 1993. *Handbook of the fruit flies (Diptera: Tephritidae) of America north of Mexico*. Ithaca, NY, Comstock. 576 pp. (pp. 37–41).

Heteroptera

Slater, J.A. & Baranowski, R.M. 1978. *How to know the true bugs (Hemiptera–Heteroptera)*. Dubuque, IA, W.C. Brown. (pp. 12–15).

Aleyrodidae:

Martin, J.H. 1999. *The whitefly fauna of Australia (Sternorrhyncha: Aleyrodidae): a taxonomic account and identification guide*. Canberra, Commonwealth Scientific and Industrial Research Organisation. (pp. 124–125).

Aphididae:

Blackman, R.L. & Eastop, V.F. 2000. *Aphids on the worlds crops: an identification and information guide*, 2nd edn. UK, Wiley. 250 pp. (pp. 363–365).

Coccidae:

Gill, R.J. 1988. *The scale insects of California*. Part 1. *The soft scales (Homoptera: Coccoidea: Coccidae)*. Technical Series in Agricultural Biosystematics and Plant Pathology No. 1. Sacramento, California Department of Food and Agriculture. 132 pp. (pp. 126–127).

Coccoidea:

Gill, R.J. 1993. *The scale insects of California*. Part 2. *The minor families (Homoptera: Coccoidea)*. Sacramento, California Department of Food and Agriculture. 241 pp.

Diaspididae:

Gill, R.J. 1997. *The scale insects of California*. Part 3. *The armored scales (Homoptera: Diaspididae)*. Technical Series in Agricultural Biosystematics and Plant Pathology No. 3. Sacramento, California Department of Food and Agriculture. 307 pp.

Williams, D.J. & Watson, G.W. 1988. *The scale insects of the tropical south Pacific region*. Part 1. *The armoured scales (Diaspididae)*. Wallingford, UK, CAB International Institute of Entomology. (p. 13).

Pseudococcidae:

Williams, D.J. & de Willink, M.C.G. 1992. *Mealybugs of Central and South America*. Wallingford, UK, CAB International. 635 pp. (p. 22).

Williams, D.J. & Watson, G.W. 1988. *The scale insects of tropical south Pacific region*. Part 2. *Mealybugs (Pseudococcidae)*. CAB International Institute of Entomology. 260 pp. (p. 11).

Lepidoptera

Holloway, J.D., Bradley, J.D. & Betts, C.R., eds. 1987. *CIE guides to insects of importance to man*. Vol. 1. *Lepidoptera*. Wallingford, UK, CAB International Institute of Entomology. (pp. 11–14).

Thysanoptera

Palmer, J.M., Mound, L.A. & du Heume, G.J. 1989. *CIE guides to insects of importance to man*. Vol. 2. *Thysanoptera*. Wallingford, UK, CAB International Institute of Entomology; London, British Museum of Natural History. 73 pp. (pp. 3–5).

Thrips Wiki. 2013. Collecting and preparing thrips for study. Available at http://thrips.info/wiki/Collecting_and_preparing_thrips_for_study (last accessed on 22 September 2015).

8.3 Nematology reference collection

8.3.1 Equipment and reagents

- ◆ desiccant (e.g. silica gel, calcium chloride, 95 percent ethanol)
- ◆ ethanol
- ◆ formalin
- ◆ glycerol
- ◆ parafilm
- ◆ desiccator
- ◆ dissecting needle
- ◆ glass lid to cover watch glass
- ◆ heater
- ◆ microscope slide
- ◆ microscope cover-slip
- ◆ pipette
- ◆ small glass beads, fine wire or wax to support cover-slips
- ◆ vial
- ◆ watch glass.

8.3.2 Nematode permanent mounting procedure

- ◆ Extract different life stages of nematodes as described in Chapter 5.
- ◆ Transfer live specimens to a watch glass.
- ◆ Reduce water in the watch glass to 1 ml.
- ◆ Add 2 ml of 3 percent hot formalin to the watch glass and leave to harden for at least 2 weeks.

Note: Do not use fixatives that contain ethanol because this causes distortion.

8.3.2.1 Permanent slides

- ◆ After killing and fixation, transfer specimens from fixative to 1 ml Seinhorst I solution (20 parts 95 percent ethanol, 1 part glycerol and 79 parts water) in a watch glass.
- ◆ Place the watch glass with specimens in a desiccator containing desiccant for 2–3 days.
- ◆ Add at least twice the volume of Seinhorst II solution (5 parts glycerol and 95 parts 95 percent ethanol) to the existing solution in the watch glass.
- ◆ Cover the watch glass with a glass lid, leaving a small gap of about 1–2 mm wide.
- ◆ Keep specimens at room temperature to allow slow evaporation of alcohol for 2 weeks.

- ◆ Transfer specimens with a dissecting needle (alternatively mount eyelash or feather on penholders) to a glass slide with a drop of glycerol on a microscope slide.
- ◆ Arrange nematodes if required.
- ◆ Add small glass beads, fine wire or wax to support cover-slips for large nematodes.
- ◆ Place a cover-slip gently and seal it with nail varnish.

8.3.2.2 Unmounted specimens

- ◆ After killing and fixation, transfer specimens into a vial.
- ◆ Add 3 percent formalin with 2 percent glycerol to prevent deterioration of specimens if the preservative evaporates.
- ◆ Seal the vial with parafilm and label.

8.4 Plant pathogen reference collection and herbarium

A plant pathogen reference collection and herbarium may contain any of the following types of specimens:

- ◆ fungi
- ◆ invasive plants
- ◆ parasitic plants
- ◆ plant parts affected by pathogens (e.g. bacteria, fungi, nematodes, viruses, viroids, phytoplasmas, *Liberibacter*)
- ◆ plant parts affected by abiotic factors.

A good plant pathogens reference collection and herbarium (in addition to those listed in the introduction) should keep:

- ◆ specimens with adequate amount of material
- ◆ specimens with different plant parts with different stages of symptoms
- ◆ specimens with different morphological states for fungi (e.g. anamorph and teleomorph, or aecial, pycnidial, uredinial and telial states)
- ◆ specimens from different countries
- ◆ specimens from different administrative regions
- ◆ multiple specimens to include variation between populations
- ◆ additional information, e.g. pictures of symptoms in the field and pictures of specimens prior to drying.

Note: Specimens can be mounted on microscope slides and curated in herbaria.

8.4.1 Method selection

Most plant specimens, including those infected with pathogens, can be preserved by air drying, drying with desiccants, pressing, freeze-drying, freezing and laminating. Very fleshy and delicate specimens can be preserved by pickling to maintain the shape of the specimen. The advantages and disadvantages of each of these methods are summarized in Table 3.

8.4.2 Sample requirements

Samples should be processed as soon as possible. If not, label the samples and keep them in the appropriate conditions, in a fridge or in a cool shaded place. *Note:* Some tropical plants turn dark brown when kept in a fridge.

Samples should contain adequate material in good condition for preservation. Images of samples should be taken before processing, if required. *Note:* Drying will change the colour of disease symptoms and shape of fleshy plant parts.

It is recommended that plant parts be selected with different stages of symptoms or different states of pathogens. Plant parts should be cut to fit containers. Bulky material can be cut in half or quarters, or sliced to facilitate drying.

Disposable gloves should be worn and bench surfaces and tools should be wiped clean with disinfectant. This is particularly important when handling material infected with diseases that can cause adverse effects.

Table 3: Advantages, disadvantages and types of specimens suitable for each method of preservation in herbarium

Method of preservation	Types of specimen	Advantages	Disadvantages
Air drying	Dry and less fleshy specimens	Low set-up and running costs. Symptoms are well preserved	Nucleic acids are poorly preserved
Drying with desiccants	Dry and less fleshy specimens	Low set-up and running costs. Nucleic acids are fairly well preserved	May need to replace desiccants. Symptoms are not well preserved
Press drying	Dry, less fleshy and less bulky specimens	Low set-up and running costs. Symptoms are well preserved	Nucleic acids are poorly preserved
Freeze-drying	Most specimens are suitable	Nucleic acids are well preserved	Freezer-drying ampoules limit the size of specimens. Set-up and running are more costly. Symptoms are not well preserved
Freezing	All types of specimens are suitable	Nucleic acids are well preserved	Freezer space can be limited. Set-up and running are more costly. Nucleic acids may degrade on multiple freeze-thaw processes. Symptoms are not well preserved
Laminating	Dry and relatively flat specimens	Low set-up and running costs. Fast and easy to prepare. Symptoms are well preserved	Difficult to examine specimens with hand lens or microscope. Difficult to extract specimens for additional testing. Nucleic acids are poorly preserved
Pickling	Very fleshy or delicate specimens	Low set-up and running costs. Symptoms are well preserved	Nucleic acids are poorly preserved in some preservatives

8.4.2.1 Preserving samples infected with viruses, viroids and non-culturable bacteria-like organisms such as phytoplasmas and *Liberibacter*

- ◆ Wherever possible, the original plant tissue should be stored (e.g. plant tissue infected with a virus).
- ◆ For mechanically transmissible viruses and viroids, infected herbaceous plants or infected grafted plants could be stored instead of, or in addition to, the original plant tissue.
- ◆ For all viruses, viroids and non-culturable micro-organisms (e.g. phytoplasmas, *Liberibacter*), infected grafted plants could be stored as well.
- ◆ Most viruses, viroids and non-culturable micro-organisms are well preserved. However, some of these organisms may be degraded during this process due to their unstable nature – e.g. plant tissues stored in the freezer degrade over time following multiple cycles of thawing and freezing.
- ◆ The best method of preservation is to freeze-dry plant tissue; however, drying plant tissue on desiccants (e.g. calcium chloride) is also a good method. Frozen plant tissue is also good, as long as the material is not subject to thawing and freezing cycles too many times.
- ◆ Non-culturable micro-organisms (e.g. phytoplasmas, *Liberibacter*) can only be retained in their living plants for biological work (e.g. graft or insect transmission).

8.4.3 Methods of preservation

General equipment, consumables and reagents:

- ◆ stationery
- ◆ label paper
- ◆ tissue paper
- ◆ dissecting kit (forceps, scalpels, scissors)
- ◆ chopping board
- ◆ specimen containers (e.g. large plastic bags, paper envelopes, vials)
- ◆ disinfectants: ethanol, isopropyl alcohol or commercial disinfectant (e.g. Trigene, Virkon)
- ◆ personal protection equipment (e.g. laboratory coats, disposable gloves, masks, eye goggles)
- ◆ camera, photographic stage and scanner
- ◆ computer and printer
- ◆ fridge.

8.4.3.1 Air drying

Specimens can be dried using a food dehydrator, vented oven or simply a box or cupboard with a fan inside the box to maintain air circulation to facilitate drying.

Note: Some plant pathogen reference collections and herbaria use microwaves to dry specimens; however, this is not recommended because some plant parts may explode and the heating process damages DNA of the specimen, which could be useful for molecular work in the future.

Specimen containers can be tailor-made herbarium envelopes, postal envelopes, airtight plastic boxes or resealable plastic bags.

Procedure to air dry specimens

- ◆ Spread specimens on trays to facilitate drying.
- ◆ Put trays into the dryer and turn on the dryer.
- ◆ Collect specimens when they are thoroughly dried.
- ◆ Label containers for each specimen with a unique specimen number.
- ◆ Place dried specimens into labelled containers.
- ◆ Freeze containers with specimens at -18°C for 2 days to kill herbarium pests. *Note:* put paper containers (e.g. herbarium envelopes, envelopes) in resealable plastic bags before freezing to avoid build up of water condensation.
- ◆ Wipe any water condensation from the surface of containers.
- ◆ Store specimen containers in storage system.
- ◆ Update the herbarium database.

8.4.3.2 Pressing

List of equipment, consumables and reagents:

- ◆ newspaper or blotting paper
- ◆ wooden board (commercially produced plant presses are available)
- ◆ straps
- ◆ weight
- ◆ paper folder
- ◆ strings (e.g. dental floss, cotton thread)
- ◆ tapes (use high-quality tapes to avoid them detaching from the specimen after a few years).

Procedure to press dry specimens

- ◆ Lay and arrange specimens between sheets of newspapers (or blotting paper). *Note:* Bend long stems into V or W shape.
- ◆ Write specimen number on a piece of paper and place it with the specimen.
- ◆ Add extra newspapers between specimens to absorb moisture.
- ◆ Sandwich the stack of newspapers with specimens between two pieces of wooden board.
- ◆ Tie straps around the stack and tighten the straps.
- ◆ Place heavy weight on top of the stack to apply additional pressure.
- ◆ Keep the stack in a warm and dry atmosphere.
- ◆ Position specimens properly after a few hours or a day of pressing.
- ◆ Change the newspaper daily for the first few days and then less frequently, depending on the conditions of the specimens and relative humidity. *Note:* Some plant specimens turn dark brown if the newspapers were not changed frequently to dry specimens quickly enough. Used newspapers should be discarded or thoroughly dried before reuse.
- ◆ Examine the specimens regularly to prevent mould.
- ◆ Collect samples that are thoroughly dried.
- ◆ Label paper folders with specimen number.
- ◆ Lay and fix specimens onto paper folders with strings and tapes.
- ◆ Update the plant pathogen reference collection and herbarium database.

8.4.3.3 Drying with desiccants**List of equipment, consumables and reagents:**

- ◆ desiccant products, e.g. calcium chloride or hydrosorbant silica gel beads (*note:* refer to country guidelines for appropriate disposal of these toxic chemicals)
- ◆ storage containers with screw caps (plastic or glass)
- ◆ cotton wool or paper towel (for keeping calcium chloride away from plant tissue).

Container preparation

- ◆ Add desiccant at the bottom of the plastic container. *Note:* wear gloves, face mask and goggles when handling this chemical.
- ◆ Insert cotton wool or paper towel firmly at the bottom of the container to create a barrier between the calcium chloride and plant tissue.
- ◆ Insert a label containing at least a unique identification number.

Sample preparation

- ◆ Select plant tissue for preservation. This should be as fresh as possible and contain the symptoms. *Note:* Dry or decayed tissue must be avoided. Wear gloves when handling infected plant materials. Keep samples on ice at all times.
- ◆ Remove any dampness by gently drying the surface with a paper towel.
- ◆ Cut the plant tissue into pieces that fit the container using a scalpel blade or razor blade on a clean and disinfected chopping board. *Note:* Between plant material infected with different organisms, change gloves, dispose of disposable razor blade, disinfect chopping board and blade with disinfectant such as solution of 70 percent ethanol, Virkon or bleach, or wipes such as Isowipes, Mediowipes, Trigene or V-wipes.

Sample drying and storage

- ◆ Place plant tissue pieces in the plastic container.
- ◆ Close the plastic container tightly.
- ◆ Label container using a sticker written with permanent ink or pencil or directly write on the container with permanent ink. *Note:* Minimum information to be put on the label is the scientific name of the plant material and a unique identification number.
- ◆ Store at room temperature in a dry cool place.
- ◆ Update herbarium database.

8.4.3.4 Freeze-drying**List of equipment, consumables and reagents:**

- ◆ 4 °C fridge or cool-store
- ◆ freeze-drier
- ◆ vials, rubber seals and screw caps for freeze-drier
- ◆ hydrosorbent silica gel beads

- ◆ cotton wool or paper towel
- ◆ scalpel blades or razor blades
- ◆ disposable gloves
- ◆ chopping board
- ◆ disinfectant (e.g. 70 percent ethanol; wipes, e.g. Trigene)
- ◆ paper labels (to be inserted inside the vials)
- ◆ sticky labels (to stick on the cap of the vials)
- ◆ markers, pen
- ◆ computer database or recording paper sheet.

Vial preparation

- ◆ Pour a few beads of hydrosorbent silica gel into each vial (3–5 vials per sample).
- ◆ Insert cotton wool or paper towel firmly into the bottom of the vial to create a barrier between the silica gel beads and the sample.
- ◆ Insert a label containing at least a unique identification number.

Sample preparation

- ◆ Select plant tissue for preservation. It should be as fresh as possible and symptomatic. *Note:* Dry or decayed tissue must be avoided. Wear gloves when handling infected plant materials. Keep samples on ice at all times.
- ◆ Cut plant tissue into small pieces using a scalpel blade or razor blade on a clean and disinfected chopping board. *Note:* Between plant material infected with different organisms, change gloves, dispose of disposable razor blade, disinfect chopping board and scalpel blade with disinfectant such as solution of 70 percent ethanol, Virkon or bleach, or wipes of Isowipes, Mediwipes, Trigene or V-wipes.
- ◆ Transfer approximately 0.5 g chopped plant tissue into a prepared glass vial with label.

Sample drying and storage

- ◆ Place all vials onto the freeze-dryer metal rack.
- ◆ Put rubber seal loosely on the mouth of the vial.
- ◆ Put the rack with prepared samples into the chamber of the freeze-drier.
- ◆ Run the freeze-drier overnight. *Note:* The colour of silica gel should be dark blue when sample is thoroughly dry.

- ◆ Seal the vials by winding down the stoppering mechanism.
- ◆ Screw on plastic cap.
- ◆ Put a stock number on the top of the cap.
- ◆ Move the vials into a 4 °C fridge for long-term storage. *Note:* If the silica gel turns from blue to pink, it indicates the vial seal is probably leaking moisture and the plant tissue inside needs to be redried immediately or discarded.
- ◆ Update plant pathogen reference collection and herbarium database.

8.4.3.5 Freezing

List of equipment, consumables and reagents:

- ◆ storage containers (plastic or glass) with screw caps or sealable plastic bags
- ◆ cotton or paper towel to keep calcium chloride away from plant tissue
- ◆ sticky labels suitable for freezing
- ◆ markers or pencils
- ◆ scalpel blades or razor blades
- ◆ disposable gloves
- ◆ chopping board
- ◆ disinfectant (e.g. solution of 70 percent ethanol, Virkon, bleach; wipes, e.g. Trigene).
- ◆ computer/database or recording paper sheet.

Container preparation

- ◆ Prepare a sticky label containing at least a unique identification number or directly write on the container or plastic bag with permanent ink. *Note:* Marker on the outside may degrade over time.
- ◆ *Optional:* A paper label may also be inserted inside the container or plastic bag.

Sample preparation

- ◆ Select plant tissue for preservation: It should be as fresh as possible and symptomatic. *Note:* Dry or decayed tissue must be avoided. Wear gloves when handling infected plant materials. Keep samples on ice at all times.
- ◆ Remove any dampness by gently drying the surface with a paper towel.
- ◆ Flatten the plant tissue, if possible.
- ◆ Place the plant tissue in the labelled container or plastic bag and close it. *Note:* Between plant

tissue infected with different organisms, change gloves, dispose of disposable razor blade, disinfect chopping board and scalpel blade with disinfectant such as solution of 70 percent ethanol, Virkon or bleach, or wipes of Isowipes, Mediwipes, Trigene or V-wipes.

Sample storage

- ◆ Store sample in a $-20\text{ }^{\circ}\text{C}$ freezer or, preferably, a $-80\text{ }^{\circ}\text{C}$ freezer. *Note:* Avoid multiple thawing and freezing as it will damage the plant tissue.
- ◆ Update the plant pathogen reference collection and herbarium database.

8.4.3.6 Laminating

List of equipment, consumables and reagents:

- ◆ laminator
- ◆ lamination sheets.

Procedure to laminate dry specimens

- ◆ Lay and arrange specimens between lamination sheets.
- ◆ Write specimen number on a piece of paper and place it with the specimen. Alternatively write on the lamination sheet after lamination.
- ◆ Turn the laminator on and wait until the laminator is heated and ready for lamination.
- ◆ Run the lamination sheets with specimen through the laminator.
- ◆ Turn off the laminator.
- ◆ Update the plant pathogen reference collection and herbarium database.

8.4.3.7 Pickling

List of equipment, consumables and reagents:

- ◆ airtight glass containers
- ◆ preservatives (e.g. 70 percent ethanol).

Procedures to prepare pickled specimens

- ◆ Drain away excess liquid if applicable.
- ◆ Put the specimen into a glass container filled with preservative. *Note:* Make sure that the specimen is immersed in preservative and the lid of the glass container is sealed tight.
- ◆ Label the glass container with the specimen number and type of preservative used.

- ◆ Replace the preservative once or a few times for specimens that have a high water content.
- ◆ Update the plant pathogen reference collection and herbarium database.

8.4.3.8 Living cultures

Living cultures of fungi and bacteria can be maintained by frequent transfer of cultures onto fresh growing medium. Keeping cultures in the fridge ($4\text{ }^{\circ}\text{C}$) or immersed under mineral oil can extend the time between subculturing. Fungal cultures that form spores can be preserved by freeze-drying in sealed glass ampoules. Most cultures can be preserved for years in freezers ($-20\text{ }^{\circ}\text{C}$ or $-40\text{ }^{\circ}\text{C}$) and deep freezers ($-80\text{ }^{\circ}\text{C}$); and decades in cryogenic freezers (below $-135\text{ }^{\circ}\text{C}$) and in vapour of liquid nitrogen, with highest survival rate using the latter two methods. However, oomycetes, such as *Pythium* and *Phytophthora*, are best preserved in glass vials with water and kept in a fridge ($4\text{ }^{\circ}\text{C}$). Details of these methods are available in the references below.

Kirsop, B.E. & Doyle, A. 1991. *Maintenance of microorganisms and cultured cells: a manual of laboratory methods*, 2nd edition. Academic Press. 288 pp.

Smith, D. & Onions, A.H.S. 1994. *The preservation and maintenance of living fungi*, 2nd edition. UK, International Mycological Institute. 132 pp.

Waller, J.M., Lenné, J.M. & Waller, S.J., eds. 2001. *Plant pathologist's pocketbook*, 3rd edn. Wallingford, UK, CAB International. 528 pp.

8.4.4 Labelling and data recording

Each plant pathogen reference collection and herbarium specimen must be labelled with a unique specimen number. Additional information as below can be recorded on the label or in a separate database:

- ◆ scientific name of plant
- ◆ scientific name of pathogens
- ◆ plant part or substratum
- ◆ address and GPS coordinates of collection site
- ◆ name of collector
- ◆ date of collection
- ◆ name of identifier

- ◆ date of identification
- ◆ technique used for identification
- ◆ date of preservation
- ◆ images
- ◆ symptoms observed on plants
- ◆ field notes
- ◆ ephemeral features (colour, smell, shape) before drying.

Each microscope slide must be labelled with the unique specimen number of the specimen from which it was prepared. Additional information below is recommended to be recorded on the label as well:

- ◆ pathogen name
- ◆ plant host name
- ◆ name of identifier
- ◆ date of identification.

8.5 Other reference collections

8.5.1 Miscellaneous reference material

Nucleic acid extracts obtained from pests, pathogens and infected plant material can be stored in a -80°C freezer as genetic reference material. DNA can be kept for much longer as it is more stable than RNA. In any case, avoid multiple thawing and freezing as this will affect the quality of the nucleic acids.

Grids used in transmission electron microscopy to visualize pathogens can be stored as reference material. These grids can be kept for many years in a dry and cool environment.

8.5.2 International reference collections

Wherever possible, a sample of the preserved specimens should be sent to international reference collections. Some countries may also have their own reference collections in museums or universities. Institutes willing to have a reference collection should maintain a clear database which should be easily accessible to other researchers nationally and internationally.

Examples of international arthropod collections and their abbreviations:

- BMNH Natural History Museum, London
- BPBM Bishop Museum, Honolulu, Hawaii
- NMNW Namibian National Insect Collection, Windhoek, Namibia (E. Marais)

- NMSA Natal Museum, Pietermaritzburg, KwaZulu-Natal, South Africa (M. Mostovski)
- PCV P Cerretti collection, Verona, Italy
- SMNS Staatliches Museum für Naturkunde, Stuttgart, Germany (H.-P. Tschorsnig)
- TAU Department of Zoology, Tel Aviv University, Tel Aviv, Israel (A. Freidberg)
- TZC Theo Zeegers collection, Soest, The Netherlands
- ZMAN Zoölogisch Museum, Amsterdam, The Netherlands (B. Brugge)
- NZAC New Zealand Arthropod Collection, Auckland.

A comprehensive list of plant pathogen reference collections and herbaria is available at the website of the [Index Herbariorum maintained by the New York Botanical Garden](#).

Some further examples of international herbaria for micro-organisms on plant are:

- ◆ Purdue Agriculture, Purdue Herbaria – Arthur Fungarium (PUR): <https://ag.purdue.edu/btny/Herbaria/Pages/arthur.aspx>
- ◆ Cornell University Plant Pathology Herbarium (CUP): <http://www.plantpath.cornell.edu/CUPpages/index.html>
- ◆ New South Wales Department of Primary Industries Plant Pathology Herbarium (HERB-DAR): <http://www.dpi.nsw.gov.au/aboutus/services/collections/herbarium>
- ◆ The New York Botanical Garden (NYBG): <http://sciweb.nybg.org/science2/Mycology.asp>
- ◆ Landcare Research. New Zealand Fungal & Plant Disease Collection (PDD): <http://www.landcareresearch.co.nz/resources/collections/pdd>
- ◆ Natural Resources Canada. Pacific Forestry Centre's Forest Pathology Herbarium (DAVFP): <http://www.nrcan.gc.ca/forests/research-centres/pfc/13493>
- ◆ DAFF Plant Pathology Herbarium and Insect Collection, Australia (BRIP): <http://collections.daff.qld.gov.au/web/home.html>
- ◆ Farr, D.F. & Rossman, A.Y. *Fungal databases*. Systematic Mycology and Microbiology Laboratory, Agricultural Research Service, United States Department of Agriculture. <http://nt.ars-grin.gov/fungaldatabases/>

A comprehensive list of culture collections is available at the website of the World Federation for Culture Collections (WFCC). The Global Catalogue of Microorganisms provides an online database for microbial strains curated in many international collections.

Some further examples of international culture collections for micro-organisms on plants are:

- ◆ Agdia: <http://www.agdia.com/>
- ◆ American Type Culture Collection (ATCC): <http://www.atcc.org/>
- ◆ Centraalbureau voor Schimmelcultures – CBS-KNAW Collections: <http://www.cbs.knaw.nl/collections/>
- ◆ CABI Microbial services: <http://www.cabi.org/services/microbial-services/culture-collection/>
- ◆ Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures: <http://www.dsmz.de/>
- ◆ International Collection of Microorganisms from Plants (ICMP): <http://www.landcareresearch.co.nz/resources/collections/icmp>
- ◆ Japan Collection of Microorganisms (JCM): <http://www.jcm.riken.jp/>
- ◆ LOEWE: http://www.loewe-info.com/index.php?cp_sid=142506643c4&cp_tpl=5501
- ◆ CAB International. The United Kingdom National Culture Collection (UKNCC): <http://www.ukncc.co.uk/>

8.5.3 Virtual reference collections

Some examples of virtual collections are:

- ◆ CABI Crop Protection Compendium: <http://www.cabi.org/cpc>
- ◆ CABI Forestry Compendium: <http://www.cabi.org/fc/>
- ◆ PaDIL – High quality images and information tools designed for biosecurity and biodiversity: <http://www.padil.gov.au/>
- ◆ Plantwise Knowledge Bank: <http://www.plantwise.org/KnowledgeBank/home.aspx>
- ◆ Forestry Images: <http://www.forestryimages.org/>
- ◆ University of Maine plant disease images: <http://extension.umaine.edu/ipm/ipddl/plant-disease-images/>

- ◆ AntWeb – the world's largest online database of images, specimen records and natural history information on ants: http://www.antweb.org/user_guide.jsp

8.5.4 DNA sequence reference collection

Several sequence databases are available.

All organisms

- ◆ NCBI (National Center for Biotechnology Information) GenBank sequence database: <http://www.ncbi.nlm.nih.gov/genbank/>
- ◆ BOLDSYSTEMS (Barcode of Life Data Systems): <http://www.boldsystems.org/>
- ◆ Q-Bank – Comprehensive databases on quarantine plant pests and diseases: <http://www.q-bank.eu/>

Fungi

- ◆ MycoBank: <http://www.mycobank.org/>
- ◆ Fusarium-ID: <http://isolate.fusariumdb.org/index.php>
- ◆ Phytophthora database: <http://www.phytophthoradb.org/>
- ◆ Phytophthora-ID: <http://phytophthora-id.org/seq-id.html>
- ◆ Pythium Genome Database: <http://pythium.plantbiology.msu.edu/>

Bacteria

- ◆ Plant Associated and Environmental Microbes Database (PAMDB.org): <http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl>

Nematodes

- ◆ WormBase for the genetics of nematodes: <http://www.wormbase.org>

Insects

- ◆ BeeBase for bee research: <http://hymenoptera-genome.org/beebase/>
- ◆ FlyBase: a database of *Drosophila* genes and genomes: <http://flybase.org>
- ◆ BeetleBase for *Tribolium castaneum* genetics, genomics and developmental biology: <http://beetlebase.org>

9. Reporting

Introduction

Upon completion of the diagnostic work, the identification details need to be reported to the submitter within the agreed time frame. It is important that another competent staff member checks the final report before sending it off to the submitter, to avoid errors.

The specimen replies should:

- ◆ be as brief as possible
- ◆ be phrased in a careful manner
- ◆ give the information requested by the submitter, wherever possible
- ◆ not exceed the level of the staff member's competency
- ◆ not exceed the level of the authoritativeness of the identification.

The final report should contain:

- ◆ all the submitter information
- ◆ all the sample information
- ◆ all tests done
- ◆ scientific name of pest and disease by following accepted format, e.g. for entomology – *Genus species* [ORDER: Family]
- ◆ signatures of identifiers
- ◆ details of additional samples found (if any)
- ◆ and, if applicable:
 - live/dead status
 - pest/regulatory status
 - number of organisms and their life stages
 - date and time of reply
 - printed identifier names
 - charges.

It is important to demonstrate proof that a reply was sent to the submitter, in particular:

- ◆ what was sent
- ◆ how it was sent
- ◆ when it was sent
- ◆ who sent it.

Interim report

It is advisable to send an interim reply when the identification cannot be completed within the agreed reporting time frame.

Amendment to final report

When the contents of a report are missing or incorrect (e.g. the identification of organisms or test result is incorrect), an amended report should be sent to the submitter. Re-generate the final report and ensure that this new report contains an additional message stating that this report replaces Report Number: XXXXXX or that it replaces the report issued on dd/mm/yyyy.

New record

It is the responsibility of the diagnostic staff to ensure that laboratory management is notified as soon as possible of the following categories of identification:

- ◆ any identification suspected to be new to the country
- ◆ any identification of an unwanted organism that is pending confirmation
- ◆ any significant find of a response organism.

These new identifications must be confirmed by either another scientist or a nationally or internationally recognized expert.

10. Fate of Sample

Introduction

Diagnostic laboratories need to consider the best means of treating a sample once it has been fully analysed and the final diagnostic report released.

Samples may be either disposed of in a manner appropriate to their biosecurity risk or retained for future use.

10.1 Disposal

Before a decision is made to dispose of a sample the lab should consider the need to retain it as evidence. Refer to section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*) for further guidance in this area. The sample should also consider a sample's value for future use as described below.

If a sample is deemed to be of no further value, it should be disposed of in a manner that renders it inert with respect to pest risk. Facilities to do this should exist within the lab as described in Chapter 2.

10.2 Sample or specimen retention

A lab may choose to retain samples and their related specimens for many reasons.

In the phytosanitary context, sample retention may be important in order to provide

evidence for cases of non-compliance, legal actions resulting from phytosanitary action or trade disputes. see section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

Samples may also be retained because of their diagnostic value as reference specimens, or they may represent the first record of a pest in an area. In the latter case, this evidence could be used in an official capacity by an NPPO for purposes such as pest reporting (ISPM 17), establishing a country's pest status (ISPM 8) and pest free areas (ISPM 4).

If a sample or specimen has good diagnostic reference value it may be retained in the lab or another collection to aid in future diagnoses or for training purposes. Criteria for selection of additional reference specimens include, but are not limited to, the following:

- ◆ new species to the collection
- ◆ specimen is in better condition than those already in the collection
- ◆ is a new country-of-origin record
- ◆ for training material, e.g. from projects
- ◆ for photo image library.

More details on preserving samples and specimens for these uses can be found in Chapter 8.

Section 3 – Other Information Sources

Introduction

The Internet provides access to a vast array of information to underpin diagnosis and development of expertise. Section 5.5 provides information on key reference materials for plant pests as well as centres of excellence and expert databases.

Bibliography

- Barnet, H.L. & Hunter, B.B.** 1972. *Illustrated genera of imperfect fungi*, 3rd edn. Minneapolis, MN, Burgess.
- Boonham, N., Glover, R., Tomlinson, J. & Mumford, R.** 2008. Exploiting generic platform technologies for the detection and identification of plant pathogens. *European Journal of Plant Pathology*, 121: 355–363. DOI: 10.1007/978-1-4020-8780-6_15.
- Boonham, N., Tomlinson, J. & Mumford, R.** 2007. Microarrays for rapid identification of plant viruses. *Annual Review of Phytopathology*, 45: 307–328. DOI: 10.1146/annurev.phyto.45.062806.094349.
- Bradbury, J.F.** 1985. *Guide to plant pathogenic bacteria*. Wallingford, UK, CAB International. 332 pp.
- CABI & EPPO.** 1997. *Quarantine pests for Europe*, 2nd edn. Wallingford, UK, CAB International and Paris, European and Mediterranean Plant Protection Organization. 1425 pp.
- Cannon, R.J.C., Pemberton, A.W. and Bartlett, P.W.** 1999. Appropriate measures for the eradication of unlisted pests. *Bulletin OEPP / EPPO Bulletin*, 29: 29–36.
- Coolen, W.A. & D'Herde, C.J.** 1972. *A method for the quantitative extraction of nematodes from plant tissue*. Ghent, Belgium, Ministry of Agriculture, State Agricultural Research Centre. 77 pp.
- Decraemer, W. & Hunt, D.J.** 2006. Structure & classification. In R.N. Perry & M. Moens, eds. *Plant nematology*, 1st edn, pp. 3–32. Wallingford, UK, CAB International.
- Ellis, M.B. & Ellis, J.P.** 1997. *Microfungi on land plants: an identification handbook*. Slough, UK, Richmond Publishing. 868 pp.
- EPPO.** 2013a. Diagnostic: PM 7/119 (1) Nematode extraction. *EPPO Bulletin*, 43: 471–495.
- EPPO.** 2013b. *Diagnostic protocols for regulated pests: pictorial glossary of morphological terms in nematology*. EPPO Technical Document No. 1056 (rev. 4). Paris, European and Mediterranean Plant Protection Organization. Available at http://www.eppo.int/QUARANTINE/diag_activities/EPPO_TD_1056_Glossary.pdf (last accessed on 16 September 2015).
- Erwin, D.C. & Ribeiro, O.K.** 1996., *Phytophthora diseases worldwide*. St Paul, MN, APS Press, American Phytopathological Society. 592 pp.
- Fenwick, D.W.** 1940. Methods for the recovery and counting of cysts of *Heterodera schachtii* from soil. *Journal of Helminthology*, 18: 155–172.
- Flegg, J.J.M. & Hooper, D.J.** 1970. Extraction of free-living stages from soil. In J.F. Southey, ed. *Laboratory methods for work with plant and soil nematodes*, pp. 5–22. Technical Bulletin 2. London, Ministry of Agriculture, Fisheries and Food.
- Hockland, S., Inserra, R.N., Millar, L. & Lehman, P.S.** 2006. International plant health – putting legislation into practice. In R.N. Perry & M. Moens, eds. *Plant nematology*, 1st edn, pp. 327–345. Wallingford, UK, CAB International.
- Hooper, D.J. & Evans, K.** 1993. Extraction, identification and control of plant parasitic nematodes. In K. Evans, D.M. Trudgill & J.M. Webster, eds. *Plant parasitic nematodes in temperate agriculture*, pp. 1–60. Wallingford, UK, CAB International.
- Hooper, D.J.** 1986. Extraction of nematodes from plant tissue. In J.F. Southey, ed. *Laboratory methods for work with plant and soil nematodes*. Reference Book 402, 6th edn, pp. 51–58. London, Ministry of Agriculture, Fisheries and Food.
- Hooper, D.J., Hallmann, J. & Subbotin, S.A.** 2005. Methods for extraction, processing and detection of plant and soil nematodes. In M. Luc, R.A. Sikora & J. Bridge, eds. *Plant parasitic nematodes in subtropical and tropical agriculture*, 2nd edn, pp. 53–86. Wallingford, UK, CAB International.
- IMI descriptions of fungi and bacteria*. Wallingford, UK, CAB International. Available at <http://www.cabi.org/dfb/> (last accessed on 16 September 2015).
- Janse, J.D.** 2010. *Phylobacteriology: principles and practice*. Wallingford, UK, CAB International. 368 pp.
- Lelliott, R.A. & Stead, D.E.** 1991. *Methods for the diagnosis of bacterial diseases of plants*. Oxford, Wiley. 224 pp.
- Leslie, J.F. & Summerell, B.A.** 2006. *The Fusarium laboratory manual*. Oxford, Blackwell. 388 pp.

- Luc, M., Sikora, R.A. & Bridge, J., eds.** 2005. *Plant parasitic nematodes in subtropical and tropical agriculture*, 2nd edn. Wallingford, UK, CAB International. 896 pp.
- Manzanilla-López, R.H. & Marbán-Mendoza, N., eds.** 2012. *Practical plant nematology*. Mexico City, Biblioteca Básica de Agricultura, Grupo Mundi-Prensa. 883 pp.
- Monger, W.A., Alicai, T., Ndunguru, J., Kinyua, Z.M., Potts, M., Reeder, R.H., Miano, D.W., Adams, I.P., Boonham, N., Glover, R.H. & Smith, J.** 2010. The complete genome sequence of the Tanzanian strain of *Cassava brown streak virus* and comparison with the Ugandan strain sequence. *Archives of Virology*, 155: 429–433. DOI:10.1007/s00705-009-0581-8.
- Seinhorst, J.W.** 1962. On the killing, fixing and transferring to glycerin of nematodes. *Nematologica*, 8: 29–32.
- Siddiqi, M.R.** 2000. *Tylenchida parasites of plants and insects*, 2nd edn. Wallingford, UK, CAB International. 864 pp.
- Smith, J.J., Waage, J., Woodhall, J.W., Bishop, S.J. & Spence, N.J.** 2008. The challenge of providing plant pest diagnostic services for Africa. *European Journal of Plant Pathology*, 121: 365–375.
- Snowdon, A.L.** 2010a. *A colour atlas of post-harvest diseases and disorders of fruits and vegetables*. Vol. 1. *General introduction and fruits*. Boca Raton, CRC Press. 320 pp.
- Snowdon, A.L.** 2010b. *A colour atlas of post-harvest diseases and disorders of fruits and vegetables*. Vol. 2. *Vegetables*. Boca Raton, CRC Press. 416 pp.
- Tarjan, A.C.** 1967. Influence of temperature and hydrogen peroxide on the extraction of burrowing nematodes from citrus roots. *Plant Disease Reporter*, 51: 1024–1028.
- Tarjan, A.C.** 1972. Observations on extracting citrus nematode, *Tylenchulus semipenetrans*, from citrus roots. *Plant Disease Reporter*, 56: 186–188.
- Tomlinson, J. & Boonham, N.** 2008. Potential of LAMP for detection of plant pathogens. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, 3: 1–7.
- Weller, S.A., Aspin, A. & Stead, D.E.** 2008. Classification and identification of plant-associated bacteria by fatty acid profiling. *EPPO Bulletin*, 06/2008; 30(3–4): 375–380. DOI: 10.1111/j.1365-2338.2000.tb00914.x.
- Weigers, A.L.** 2003. Valid methods: the quality assurance of test method development, validation, approval, and transfer for veterinary testing laboratories. *J. Vet. Diagn. Invest.*, 15(4): 303–310.
- Winfield, A.L., Enfield, M.A. & Foremann, J.H.** 1987. A column elutriator for extracting cyst nematodes and other small invertebrates from soil samples. *Annals of Applied Biology*, 111: 223–231. DOI: 10.1111/j.1744-7348.1987.tb01449.x.

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IPPC

The International Plant Protection Convention (IPPC) is an international plant health agreement that aims to protect cultivated and wild plants by preventing the introduction and spread of pests. International travel and trade are greater than ever before. As people and commodities move around the world, organisms that present risks to plants travel with them.

Organization

- ◆ The number of contracting party signatories to the Convention exceeds 181.
- ◆ Each contracting party has a national plant protection organization (NPPO) and an Official IPPC contact point.
- ◆ 10 regional plant protection organizations (RPPOs) have been established to coordinate NPPOs in various regions of the world.
- ◆ IPPC liaises with relevant international organizations to help build regional and national capacities.
- ◆ The Secretariat is provided by the Food and Agriculture Organization of the United Nations (FAO).



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