Practical guide to detection and identification of *Phytophthora*

Version 1.0

André Drenth & Barbara Sendall

CRC for Tropical Plant Protection
Brisbane Australia

Practical guide to detection and identification of *Phytophthora*

Version 1.0

André Drenth and Barbara Sendall

CRC for Tropical Plant Protection
Brisbane Australia

Practical guide to detection and identification of *Phytophthora*

Preface

This guide has been written as a first step to help your foray into the world of Phytophthora. It is not meant to be exhaustive or comprehensive but it is aimed at making you familiar with the basics of identifying Phytophthora diseases, give you useful hints for the choice and preparation of selective media and tips on isolation and culturing *Phytophthora.* It also gives very basic methods and procedures for identification of different species of *Phytophthora* using a limited set of morphological characters. This guide has been prepared as a working and training manual for project PHT/1996/193 "Survey of the presence and importance of *Phytophthora* in South-east Asia" funded by the Australian Centre for International Agricultural Research (ACIAR). It therefore focuses on only eight species of *Phytophthora,* which are most commonly found in Southeast Asia.

This guide is meant to be practical and should be seen as an introductory laboratory manual only. It does not contain any mycological novelties or in depth treatments. It is simply aimed at helping students and plant pathologists to take the first steps into the world of Phytophthora. And we speak from personal experience when we say what a fascinating world that is. For more details and in depth descriptions of all species of *Phytophthora* and their general biology we strongly recommend that you consult the most excellent book "Phytophthora diseases worldwide" written by Donald Erwin and Olaf Ribeiro (1996). This book contains the descriptions of all *Phytophthora* species and vast amounts of information concerning Phytophthora diseases.

This is the first draft of this practical guide and mistakes are solely our responsibility. However, we would very much appreciate any comments on how to improve its usefulness and incorporate any tips you may have and want to share with other Phytophthora workers.

André Drenth and Barbara Sendall

Brisbane, June 2001

CRC for Tropical Plant Protection
Indooroopilly Research Centre
Plant Pathology Building
80 Meiers Road
Indooroopilly QLD 4068
Australia
Email: drentha@dpi.qld.gov.au

Table of contents

1. Introduction to the genus *Phytophthora*

1.1 The genus *Phytophthora*

Phytophthora de Bary 1887 is a cosmopolitan genus of Oomycete obligate plant pathogens containing approximately 60 described species (Erwin and Ribeiro 1996). *Phytophthora* species attack a wide range of plants, and are responsible for some of the world's most destructive plant diseases - examples include the European potato famine of the 19th century caused by *P. infestans* (Bourke 1964). Phytophthora diseases have been well studied in the temperate regions of the world. However, Phytophthora diseases are very common throughout the wet tropical regions of the world and cause significant diseases losses in many tropical fruit crops in the form of root rots, collar rots, stem cankers, leaf blights and fruit rot. For example, *P. palmivora* alone causes a myriad of severe diseases on many different crops including: black pod of cocoa; root, stem and fruit rot of pawpaw; root rot and blight of citrus; bud rot in palms; black stripe in rubber; and root rot, trunk canker, and fruit rot in durian.

1.2 Evolutionary placement

Phytophthora is a member of the Order Peronosporales within the Class Oomycetes in the Kingdom Chromista (Hawksworth *et al.* 1995; van de Peer *et al.* 1996) see Table 1.1). The Oomycetes includes four orders, two of which, the Saprolegniales and the Peronosporales, contain important plant pathogens. The other two orders contain small groups of mainly aquatic fungal-like organisms. Within the Peronosporales, the family Pythiaceae contains a number of genera, the best-known of which are *Phytophthora* and its sister group, *Pythium*, a genus of approximately 120 species (van der Plaats-Niterink 1981).

Table 1.1. Classification of the Oomycetes (Hawksworth *et al*. 1995).

Kingdom	Class	Order	Family	Genus
Chromista	Oomycetes	Lagenidiales		
		Leptomitales		
		Saprolegniales	Saprolegniaceae	*Achlya* *Saprolegnia*
		Peronosporales	Pythiaceae	*Pythium* ***Phytophthora***
			Peronosporaceae	*Bremia* *Peronospora*
			Albuginaceae	*Albugo*

1.3 Biology

The Oomycetes share many characteristics of ecology and life history with the true fungi. However, they are clearly distinguished from the Basidiomycetes and Ascomycetes by their genetics and reproductive mechanisms (Erwin and Ribeiro 1996). Their placement in the Kingdom Chromista (Cavalier-Smith 1986) is supported by a large number of characteristics, including variation in metabolic pathways (Elliott 1983; Hendrix 1970; Wang and Bartnicki-Garcia 1973), the presence of β-glucans rather than chitin in cell walls (Bartnicki-Garcia and Wang 1983), production of motile heterokont zoospores (Desjardins *et al.* 1969), and predominance of the diploid stage in the lifecycle (Erwin and Ribeiro 1996).

1.3.1 Life cycle

The life cycle of *Phytophthora* may involve up to three asexual spore forms in addition to the sexual spore form (Figure 1.1). Diploid vegetative mycelium produces asexual sporangia, which may germinate directly, or differentiate to produce 8-32 zoospores, each of which pass through a cycle of dispersal and encystment before germinating. Some species, such as *P. cinnamomi*, also produce asexual chlamydospores from the mycelium. Sexual reproduction results in the production of oospores. All spore types are potentially infective, and chlamydospores and oospores also function as overwintering or resting structures.

All species of *Phytophthora* have a soil-borne resting stage. Some, such as *P. sojae*, are also dispersed primarily in the soil, via the release of zoospores from infected plant material. Others, such as *P. palmivora*, are aerially dispersed, primarily as caducous (deciduous) sporangia.

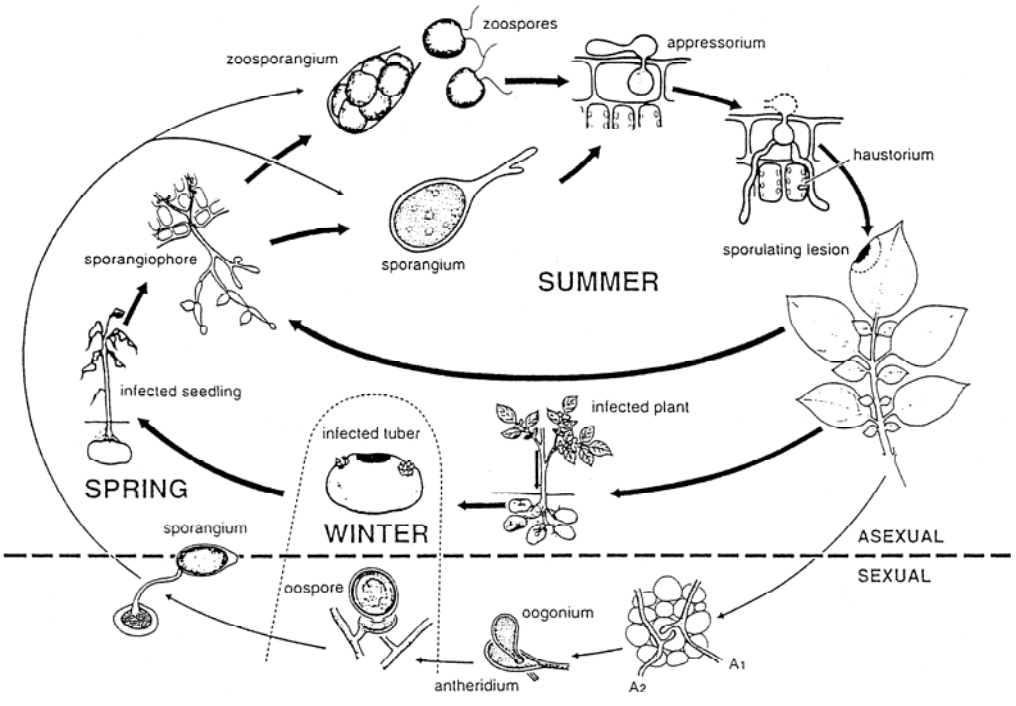


Figure 1.1 Life cycle of *Phytophthora infestans*. Reprinted from Drenth (1994)

1.3.2 Host range

Species of *Phytophthora* vary greatly in their degree of host specificity. *P. fragariae* var. *rubi* infects a single host species (Kennedy and Duncan 1995), while *P. cinnamomi* is able to attack over 1000 different host plant species (Erwin and Ribeiro 1996), and other species occupy a continuum between these two extremes. Broad host range *Phytophthora* species tend to attack their hosts using enzymes which affect relatively unspecialised host chemical and mechanical resistance mechanisms (Brasier 1983), whereas some host specific species are known to possess virulence genes which interact specifically, in a gene-for-gene system, with host resistance genes (reviewed in Thompson and Burdon 1992).

1.3.3 Mating system

All isolates of *Phytophthora* are potentially bisexual, that is, they are able to produce both male and female sexual structures, or gametangia (Galindo and Gallegly 1960). However, only about half of the species of *Phytophthora* are homothallic, and able to produce oospores rapidly and abundantly in single culture. The remaining species are heterothallic, and produce gametangia only in response to chemical stimulation from an isolate of the opposite mating type (Brasier 1992; Ko 1978).

The system of heterothallism involving A1 and A2 mating types is universal throughout the genus. Isolates of opposite mating types from different species are often able to reciprocally stimulate gametangial formation (Ko 1978, Yu *et al*. 1981). The mating system of a *Phytophthora* species determines its ability to outbreed: homothallism allows frequent selfing, whereas heterothallism encourages outbreeding. However, both homothallic and heterothallic species do have a range of reproductive options. Homothallic species have recently been shown to undergo low levels of outbreeding *in vitro* (Whisson *et al*. 1994), while heterothallic species have been shown to inbreed at low levels (Goodwin 1997, Judelson 1997).

1.4 *Phytophthora* systematics

The genus *Phytophthora* has been widely acknowledged as taxonomically 'difficult' (Brasier 1983), as many of the characters used for species identification are plastic, highly influenced by environment, show overlap between species, and have an unknown genetic basis. Nonetheless, since a major review of the genus was performed by Waterhouse (1963), morphological characters have remained the basis for species identification and taxonomy (Newhook 1978; Stamps *et al.* 1990). Waterhouse classified species based primarily on papillation (Figure 1.2) and caducity (easy detachment) of sporangia, type of antheridial attachment (Figure 1.3), and mating system. Based on this analysis, the genus was divided into six major groups (Table 1.2), which were intended solely as an aid to species identification, and were not meant to imply a natural classification (Waterhouse 1963).

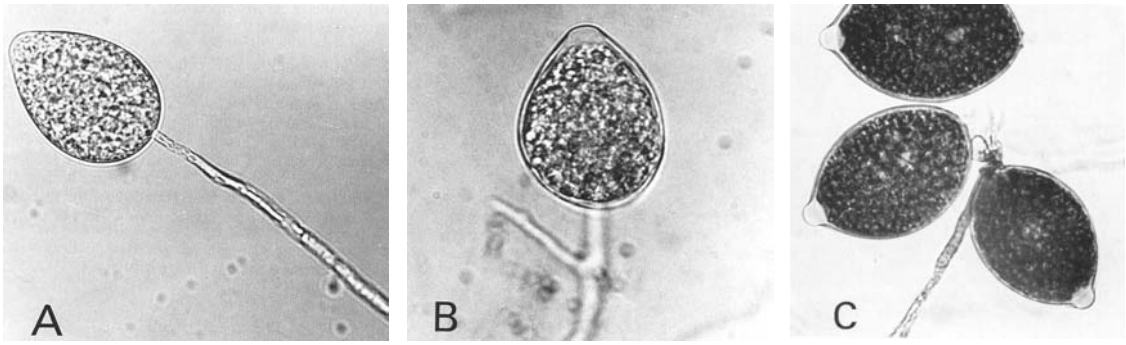


Figure 1.2 (a) Non-papillate, (b) semi-papillate and (c) papillate sporangia. Photographs from Erwin and Ribeiro 1996.

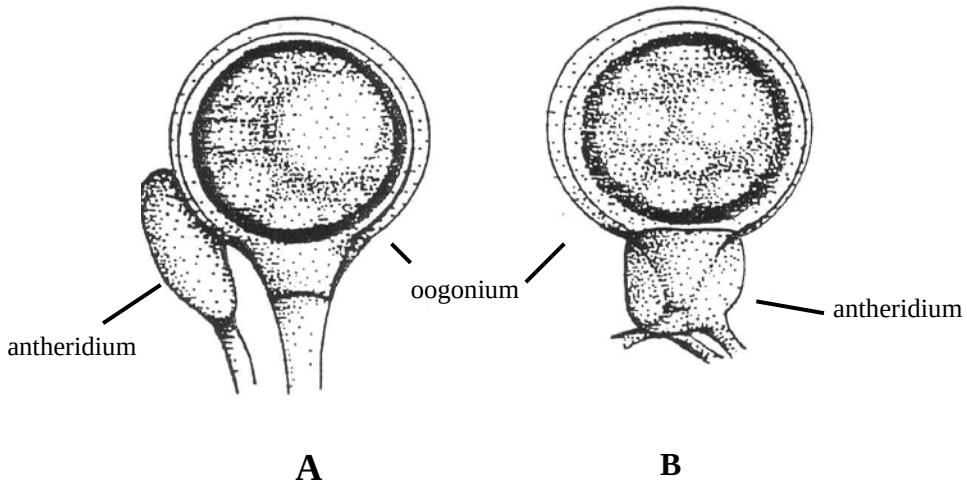


Figure 1.3. Antheridial attachment – (a) paragynous, (b) amphigynous. Illustration from Erwin and Ribeiro 1996.

Table 1.2. Classification of *Phytophthora* into six groups by Waterhouse (1963).

Group	Sporangia	Antheridial attachment	Examples
1	papillate	paragynous	*P. cactorum, P. clandestina*
II	papillate	amphigynous	*P. capsici, P. palmivora*
III	semi-papillate	paragynous	*P. inflata, P. multivesiculata*
IV	semi-papillate	amphigynous	*P. infestans, P. ilicis*
V	non-papillate	paragynous	*P. megasperma, P. sojae*
VI	non-papillate	amphigynous	*P. cinnamomi, P. drechsleri*

1.5 Pathogenicity of *Phytophthora*

Almost all species within the genus *Phytophthora* are formidable plant pathogens. Hence, we have to ask the question what makes these organisms such effective plant pathogens. The following factors are involved:

- The ability to produce different types of spores such as sporangia and zoospores for short-term survival and spread, and chlamydospores and oospores for more long-term survival.
- Rapid sporulation on host tissue such as leaves typically within 3-5 days after infection. This results in a rapid build up of inoculum in a multicyclic fashion, leading to epidemics under favourable environmental conditions.
- Ability of zoospores of *Phytophthora* to be attracted to root tips through a chemical stimulus (positive chemotaxis) coupled with the mobility of zoospores to actually swim to the actively growing root tips, encyst, and infect young, susceptible root tissue.
- Ability to survive in or outside the host tissue as oospores or chlamydospores for long periods of time. Oospores are also known to survive passage of the digestive systems of animals (for example, snails).
- Production of sporangia, which can be airborne and may travel reasonable distances on wind currents and infect neighbouring fields. These sporangia can directly infect host tissue. These same sporangiospores also have the ability do differentiate into 4-32 zoospores under humid and cool conditions and cause multiple infections from the one sporangium. However, zoospores can travel only short distances as they are susceptible to desiccation.
- Phytophthora pathogens belong to the Kingdom Chromista and as such have different biochemical pathways to the true fungi. Therefore many fungicides are not very effective against Phytophthora pathogens.
- Phytophthora pathogens thrive under humid and wet conditions, which makes them difficult to control, as protectant fungicides are difficult to apply and least effective under such conditions.

Phytophthora pathogens can cause many different diseases and disease symptoms on a wide range of plant species. In the next section, the disease symptoms most often encountered are discussed.

1.5.1 Root rot

In general seedlings of many plants are very susceptible to root rot and damping off caused by *Phytophthora*. The early symptoms are the wilting and yellowing of young seedlings. General symptoms of root rot are that plants appear water stressed, chlorotic, and are often stunted in their growth. Affected root tissue is soft, watersoaked and discoloured to dark brown compared to the creamy white colour of healthy roots. Advanced root rot leads to the lack of secondary and tertiary roots and a lack of healthy root tips.

1.5.2 Collar rot

Collar rot often develops at or just below ground level. The infection moves upwards from the roots, rotting the lower bark tissue and discolouring the lower stem. Above ground symptoms appear as wilting, reduction of foliage and dieback of branches. Bark

and cortex tissue often have a swollen and cracked appearance, separating easily from the underlying tissue.

1.5.3 Tree canker

Many species of *Phytophthora* can form cankers on the stems of host plants. These cankers have various names, including: stripe canker (cinnamon), patch canker (durian) or trunk canker. The first sign of canker is often the appearance of wet lesions on the bark surface, often close to the branch points at the lower end of the trunk. Bark discolouration and exudation of reddish brown, resinous substance often accompany necrosis. When the bark is stripped away, the cortical tissues and wood appear dull and discoloured from cream coloured to reddish brown. Wood lesions are often very irregular in shape but are well defined. Expanding lesions severely restrict water and nutrient flow to the connecting branches giving the appearance of wilting. If the lesion girdles the tree branch, die back is more widespread in the crown and the entire tree may become defoliated.

1.5.4 Stem lesions

Some species of *Phytophthora* attack leaves as well as stems. For example, *P. infestans* on potato and tomato, *P. sojae* on soybean, and *P. nicotianae* on tobacco. Young immature stems are often most susceptible. In advanced stages dry, dark brown or black lesions develop in the cortical tissue on the stem near the soil line. Such lesions expand upward and may cover as much as half the length of the stem in the case of black shank on tobacco. Expanded lesions often girdle the stem and give rise to wilting and death of the upstream branches and leaves.

1.5.5 Bud rot

Bud rot (sometimes called heart rot), is a serious problem in many species of palms. It is caused predominantly by *P. palmivora*. The symptoms of bud rot of palm are exhibited over a period of months often following severe storms, which facilitate infection and spread of *Phytophthora.* Symptoms first appear as discolouration of the spear leaf and one or more of the newest leaves. These new leaves may exhibit lesions from infection that has occurred in the spear. As the infection in the bud of the palm progresses new emerging leaves show increasing amounts of damage. Eventually the spear leaves can be pulled out easily because they are rotted at the base and some white mycelial growth may be observed at the leaf bases. The fronds will turn yellow and then brown, and will fall off, finally leaving only a dead naked trunk. In the base of the bud, secondary invaders move in and fluid starts to collect giving off a foul smell. The tissue below the bud shows discolouration from reddish brown to brown. It is hard to isolate *Phytophthora* from palms with advanced bud rot due to the decay of the bud. Trees which are beginning to show symptoms, with an advancing margin on the bud, should be used instead as they are often still relatively free of secondary invaders.

1.5.6 Leaf blight

A number of *Phytophthora* species cause leaf blight. These include: *P. infestans* on potato and tomato; *P. palmivora* on a large number of tropical fruit species including rubber, durian and macadamia; and *P. colocasiae* on taro. These blights on leaves are first seen as small flecks but within 3-5 days they expand to produce large lesions. Initially, infected tissue is watersoaked but becomes necrotic (brown or black) in a few days. Often the lesions are surrounded by a halo of light green tissue. Spores appear as white velvety growth at the edge of the lesions, primarily at the underside of the leaf. It is

this white growth that distinguishes Phytophthora leaf blight from several other foliar diseases. Often large amounts of sporangiospores are produced as 1-4 sporangiophores extend from the stomata at the underside of the leaf and produce large numbers of sporangiospores which can either be airborne under relatively dry conditions or differentiate into numerous zoospores under wet and humid conditions. These zoospores can encyst and form new lesions on the same leaf or plant and can spread to neighbouring plants through leaf to leaf contact.

1.5.7 Fruit rot

Fruit rot caused by *Phytophthora* appears as watersoaked lesions with light brown centres 3-5 days after infection, depending on the host. The lesions expand rapidly and can completely rot an entire fruit. Under conditions of high humidity, white/grey mycelium may be found behind the advancing margin of the lesions. Often the fruit does not drop and may mummify on the tree. The infection can also be internal as in the case of *P. palmivora* in papaya where mycelial growth can be seen on the seeds after cutting open infected fruit.

1.5.8 Tuber and corm rot

Tubers of potato and corms of taro are considered to be enlarged stem pieces and are susceptible to infection by *Phytophthora*. Potato tubers can be infected by zoospores of *P. infestans* washed down by rain from the leaves. Tuber infections are characterised by patches of brown to purple discolouration on the potato skin. Cutting just below the skin reveals a dark reddish brown, dry corky rot. Heavy infection can give rise to total loss of the tubers. Light infections can occur and are difficult to detect. However, if such potatoes are used as seed potatoes they can infect the emerging stems and start off a new epidemic in the next planting season. This is probably how most late blight epidemics start. Potato can also be infected by *P. erythroseptica* causing the so-called pink rot disease. Infected tubers have a dull brown appearance and exude water under pressure. The cut surface of tubers becomes faint pink after exposure to air. After 30 min the entire cut surface of the tuber turns bright pink. If corms of taro are infected, they stay firm and leathery, which is typical of Phytophthora dry rot. Under favourable conditions, the corms may rot completely after about one week.

2. Media and antibiotics for isolation of *Phytophthora* from diseased plant tissue and soil

The Oomycetes are not true fungi, and therefore special techniques are required for their isolation. Most species of *Phytophthora* grow rather slowly *in vitro* compared with saprophytic fungi and bacteria. In addition, bacterial populations need to be kept low because they may suppress the growth of *Phytophthora* by direct competition, by antagonism caused by antibiotic production, or by direct parasitism. The use of selective media usually overcomes these problems. Antibiotics are added to isolation media in order to suppress the growth of bacteria. Also, because *Phytophthora* spp. are out-competed by many fungi, it is desirable to choose media which are "weak" in nutritional terms. This reduces the growth rate of fungal contaminants, allowing colonies of *Phytophthora* to become established. Synthetic cornmeal agar (manufactured by Difco) is the most frequently used basic medium for isolation of *Phytophthora* from infected plant tissue. However, other desirable basal media include: water agar, or 2% or 4% (v/v) V8 juice agar. Suitable antibiotics that are effective against bacteria include ampicillin, penicillin, rifampicin, and vancomycin, alone or in combination. Suitable antibiotics with antifungal activity include nystatin and pimaricin. Nystatin is usually less inexpensive and more readily available than pimaricin.

2.1 Basic media for isolation from diseased tissue

V8 juice Media

Empty the contents of two 665 ml well-shaken cans of V8 juice into a 2 litre beaker. Add 10 g calcium carbonate (analytical grade), and stir for 20 minutes to adjust acidity. Dilute for media as described below. Unused V8 juice can be stored at –20°C. It must be completely thawed prior to use.

V8 juice agar – for routine growth and maintenance of *Phytophthora* cultures

Dilute amended V8 juice to 20% (v/v) final concentration:

100 ml $CaCl_2$-amended V8 juice
400 ml distilled or deionised water
7.5 g agar

Autoclave at 121°C for 20 minutes. Cool to 55-60°C before pouring plates. Dry plates in the laminar flow cabinet for 30-40 minutes. Wrap in plastic wrap prior to storing in the fridge. Avoid having condensation on the lids of the plates.

Diluted V8 juice agar – for isolation of *Phytophthora* from infected plant material

Amended V8 juice can also be diluted to 2% or 4% (v/v) final concentration
Autoclave as above and cool before adding antibiotics as described in section 2.2.

Cornmeal agar (CMA)

Because CMA is not rich in nutrients, it is very suitable for isolation of *Phytophthora* from infected tissue. Amend with antibiotics as described in section 2.2 once the media has cooled to 50-55°C. If commercially produced cornmeal agar is not available, fresh CMA can be prepared as follows:

Method 1
Cornmeal (polenta) (60 g/litre distilled or deionised water) is autoclaved at 121°C for 15 minutes and filtered. Add 15 g agar and autoclave.

Method 2
Boil 200 g corn (maize) in 200 ml distilled or deionised water for 1 hour, stirring occasionally. Filter through a layer of muslin. Make filtrate up to 1 litre, add 15 g agar and autoclave.

Water agar
Add 15 g agar to 1 litre distilled or deionised water. Autoclave, and add antibiotics (as per section 2.2) once the media has cooled to 50-55°C.

2.2 Selective media for isolation from diseased tissue

The following recipes for selective media are based on commercially prepared cornmeal agar. Diluted V8, fresh CMA, or water agar can also be used as basal media. Add antibiotics as desired from one of the formulations listed below. Most antibiotic stock solutions require filter sterilisation (0.22-0.45 μm) prior to aseptically adding them to autoclaved media cooled to 50-55°C. Stock solutions of antibiotics should be kept at 4°C or –20°C (frozen). Table 2.8 lists common antibiotics, their properties and preparation, and some possible alternatives.

Plates of selective media used for isolations should not contain any free water or condensation on the lids as water encourages the growth and spread of bacterial contaminants. Therefore, dry the plates with the lids half-off in the laminar flow for 20-30 minutes. Store the plates wrapped in plastic in the refrigerator. The plates should be stored upside-down. Note that pimaricin and nystatin are light-sensitive. Media containing these anti-fungal agents should wrapped in foil or black plastic and stored in the refrigerator. Ideally, selective media containing antibiotics should be made fresh before use. Otherwise, they should be used within 2-4 weeks of preparation.

3-P Medium (Eckert and Tsao 1960, 1962)

Add 17 g cornmeal agar (for example, Difco brand) to 1 litre distilled or deionised water. Autoclave at 121°C for 20 minutes and cool to 50-55°C in a waterbath. Add antibiotics to the following concentrations (μg/ml=mg/l=ppm): pimaricin (100), penicillin (50), and polymyxin B (50). Table 2.1 explains which antibiotic stock solutions are required, and what amounts to add to the media. Plates should be stored in a refrigerator (4°C), wrapped in foil or black plastic as pimaricin is light sensitive.

Table 2.1 3-P Medium

Antibiotic	Stock (mg/ml)	Amount (ml) to add to 1 litre media	Final concentration (μg/ml)
Pimaricin	25	4	100
Penicillin	50	1	50
Polymixin B	50	1	50

Note: this medium is suitable for isolation of *Phytophthora* from freshly diseased tissue but not from old, decayed tissue or freshly infested soil in which the propagules are likely to be spores. This is because spore germination can be inhibited by high levels of pimaricin. More suitable concentrations of pimaricin for the purpose of isolation from old plant tissue or soil are 5-10 ppm (Table 2.2).

Table 2.2 3-P Medium with 10 μg/ml pimaricin

Antibiotic	Stock (mg/ml)	Amount (ml) to add to 1 litre media	Final concentration (μg/ml)
Pimaricin	25	0.4	10
Penicillin	50	1	50
Polymixin B	50	1	50

P_{10}VP Medium (Tsao and Ocana 1969)

Cornmeal agar is prepared as above, autoclaved, cooled to 50-55°C and amended with the following (μg/ml): pimaricin (10), vancomycin (200), and pentachloronitrobenzene (PCNB; 100). This medium is suitable for isolating *Phytophthora* from the soil and infected plant tissue (Table 2.3). Hymexazol (see below) can also be added to a final concentration of 25-50 μg/ml.

Table 2.3 P_{10}VP Medium

Antibiotic	Stock (mg/ml)	Amount (ml) to add to 1 litre media	Final concentration (μg/ml)
Pimaricin	25	0.4	10
Vancomycin	100	2	200
PCNB	Not readily soluble	100 mg powder	100

P_{10}ARP Medium (Kannwischer and Mitchell 1978)

Cornmeal agar is prepared as above, autoclaved, cooled to 50-55°C and amended with the following (μg/ml): pimaricin (10), ampicillin (250), rifampicin (10), and pentachloronitrobenzene (PCNB; 100) (refer to Table 2.4). Hymexazol (see below) can also be added to a final concentration of 25-50 μg/ml.

Table 2.4 P_{10}ARP Medium

Antibiotic	Stock (mg/ml)	Amount (ml) to add to 1 litre media	Final concentration (μg/ml)
Pimaricin	25	0.4	10
Ampicillin	100	2.5	250
Rifampicin	10	1	10
PCNB	Not readily soluble	100 mg powder	100

P_5ARP Medium (Jeffers and Martin 1986; Papavizas *et al.* 1981)

As above, with pimaricin reduced to 5 μg/ml (refer to Table 2.5). Hymexazol (see below) can also be added to a final concentration of 25-50 μg/ml.

Table 2.5 P_5ARP Medium

Antibiotic	Stock (mg/ml)	Amount (ml) to add to 1 litre media	Final concentration (μg/ml)
Pimaricin	25	0.2	5
Ampicillin	100	2.5	250
Rifampicin	10	1	10
PCNB	Not readily soluble	100 mg powder	100

Note: P_{10}ARP and P_5ARP are the media of choice for isolating most species of *Phytophthora.*

Hymexazol-amended Medium (Masago *et al.* 1977)

Hymexazol (Tachigaren) is a fungicide which has been found to suppress most *Pythium* spp. except for *P. irregulare* and *P. vexans.* It can also inhibit some *Phytophthora* spp., including *P. cinnamomi, P. citrophthora* and *P. palmivora.*

One litre basic medium is autoclaved, cooled to 50-55°C and amended with the following (μg/ml): benomyl (10), pentachloronitrobenzene (PCNB; 25), nystatin (25), ampicillin (500), rifampicin (10) and hymexazol (25-50) (refer to Tables 2.6 and 2.7).

Hymexazol can also be added to other selective media at a final concentration of 25-50 μg/ml. Make up stock as described in Table 2.8. Add 0.5 ml of this stock to 1 litre of selective media to give a final concentration of 25 μg/ml, or 1.0 ml to 1 litre of selective media to give a final concentration of 50 μg/ml.

Table 2.6 Hymexazol Medium

Antibiotic	Stock (mg/ml)	Amount (ml) to add to 1 litre media	Final concentration (μg/ml)
Benomyl	Not readily soluble	10 mg powder	10
PCNB	Not readily soluble	25 mg powder	25
Nystatin	100	0.25	25
Ampicillin	100	5	500
Rifampicin	10	1	10
Hymexazol	50	0.5	25

Table 2.7 Hymexazol Medium

Antibiotic	Stock (mg/ml)	Amount (ml) to add to 1 litre media	Final concentration (μg/ml)
Benomyl	Not readily soluble	5 mg powder	5
PCNB	Not readily soluble	25 mg powder	25
Nystatin	100	0.25	25
Ampicillin	100	5	500
Rifampicin	10	1	10
Hymexazol	50	1	50

Table 2.8 Some common antibiotics, their properties, preparation and alternatives

Chemical	Activity	Target organisms	Preparation of stock solution[a]	Stock (mg/ml)	Range used (µG/ML)	Comments	Alternative
Ampicillin	Antibacterial	Gram +ve bacteria	Dissolve 1000 mg (1g) powder in 10 ml distilled water. Filter-sterilise	100	250-500		Penicillin G
Benomyl	Antifungal	Most fungi except Zygomycetes and Oomycetes	Benomyl is relatively insoluble in water. Add powder to media *prior* to autoclaving. Agitate media during pouring to ensure even distribution of suspension	-	10-25	Does not suppress many undesirable fungi	
Hymexazol (also called HMI or Tachigaren)	Antifungal	Most *Pythium* spp.	Dissolve 500 mg powder in 10 mL distilled water. Filter sterilise	50	25-50	May inhibit some *Phytophthora* spp.	
Nystatin (Mycostatin)	Antifungal	Most fungi except the Peronosporales	Dissolve 500 mg powder in 5 mL distilled water. Filter-sterilise	100	10-100	Not active against some *Mortierella* spp. Not as active as Pimaricin; used at a higher concentration	Pimaricin
Penicillin G	Antibacterial	Gram +ve and Gram –ve cocci; Gram +ve bacilli	Dissolve 500 mg powder in 10 ml distilled water. Filter sterilise	50	50-100	Not active against Gram –ve bacilli	Supplement with polymixin B at 50–100 µg/ml
Pentachloronitro-benzene (PCNB)	Antifungal	Narrow antifungal spectrum	PCNB is not soluble in water. However, it is heat stable so that the powder can be added to the media *prior* to autoclaving	-	10-100	Does not suppress many undesirable fungi	
Pimaricin	Antifungal	Most fungi except the Pythiaceae	Does not dissolve in water. Mix 250 mg powder in 10 ml sterile distilled water. *Do not* filter-sterilise	25	2-100 Used most frequently at 5-10 µg/ml	Not active against some *Mortierella* spp. Dosage must be restricted to ≤ 10 µg/ml	Nystatin
Polymixin B	Antibacterial	Gram –ve bacteria	Dissolve 500 mg powder in 10 ml distilled water. Filter sterilise	50	20-50		
Rifampicin	Antibacterial	Gram +ve bacteria;Gram -ve bacteria to a lesser extent	Dissolve 100 mg powder in 10 ml ethanol (95%). Filter-sterilise	10	10		Penicillin G and polymixin B
Vancomycin	Antibacterial	Gram+ve bacteria; Gram-ve bacteria to a lesser extent	Dissolve 1000 mg (1g) powder in 10 ml distilled water. Filter-sterilise	100	100-200	Very expensive	Penicillin G and polymixin B

[a] Consider how many litres of Selective Media are required before making up stock solutions because antibiotics are often expensive. Reduce or increase the quantity of stock solution you make, accordingly.

3 Detection and isolation of *Phytophthora* from infected plant tissue and soil

It is estimated that *Phytophthora* species cause 90% of the crown rots of woody plants. However, lack of knowledge on how to isolate *Phytophthora* often leads to negative results and hence other pathogens such as *Fusarium*, *Pythium*, *Rhizoctonia* and nematodes are frequently blamed for root and crown rots (Tsao 1990). Unlike species of *Pythium* and *Fusarium*, which are generally associated with plants and soil, *Phytophthora* species associated with diseased plants are likely to be the causal agent of the disease. This is because most *Phytophthora* species attack only living or freshly wounded tissue. They are primary invaders and hence do not invade plant tissue already invaded by other microorganisms. Detection and/or isolation of *Phytophthora* from plant tissue is relatively simple and successful if the tissue is fresh and recently infected. Isolation of *Phytophthora* from necrotic plant tissue is more difficult because most species of *Phytophthora* have poor saprophytic capabilities, and there may be very little mycelia remaining once the host tissue dies. In addition, dormant propagules such as chlamydospores and oospores are slow to germinate and emerge from senescent plant tissue. Isolation of *Phytophthora* directly from soil is difficult, but the use of baiting techniques markedly increases the frequency of successful isolation of *Phytophthora* from infested soils.

3.1 Selection of samples for isolation of *Phytophthora*

If *Phytophthora* is not recovered from your samples it may be due to the simple fact that *Phytophthora* is absent in your sample. Alternatively, the absence of *Phytophthora* could be due to improper handling and storage of the samples. Negative samples should be baited two to three times to ensure that *Phytophthora* is not present in the soil. In order to successfully isolate *Phytophthora* from soil and plant samples, it is important to take the samples from the correct area of infestation and handle them appropriately.

3.2 Isolation of *Phytophthora* from infected plant material

Phytophthora species attack only healthy plant material, including roots. Thus, the pathogen can be present when no symptoms are obvious. *Phytophthora* species are difficult to isolate from necrotic tissue because the tissue often harbours many secondary pathogens. Therefore, it is very difficult to isolate *Phytophthora* from dead plants. Baiting and isolation is best approached by sampling slightly affected trees or soil taken from trees next to already dead ones.

Successful isolation of *Phytophthora* species from diseased tissue involves careful selection of freshly infected tissue. Therefore it is best to obtain material from the edge of an actively growing lesion. Leaf and stem tissue selected for isolation should ideally contain part diseased and part healthy tissue. Once the tissue has been surface-sterilised, it should be transferred to the appropriate selective medium, and the plates examined regularly for the slow emergence of non-septate hyphae.

Pythium spp. are almost invariably present on both healthy and diseased roots, crowns and lower stems of plants. There are three ways in which contamination of isolation media by *Pythium* can be minimised:

- *Pythium* is confined to roots or badly rotted lower stems – choose other parts if possible
- *Pythium* is confined to the outer cortex of the root – surface sterilisation will usually kill it; alternatively choose the centre of the root
- Hymexazol will inhibit most species except for *P. irregulare* and *P. vexans.* Care must be taken however, as it can also inhibit some *Phytophthora* spp., including *P. cinnamomi, P. citrophthora* and *P. palmivora.* When these species are suspected, it is wise to use selective media with and without hymexazol.

3.2.1 Preparation and surface sterilisation of tissue

Place well-washed roots, stems or leaves suspected to be infected with *Phytophthora* in a shallow layer of pond water or distilled water. Leave for 24-48 hours in the light, at 18-25ºC and examine for sporangial development. If sporangia are found, a small infected plant piece can be cut off, surface sterilised and transferred to selective media.

Infected fruit is easily treated by cutting off the outer parts and placing small pieces of the freshly cut fruit onto selective media. Leaf tissue which is reasonably clean may be placed immediately onto selective media but it is almost always better to surface sterilise it first. Surface sterilise leaf and stem tissue by dipping in 70% ethanol for 30-60 seconds. Blot tissue dry well between sterile filter paper before placing on selective media. If wet plant material is placed onto media, bacteria can grow rapidly and suppress the growth of *Phytophthora.* If the stems are particularly thick (0.5-1 cm wide), they can be dipped in 70% ethanol for 10-30 seconds, and then *quickly* flamed to burn off the excess ethanol. Small sections can then be taken either side of the lesion, and then plated directly onto selective media.

Diseased roots often need more preparation. Place the roots in a beaker and wash them in gently running water for several hours. This process removes the bacteria and stimulates production of sporangia. After washing, cut out small sections of advancing root lesions, surface-sterilise and blot the roots dry between sterile filter paper. Transfer to selective media. Infected root material can be surface sterilised by using either one of the two methods below:

Dip pieces of root tissue in 70% v/v ethanol for about one minute, wash for 10-20 seconds in sterile distilled water and blot dry on sterile filter paper. Cut root pieces into 0.5 cm lengths before placing onto selective media.

Dip root tissue in a 1:10 dilution of commercial bleach (sodium hypochlorite; approx. 0.5% v/v final concentration) for about 30 seconds. Rinse the roots in sterile water and blot dry on sterile filter paper. Cut root pieces into 0.5 cm lengths before placing onto selective media.

Sterilisation with ethanol results in fewer problems with bacterial contamination and gives good recovery of most species of *Phytophthora.* It is also important that root pieces are blotted dry very well and pushed just under the surface of agar instead of just being placed on top. This will ensure good contact between bacteria in the tissue and the antibiotics in the media. *Phytophthora* species will grow through the media quickly leaving bacterial contaminants behind. Refer to *Media and antibiotics for isolation of* Phytophthora *from plant tissue and soil* (section 2).

3.2.2 Isolation from infected plant material by "baiting" with cocoa pods

(method of (Chee and Foong 1968)

Phytophthora can also be isolated from infected plant tissue by baiting. This method is useful for two reasons: (i) the initial steps can be performed in the field, and (ii) surface sterilisation of the baited tissue is usually not required.

1. Core out 8 mm diameter plugs of tissue from a green (unripe) cocoa pod.
2. Insert a wedge of diseased tissue (1 cm wide×2 cm long) into the hole and push it in so that the end is flush with the outside of the fruit. Alternatively, the pod can be cut at an angle and very fine pieces of tissue such as bark inserted into the cuts.
3. Seal the pod in a plastic bag and incubate at room temperature. Up to six wedges can be inserted into a single pod.
4. After 4-5 days, brown discolouration should be obvious around the plugs. A firm rot indicates the presence of *Phytophthora*, a soft rot the presence of saprophytic organisms. Take a small amount of healthy tissue from around the discoloured patch. If the tissue is taken from inside the pod, the tissue does not require surface sterilisation.
5. Plate tissue pieces onto selective media or soil extract agar (see section 4.3 – add 15 g agar/litre soil extract prior to autoclaving).

This method has also been used to isolate *P. nicotianae.* It can also be adapted to isolate *Phytophthora* from the soil (see section 3.6).

3.3 Isolation of *Phytophthora* from soil

The best way to go about sampling soil for *Phytophthora* is as follows: where possible, samples should be taken from moist soil, near healthy roots at least 5 cm below the soil surface. The soil surface is often dry and subject to high temperatures from the sun, making it an inhospitable place for *Phytophthora.* Soil samples are often best taken during or immediately after wet weather, which typically increases *Phytophthora* activity. Sampling is often best under the edge of the plant/tree canopy as root growth is more vigorous there than immediately adjacent to the stem.

Samples should be handled carefully after collection. If soil samples are exposed to drying or high temperatures (+45°C) they will lose their viability. Therefore, samples should not be left in an enclosed vehicle in warm weather. Place your soil samples in plastic bags to prevent drying out and put them in an insulated icebox to prevent overheating. Also, avoid low temperatures as *Phytophthora* does not withstand freezing. Hence, do not put the samples in direct contact with ice. Wrap a few ice blocks in newspaper and add to an icebox to keep the temperature within normal range. In case the samples need to be stored, do not use a refrigerator but place them at 10-15°C and ensure that the samples are moist (add water if the samples are dry). It is best to process samples within a few days but soil samples can be kept like this for a few months. If soil samples dry out during storage, they can be re-moistened for 1-7 days before isolation is attempted. This can stimulate production of sporangia or germination of chlamydospores or oospores.

3.3.1 Detection and isolation of *Phytophthora* by baiting from the soil

Many plant parts can be used to selectively bait a target species of *Phytophthora*. These include: fruits, seeds, seed pods, seedlings, cotyledons, leaves, leaf discs/strips, and petals.

There are essentially three baiting techniques:

1. Insertion of soil or infected tissue into a hole made on a fleshy fruit (e.g. apple, cocoa pod, pear, watermelon). A large fruit is desirable. A detailed method for isolating *Phytophthora* from soil using unripe cocoa pods is given in section 3.6.
2. Planting seeds, seedlings or rooted cuttings into field soil, followed by heavy watering to induce infection.
3. Floating or partial immersing baits of various types in a water and soil mixture. This is the most widely used method for isolating *Phytophthora* spp. Infected plant tissue can often be mixed with the soil to maximise the chance of detection.

The choice of bait is dependent on the species of *Phytophthora* that is suspected to be the causal agent of disease, and the host plant. A list of baiting techniques is provided in Table 3.1. Detailed methods for baiting using lupin seeds or cocoa pods are given in sections 3.5 and 3.6.

For those techniques requiring partial immersion of baits in soil, or floating of baits in soil, high water/soil ratios (4:1 or greater) are desirable. It is desirable to use distilled or deionised water or some other source of water free from chloride or copper ions such as bottled drinking water. Dilution of the soil may also dilute inhibitors present in the soil, enhancing the formation of sporangia and zoospores. Isolations from infected bait material should be made from healthy tissue surrounding lesions. In the case of leaf discs/strips or petals, the entire tissue may be placed on the media. Use the guidelines in section 3.2.1

3.4 Incubation

Most *Phytophthora* species have optimal growing conditions between 15-25°C so incubation should take place within this range. Slightly cooler temperatures which tend to favour *Phytophthora* and slow down bacteria are preferred. Plates should be incubated in the dark, and if light-sensitive antibiotics are used, the plates should also be wrapped in black plastic or foil. Because of heavy sporulating fungi and mite infestations that may pose a serious contamination threat, place your plates in a separate incubator away from clean *Phytophthora* cultures. Ideally, isolations from plants and soil should be performed and stored in a “rough” laboratory away from clean cultures. It is important to note that propagules of *Phytophthora* in the soil or senescent diseased tissue are dormant and may germinate slowly. Therefore, it could take 2-20 days before colonies are visible on isolation plates.

Table 3.1 Baiting techniques for isolating *Phytophthora* from soil

Species	Bait material	Procedure	Reference
P. cinnamomi	Apple or pear	Make holes in fruit. Fill with soil. Wet soil. Incubate covered with plastic bag at 15-27ºC for 5-10 days. Isolate from the edge of the rotted area around the hole. Suitable technique for many *Phytophthora* spp.	(Campbell 1949)
	Apple slices	Immerse slices in 200 ml water to which 25 g soil has been added, for 4-10 days	(Gerrettson-Cornell 1974)
	Avocado fruit	Embed fruit partially in flooded soil. Incubate at 20-27ºC for 2-4 days.	(Zentmyer *et al.* 1960)
	Avocado seedlings	Plant seedlings in wet soil. Incubate at 21-27ºC for 2-3 days.	(Zentmyer 1980)
	Avocado leaf pieces	Float leaf pieces on water added to soil for 4 days	(Pegg 1977)
P. citrophthora	Apple, lemon or orange fruit	Insert soil or citrus tissue into fruit as per Campbell (1949) for *P. cinnamomi.* Alternatively, place lemon or orange on the surface of soil for 4 or more days	(Klotz and DeWolfe 1958)
	Lemon fruit	Immerse partially in 150 ml water to which 25 cc soil has been added. Incubate at 25ºC for 6 days	(Tsao 1960)
	Lupin radicles	see Dance *et al.* (1975) under *P. cryptogea*	
P. heveae	Apple fruit, eggplant fruit, cocoa pod	No method is given but the baits could have soil inserted into them as per the apple method of Campbell (1949), or they could be embedded in partially flooded soil as per Zentmyer *et al.* (1960) for *P. cinnamomi.*	(Lee and Varghese 1974)
P. nicotianae	Apple, lemon or orange fruit	see Klotz and DeWolfe (1958) under *P. citrophthora*	
	Citrus leaf pieces	Float small leaf pieces on water 1-2 cm above 100 cc soil. Incubate at 22-28ºC for 3-4 days	(Grimm and Alexander 1973)
	Cocoa pods	Insert soil or diseased rubber tissues into unripe green pods as per the apple method of Campbell (1949). Incubate at 26-30ºC for 4-5 days	(Chee and Foong 1968)
	Lemon fruit	see Tsao (1960) under *P. citrophthora*	
	Tobacco leaves	Immerse the petiole end of leaf in water-soil mixture as per Tsao (1960) under *P. citrophthora*	(Jenkins 1962)
P. palmivora	Apple fruit	as per the apple method of Campbell (1949)	
	Apple fruit, eggplant fruit, cocoa pod	No method is given but the baits could have soil inserted into them as per as per the apple method of Campbell (1949), or they could be embedded in partially flooded soil as per Zentmyer *et al.* (1960) for *P. cinnamomi.*	(Lee and Varghese 1974)
	Black pepper leaves	Immerse black pepper leaves or leaf discs partially in a water-soil mixture. Used for isolation from black pepper soils	(Holliday and Mowat 1963)
	Cocoa pods	Place on or in soil. Or, inoculate pod surface with a small amount of soil suspension. Incubate in a plastic bag for 1-4 days. Used for isolation from cocoa soils	(Orellana 1954)
	Cocoa pods	Insert soil beneath flaps of endocarp tissue of unripe cocoa pods. Used for isolation from cocoa soils	(Turner 1965)
	Cocoa pods	see Chee and Fong (1968) under *P. nicotianae*	
	Cocoa pods and tissues	Place pod or pod tissue on soil, or in flooded soil. Soil can also be placed inside the pod	(Newhook and Jackson 1977)
	Taro roots	Cut roots into 2.5 cm lengths, autoclave. Incubate in moistened soil (50 g/Petri dish) at 15ºC for 7 days	(Satyprasad and Ramaro 1980)

3.5 Lupin baiting of *Phytophthora* from soil samples

This method is a modification of a method from (Pratt and Heather 1972). If lupins are not available, soybean seeds are also suitable.

Materials required:
Plastic cup (225-250 ml) with lid for each sample
Lupin seeds
Vermiculite
Distilled and sterile distilled water

1. Surface sterilise lupin seeds in 70% alcohol for 2 minutes, rinse in sterile distilled water and then soak for 1 hour in sterile distilled water. Four to five seeds will be required for each sample, depending on germination.
2. Pre-germinate lupins in sterile vermiculite at room temperature. Water only with sterile distilled water and ensure that the water drains from the seeds. Lupin radicles need to be 2-3 cm long for baits - this will take 2 to 3 days.
3. Punch or melt 5 holes, 5 mm in diameter in lids of plastic cups.
4. When lupin radicles are at the desired length, place a layer of soil sample approximately 3 cm deep in the bottom of plastic cup. Fill cup with distilled water to 1 cm from top and cap with lids with pre-punched holes. Place pre-germinated lupins on top of lid, with radicle inserted through hole and into water (see Figure 3.1). Do not overfill the cups with soil. We recommend 5-10 times the volume of water compared to the volume of soil for best results.
5. Check baits after 2 days, up until 7 days. Brown, discoloured lesions on the lupin radicle should be surface sterilised and plated onto a selective medium. Soft rots are those usually associated with *Phytophthora.*

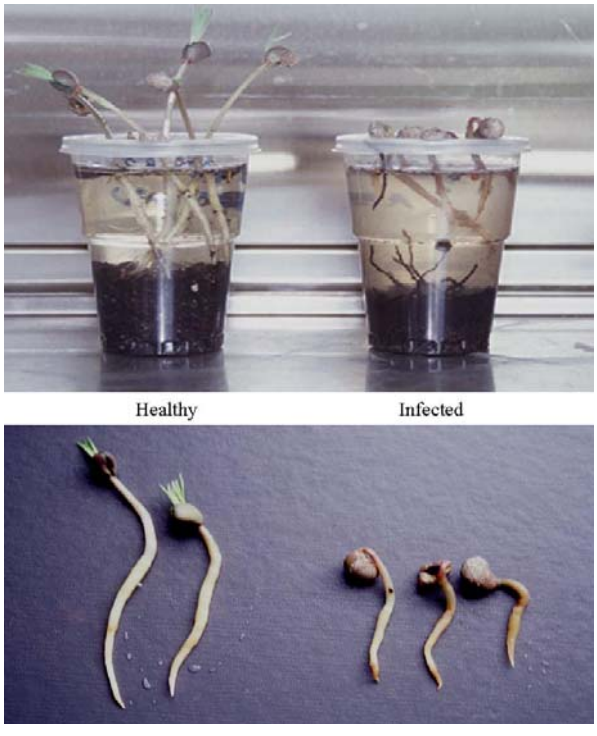


Figure 3.1 Lupin baits. *Phytophthora* can be baited out of the soils using lupins suspended in slurries made from soil samples in deionised water.

3.6 Baiting of *Phytophthora* from soil samples using cocoa pods (method of (Chee and Foong 1968)

This method has been used to isolate *P. palmivora* and *P. meadii* from soil.

1. Core out 2 cm diameter plugs of tissue from a green (unripe) cocoa pod.
2. Insert moistened soil into the hole, completely filling it.
3. Seal the pod in a plastic bag and incubate at room temperature. Up to six samples can be inserted into a single pod.
4. After 4-5 days, brown discolouration should be obvious around the plugs. A firm rot indicates the presence of *Phytophthora*, a soft rot the presence of saprophytic organisms. Take a small amount of healthy tissue from around the discoloured patch. If the tissue is taken from inside the pod, the tissue does not require surface sterilisation.
5. Plate tissue pieces onto selective media or soil extract agar (see section 4.3 – add 15 g agar /litre soil extract prior to autoclaving).

3.7 Culturing and storage of *Phytophthora*

3.7.1 Culturing

Most *Phytophthora* species grow well on a range of media including V8. Cultures of *Phytophthora* should be grown at 15-25 °C in a dark incubator. Cultures should be transferred every two to four weeks to maintain vigour. For long-term storage, water storage is recommended (see below).

The pathogenicity of *Phytophthora* cultures is known to decrease after prolonged storage on media. In case pathogenicity studies need to be performed, serial passage through the host plant is required. Another alternative is storage of cultures in liquid nitrogen (see below) which seems to overcome the problem of loss of pathogenicity.

3.7.2 Long term storage in sterile water

Phytophthora strains should be maintained as living cultures for two reasons. (i) to provide other researchers and ourselves with reference strains for various studies involving pathogenicity, virulence, mating type etc. and (ii) as a source of DNA for genetic diversity and evolutionary studies. *Phytophthora* cultures can be stored in two ways, either in sterile water or in liquid nitrogen.

To store cultures of *Phytophthora,* cut 8-10 small blocks from an actively growing plate culture, and place in small screw capped glass bottles containing autoclaved distilled water. The caps should be tightened during storage and the vials placed at room temperature in the dark. Most species of *Phytophthora* can be stored this way but keep in mind that the isolates will lose pathogenicity and aggressiveness during storage and cannot be used for studies in that area after prolonged storage. Ideally cultures should be revitalised once a year or every second year. For some species, a soybean seed or corn seed can be added to prior to autoclaving the water which seems to induce oospore formation in homothallic species.

3.7.3 Long term storage in liquid nitrogen

Cryopreservation of *Phytophthora* cultures in liquid nitrogen at –196°C is a good way to store most *Phytophthora* species. The cultures are maintained in their original genetic state and do not seem to lose their pathogenicity or aggressiveness. In addition liquid nitrogen storage is very effective and time efficient since the only requirement is maintaining the supply of liquid nitrogen. Our laboratory has a contract with a supplier of liquid nitrogen, which tops up the tanks fortnightly at minimal cost. However, the setup costs and maintenance of such a facility are much higher than storage under water and is only worthwhile for large culture collections and specialised laboratories with cheap and reliable access to liquid nitrogen.

3.7.4 Liquid nitrogen storage protocol

For storage in liquid nitrogen a cryoprotectant is needed to protect the *Phytophthora* cultures during the process of freezing. We routinely use a mixture of 5% DMSO and 5% glycerol as cryoprotectant. The key to successful cryopreservation of *Phytophthora* species is slow cooling, less than 1°C per minute to -40°C. The reason for this is that at faster cooling rates, ice nucleation damages the cell membranes causing loss of viability of the cultures. Slow cooling can be achieved either through expensive specialised controlled cooling equipment or as we do it by placing the vials with cryoprotectant in a styrofoam box. This box is then placed in a -70°C freezer overnight. The insulation of the box will ensure slow cooling down to -70°C. From the -70°C freezer the isolates can then be placed directly into a liquid nitrogen storage container. For liquid nitrogen storage, it is best to grow *Phytophthora* on media with a poor nutritional value as this promotes sporulation. Small discs of agar containing mycelium and spores can be placed into the cryoprotectant and preserved that way. For profusely sporulating species such as *P. infestans* and *P. palmivora*, plates can be flooded and the resulting spore suspension added to the vials. Then add the cryoprotectant in a concentrated form so as to give a minimal dilution of the concentrated sporangia. Sporangiospores can also be harvested from plant tissue for storage in liquid nitrogen. However, it is important to remember that they must be placed back on plant tissue after storage in liquid nitrogen, and that they probably contain bacterial contaminants.

Thawing after storage is less critical and vials can be taken out of liquid nitrogen and placed at room temperature. After 10-20 minutes they are thawed and the small agar blocks can be transferred onto culture plates. Place a block of agar on the media and then push it over the media to dilute out the cryoprotectant. Alternatively, the small blocks can be blotted dry on sterile filter paper to remove most of the cryoprotectant. This will ensure rapid recovery of the cultures. However, this may also result in a loss of sporangiospores. Up to three little blocks from one vial can be placed onto a single culture plate. Once vials have been thawed they cannot be stored again. Hence, multiple copies of the samples must be stored.

Some *Phytophthora* species store better than others in liquid nitrogen. Species that sporulate profusely such as *P. infestans* and *P. palmivora* can easily and reliably be stored using many different cryoprotectants and freezing protocols. Poorly sporulating species such as *P. cinnamomi* tend to have a lower recovery rate and are often slow to recover from liquid nitrogen storage.

4 Identification of *Phytophthora*

Many species of *Phytophthora* can be easily identified. However, the morphological differences among some species are few and variable, making it difficult to classify the species accurately. Identification of *Phytophthora* is based on the taxonomic keys of Waterhouse (1963) and Stamps *et al.* (1990). Characters which are used to classify species of *Phytophthora* include: sporangium morphology; morphology of sexual structures such as antheridia, oogonia and oospores; presence or absence of chlamydospores, and morphology of hyphae. A list of terms used to describe the morphology of *Phytophthora* is provided in section 4.7 of this chapter. In this section, a very simplified methodology for identification of a few common species of *Phytophthora* found in the tropics will be described. The information below should be considered as a first step in the identification of *Phytophthora* species.

In many cases, the suspect *Phytophthora* species isolated from diseased tissue or infested soil can be narrowed down according to the host from which it was isolated. Table 4.1 provides a list of species likely to be encountered in Southeast Asia and the hosts they attack.

Table 4.1 *Phytophthora* species commonly found in southeast Asia

Species	Hosts	Some diseases caused
P. capsici	many, including *Capsicum, Piper nigrum* (black pepper), *Theobroma cacao* (cocoa), *Macadamia, Hevea* (rubber), *Carica papaya* (papaya)	Foliar blight and root and crown rot of *Capsicum* Fruit and root rot of cucurbits (melon, cucumber, squash) Blight of foliage, flowers and nuts of macadamia Foot rot of black pepper
P. cinnamomi	numerous (> 1000 species) including *Persea* (avocado), pineapple, macadamia	Root rot of pineapple Foliar blight and trunk canker of macadamia Root rot of avocado
P. citrophthora	various fruit trees, rubber, avocado, cacao, rubber	Crown rot, gummosis, fibrous root rot, and brown rot of *Citrus* Damping off of citrus seedlings
P. colocasiae	mainly *Colocasia esculenta* (taro, dahseen, gabi); also found on *Alocasia* spp. (yam), *Piper betle* (betel),	Foliar blight of taro Foliar blight, corm rot and stem canker of other species
P. heveae	rubber, avocado, mango, cacao and *Cocos nucifera* (coconut)	Pod rot and black stripe of rubber Bud rot and nut fall of coconut Trunk canker of avocado Pod rot of cacao
P. infestans	main hosts are species of *Solanum*	Leaf and stem blights of potato and tomato
P. nicotianae	numerous; including avocado, black pepper, betel vine, coconut, citrus, macadamia, papaya, pineapple, rubber, taro, tobacco	Black shank of tobacco Brown rot, foot rot, gummosis, and root rot of citrus Heart rot of pineapple Crown rot and blight of macadamia
P. palmivora	numerous; including avocado, black pepper, betel vine, cacao, citrus, coconut, macadamia, various spp. of palms, papaya, pineapple, rubber, taro, tobacco	Black pod, stem canker and chupon wilt of cacao Root and fruit rot of papaya Bud rot and premature nut fall of coconut and other palms Patch canker of durian Black stripe, patch canker, green pod rot, abnormal leaf fall and green twig blight of rubber Foot rot of black pepper

4.1 Cultures

Ideally, the culture used for species identification should be obtained from a hyphal tip, or a single germinated zoospore cyst, sporangium or oospore. It is important to remember that on selective media most *Phytophthora* species will not sporulate and form characteristic propagules for identification. Therefore, cultures should be incubated at the optimum temperature for the suspected species, on a natural medium such as V8 juice (see section 2.1), carrot agar or Lima bean agar (see section 4.8.3). In order to identify an isolate of *Phytophthora* to species level, it is necessary to induce the production of asexual and sexual structures that will aid in species identification. In addition, characteristics of the mycelium, and whether the culture produces chlamydospores will also assist in identification. Methods for producing asexual and sexual structures are described in sections 4.3 to 4.5.

4.2 Morphological characters

There are a number of morphological characters upon which identification of *Phytophthora* species is based. These include: sporangium shape, papillation, and caducity; sporangiophore morphology; presence of chlamydospores and hyphal swellings; antheridial attachment, and whether sexual reproduction is heterothallic or homothallic.

4.3 Sporangia

Sporulation in *Phytophthora* cultures provides important clues for species identification. Important characters to observe are:

- sporangium morphology (shape, size, length:width ratio) (see Figure 4.1)
- papillation of the sporangium (see Figure 4.2)
- caducity (shedding of the sporangium at maturity)
- length of the pedicel on the sporangium
- proliferation of sporangium (production of new sporangium within a sporangium that has germinated directly)
- branching of the sporangiophores on which the sporangia are borne (see Figure 4.3)

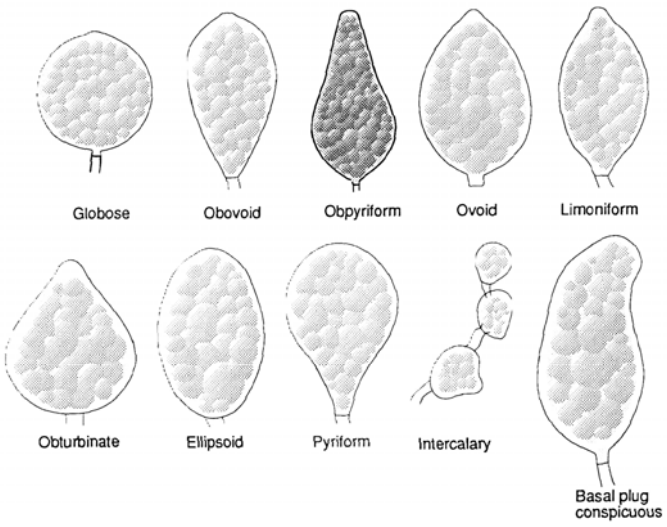
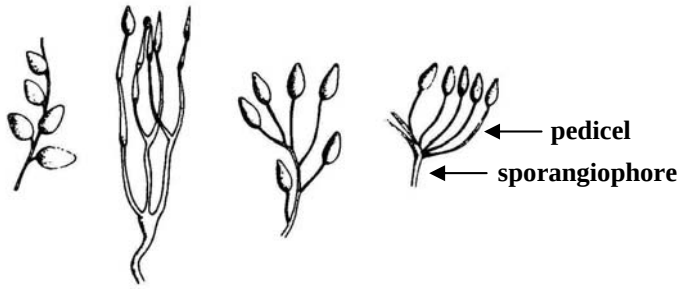


Figure 4.1 Sporangium shape

Non-papillate Semi-papillate Papillate

Figure 4.2 Sporangium papillation



Simple sympodium (left) **Compound sympodia** (middle)
Umbellate sympodium (right)

Figure 4.3 Sporangiophore morphology

Some species of *Phytophthora* produce sporangia readily on the surface of agar media. However, many species need to be cultured in water, mineral salt solutions or dilute soil extracts before they will produce sporangia. It is important to remember that sporangia production in *Phytophthora* is dependent on light (Schmitthenner and Bhat 1994). Table 4.2 provides a general guide to which species of *Phytophthora* produce sporangia on agar media.

Table 4.2 Sporangia production on solid or liquid media

Sporangia produced on agar	Sporangia produced in liquid media
P. capsici	*P. cambivora*
P. heveae	*P. cinnamomi*
P. megakarya	*P. citricola*
P. nicotianae	*P. cryptogea*
P. palmivora	*P. drechsleri*

4.3.1 Induction of sporangia in liquid culture

Distilled water

Cut 0.5 cm^2 agar discs from the edge of a colony that has been grown on V8 juice agar or carrot agar. Cultures 2-4 days old are most suitable. Incubate the discs in a shallow layer of distilled water (or pond water or Salt Solution or Soil Extract) in a Petri dish, at

room temperature (22-24°C). Incubation under continuous fluorescent light is recommended. Sporangia are produced within 12 hours in some species, and typically within 1-2 days.

Pond water
Some species of *Phytophthora* will produce sporangia when agar blocks of a culture are transferred from Lima bean agar to non-sterile pond or stream water. Incubate as per the *Distilled water* method.

Salt Washing
This method is useful for *P. cinnamomi.*
1. Grow colonies on dilute Lima bean agar (1.5%), and transfer 0.5 cm^2 agar discs from the colony edge to 25 ml Lima bean broth, removing as much agar as possible.
2. After 40-48 hours, pour off the culture medium and replace with 25 ml Chen-Zentmyer's Salt Solution (see section 4.8).
3. After an hour, tip off the Salt Solution and replace with fresh solution.
4. Repeat three more times.
5. Incubate in the light after the final wash. Sporangia should develop within 5-10 hours.

Soil Extract
1. Mix 10 g of soil with 1 litre of distilled water.
2. Filter through filter paper.
3. Autoclave extract. Autoclaving is not recommended if the suspected pathogen is *P. cinnamomi.*
4. Incubate agar blocks of culture as per the *Distilled water* method.

4.4 Chlamydospores and hyphal swellings

Chlamydospores are thick-walled spores that function as a resting spore. Chlamydospores can be intercalary (formed between hyphae) or terminal (on the ends of hyphae). They differ from hyphal swellings by having thick walls and are delimited from the mycelium by septa. The morphology of chlamydospores does not differ greatly between species and therefore these spores are of limited use in species identification. However, the presence (for example, *P. palmivora*) or absence (for example, *P. heveae*) of chlamydospores can aid species identification. Chlamydospores are generally produced readily in agar or water culture.

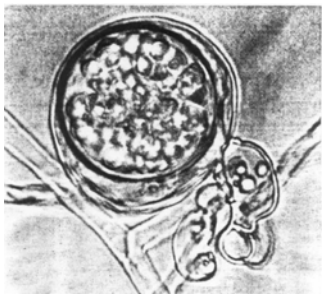
4.5 Sexual Structures

Production of oogonia, antheridia and oospores and mating type tests for heterothallic species

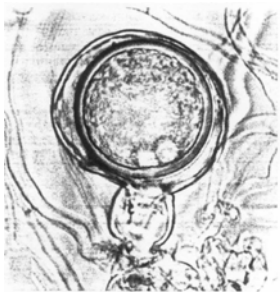
Approximately half of the species of *Phytophthora* are homothallic. They will therefore produce oogonia, antheridia, and oospores in single culture. The remainder are heterothallic, with two mating types, A1 an A2. Heterothallic species produce gametangia (oogonia and antheridia) only in the presence of an isolate of the opposite mating type on the same plate. For species identification, it is important to determine if a culture is homothallic or heterothallic, and whether the antheridium is amphigynous (around the oogonial stalk) or paragynous (next to the oogonial stalk) (see Figure 4.4). It is important that mating type tester isolates are obtained from a reliable culture

collection. Please contact us for more information about where tester isolates of the different *Phytophthora* species can be obtained.

A number of media are suitable for mating type tests, including cornmeal agar, carrot agar and Lima bean agar. Although the majority of species of *Phytophthora* produce oospores in culture, some species require specialised media containing additives such as sterols to induce oospore formation. In general it is best to start with carrot agar which works for most species. Place a 0.5 cm^2 plug of culture of the unknown isolate on one side of the Petri dish. Place an agar plug from the known A1 or A2 tester isolates on the other side of the dish. Incubate plates in the dark at the optimal temperature for the species being examined. Oospores should form at the junction of the two colonies after 7-14 days if the isolates are of different mating types.



Immature oospore with a paragynous antheridium



Mature oospore with an amphigynous antheridium

Figure 4.4 Attachment of the antheridium

4.6 Differences between *Pythium* and *Phytophthora*

Phytophthora and *Pythium* belong to the Family Pythiaceae and hence are very closely related genera. Differences between the two include:

1. Production of zoospores – in *Phytophthora*, the zoospores are produced within the sporangium, in *Pythium*, the zoospores develop within a vesicle produced by the sporangium (see Figure 4.5). This is the most important distinguishing feature between *Pythium* and *Phytophthora*. Therefore, points 2 and 3 below are provided for your information only.
2. Differences in the sporangia – the sporangia of *Phytophthora* are always terminal and usually ovoid or obpyriform in shape whereas sporangia of *Pythium* may be globulose, lobate (many lobed), or filamentous and are frequently intercalary.
3. Differences in the antheridia – in *Pythium*, the antheridia are paragynous and may be attached at any point on the oogonium whereas in *Phytophthora*, the antheridium attaches only at the lower hemisphere of the oogonium. In addition, in some species of *Pythium*, many antheridia may be attached to a single oogonium.

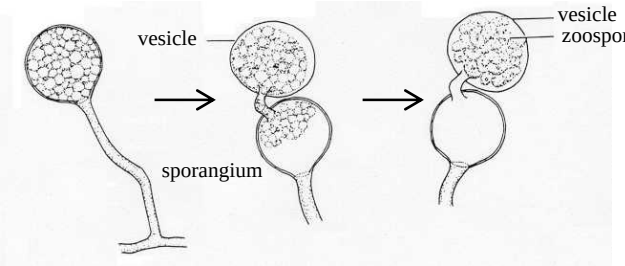


Figure 4.5 Vesicle development in *Pythium*. A vesicle develops from the sporangium, and the zoospores are produced within the vesicle. In *Phytophthora*, vesicles are not produced.

4.7 List of Terms

amphigynous the oogonium grows through the antheridium so that the antheridium surrounds the oogonial stalk

antheridium the male sex organ (gametangium)

caducous describes sporangia that separate readily from the sporangiophore

chlamydospore resting spore that is separated from the mycelium by a septum; can be terminal (on the end of a hypha) or intercalary (within a hypha) with a thickened wall. Survives for long periods in the soil

heterothallic sexual reproduction that involves cross-fertilisation of isolates of different mating types, A1 and A2

homothallic sexual reproduction by self-fertilisation (only one mating type is required)

oogonium the female sex organ (gametangium) in which the oospore forms after fertilisation by the antheridium

oospore zygote (or thick-walled spore) that forms within the oogonium after fertilisation

papilla thickening at the apex (top) of the sporangium

paragynous the antheridium is attached to the side of the oogonium; characteristic of Groups I, III and V

pedicel stalk which attaches the sporangium to the sporangiophore

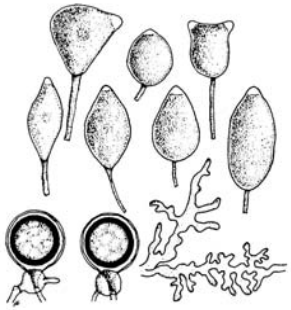
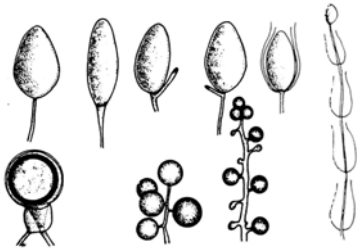
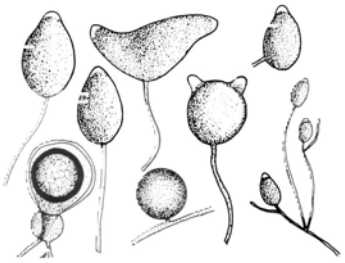
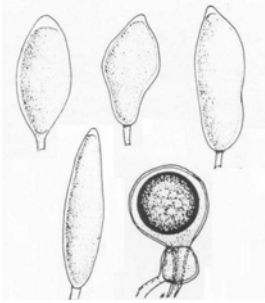
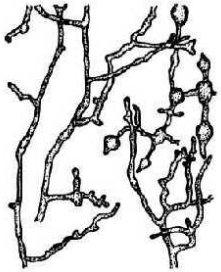
sporangiophore stalk on which the sporangium is borne; may be simple or branched

sporangiospore motile spore that emerges from a germinated sporangium (also called a zoospore)

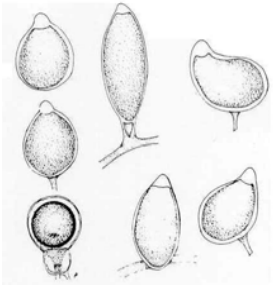
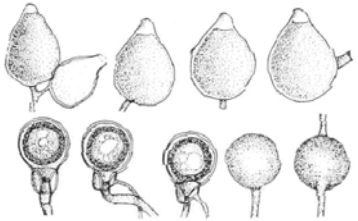
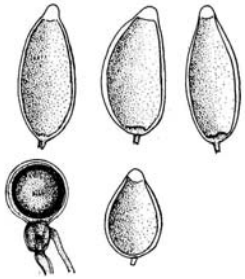
sporangium sac within which zoospores form

Table 4.3 Morphological characteristics of some *Phytophthora* species (diagrams and micrographs reproduced from Erwin and Ribeiro, 1996)

Species	*Phytophthora capsici*	*Phytophthora cinnamomi*	*Phytophthora citrophthora*	*Phytophthora colocasiae*
Waterhouse Group	Group II	Group VI	Group II	Group IV
Mating system	Heterothallic	Heterothallic	Sexual reproduction rare	Heterothallic
Optimum growth temperature	28°C	24-28°C	24-28°C	27-30°C
Morphology				
Sporangia: Papillation	Papillate or semi-papillate (sometimes with 2 or 3 apices)	Non-papillate	Papillate	Semi-papillate. A conspicuous basal plug is seen where the sporangium meets the sporangiophore
Shape	Ovoid, obovoid, ellipsoid	Includes ovoid, obpyriform, ellipsoid. Tapered at the base.	Varies from spherical, ovoid, obpyriform, obturbinate to ellipsoidal	Includes ovoid, ellipsoid, and fusiform. Tapered base, sometimes with attachments
Attachment	Caducous with long pedicels	Non caducous. Borne terminally	Non-caducous. Usually terminal, sometimes lateral or intercalary	Caducous
Sporangiophores	Sporangiophores irregularly branched	Usually unbranched	Irregularly branched	Irregularly branched
Length:width ratio of sporangia	Varies from 1.3 to 2.1	>1.5	Varies from 1.2 to 2.0	1.6
Antheridia	Paragynous	Amphigynous	Sex organs not produced in nature	Amphigynous, sub-terminal
Oogonia	Globose	Globose, with smooth, thin walls	Globose	Globose
Oospores	Spherical to subspherical 15-40 μm	Round, thin walled 19-54 μm	Produced only rarely in culture	18-30 μm
Chlamydospores	Produced abundantly by isolates from cacao, black pepper, macadamia	Produced abundantly in culture, globose, thin-walled. Terminal or intercalary, often in grapelike clusters of 3-10	Reported only in isolates from cacao	Abundant in some isolates, rare in others. Borne terminally or intercalary in the mycelium
Mycelium	Torulose, with hyphal swellings	Coralloid, with abundant hyphal swellings and vesicles	Smooth or coarse; hyphal swellings present in some isolates	Hyphal swellings are not produced
Distinguishing characters	Long pedicels on caducous sporangia, compared with round, non-caducous sporangia in *P. nicotianae* and ovoid, caducous sporangia on short pedicels in *P. palmivora*	Mycelial morphology	None reported	None reported

Species	*Phytophthora capsici*	*Phytophthora cinnamomi*	*Phytophthora citrophthora*	*Phytophthora colocasiae*
Morphological characteristics	 Papillate sporangia, caducous with long pedicels. Note bipapillate sporangia (top right). Extensive hyphal swellings on mycelium	 Non-papillate sporangia. Note the internal proliferation of sporangia. Globose oogonium with paragynous antheridium (bottom left). Note the globose chlamydospores (bottom middle)	 Papillate sporangia, some with two papillae. Note the variation in sporangial shapes. Antheridium is amphigynous (bottom left)	 Semi-papillate sporangia with short pedicels
ADDITIONAL FIGURES AND NOTES	 Torulose hyphae with hyphal swellings	 Coralloid hyphae with hyphal swellings	In the tropics and sub-tropics, *P. nicotianae*, *P. cinnamomi* and *P. palmivora* are also pathogens of citrus. *P. citrophthora* is active at moderate temps (30°C) and *P. nicotianae* at higher temps (>30°C)	

Species	***Phytophthora heveae***	***Phytophthora infestans***	***Phytophthora nicotianae***	***Phytophthora palmivora***
Waterhouse Group	Group II	Group IV	Group II	Group II
Mating system	Homothallic	Heterothallic	Heterothallic	Heterothallic
Optimum growth temperature	25°C	20°C	27-32°C	27-30°C
Morphology				
Sporangia: Papillation	Papillate	Semi-papillate	Papillate	Distinctly papillate
Shape	Irregular, obpyriform to ellipsoid	Ovoid, ellipsoid to limoniform	Varies from ellipsoid, ovoid, pyriform, obpyriform to spherical, with a prominent papilla	Mainly ellipsoid and ovoid, pyriform
Attachment	Caducous	Caducous	Non-caducous	Caducous
Sporangiophores	Sporangiophores irregularly branched. Sporangia often form laterally on sporangiophores	Sporangiophores are compound sympodial, with a small characteristic swelling just below the sporangium	Sporangia irregularly or sympodially branched	Sporangia occur in groups of up to 20 on one sympodium. Pedicels are short
Length:width ratio of sporangia	1.5	1.6	Varies from 1.1-1.7	Varies from 1.7-1.9
Antheridia	Amphigynous, spherical or cylindrical, sometimes bicellular	Amphigynous	Round or oval, amphigynous	Amphigynous
Oogonia	Globose, funnel-shaped and produced readily in culture	Spherical to obpyriform, smooth walled	Spherical and smooth-walled	Spherical
Oospores	Oospores round, smooth and thick-walled, 15-28 μm	30 μm	24 μm	23 μm
Chlamydospores	Not produced	Not recorded	Produced abundantly by most isolates, terminal or intercalary	Globose to subglobose, terminal or intercalary
Mycelium	Hyphal swellings not produced	Hyphal swellings not produced	Hyphal swellings have been noted	Hyphal swellings have been noted in some isolates
Distinguishing characters	*P. heveae* distinguished from *P. colocasiae*, *P. nicotianae* and *P. palmivora* by lack of aerial mycelium, and the absence of chlamydospores	None recorded	None recorded	*P. palmivora* is distinguished from other heterothallic species by the production of conspicuous papillate sporangia on short pedicels, and chlamydospore production

Species	*Phytophthora heveae*	*Phytophthora infestans*	*Phytophthora nicotianae*	*Phytophthora palmivora*
Morphological characteristics	 Papillate sporangia with variable shapes. Sporangia often form laterally on the sporangiophore (bottom middle)	 Semi-papillate, caducous sporangia (left). Amphigynous antheridia. Compound sympodial sporangiophore (right).	 Papillate sporangia. Amphigynous antheridia. Terminal and intercalary chlamydospores (bottom right)	 Distinctly papillate, caducous sporangia. Sporangia often clustered. Pedicels very short. Amphigynous antheridia
Additional figures and notes			 Micrograph of sporangia of various shapes	 Micrograph of an ovoid sporangium

4.8 Media

4.8.1 Carrot agar

1. Wash 200 g carrots and slice thickly.
2. Autoclave carrots in 500 ml distilled or deionised water.
3. Blend warm carrots in a blender at high speed for 40 seconds.
4. Squeeze homogenate through 4 layers of muslin.
5. Make filtrate up to 1 litre with deionised water.
6. Add 15 g agar and autoclave.
7. Pour plates thinly for easier microscopic examination. Yields 40-50 plates.

4.8.2 Dilute Lima bean agar or broth

1. Autoclave frozen (not dried) Lima beans (50 g) in 1 litre of distilled or deionised water for 15 minutes.
2. Strain broth through muslin, adjust to 1 litre. Add 15 g agar and autoclave again.
3. For Dilute Lima bean broth, do not add agar prior to autoclaving. Store in the dark.

4.8.3 Lima bean extract agar

1. Autoclave frozen (not dried) Lima beans (285 g) in 1 litre of distilled or deionised water for 15 minutes.
2. Strain broth through muslin, adjust to 1 litre. Add 15 g agar and autoclave again.
3. Broth is made by not adding agar prior to autoclaving. Store in the dark.

This medium is suitable for induction of sporangia in *P. capsici, P. erythroseptica, P. heveae* and *P. infestans.*

4.8.4 Salt Solution of Chen and Zentmyer (1970)

Dissolve the following in 1 litre distilled water:

Calcium carbonate $Ca(NO_3)_2$:	1.64 g
Potassium nitrate KNO_3:	0.05 g
Magnesium sulphate $MgSO_4$	0.48 g

Add 1 ml chelated iron (see below), and adjust pH to 7.0.

Chelated iron is made by dissolving the following ingredients in distilled water, and making the solution up to 1 litre:

Ethylenediaminetetra-acetic acid (EDTA)		13.05 g
Potassium hydroxide	(KOH)	7.5 g
Ferrous sulphate	($FeSO_4$)	24.9 g

5 Plant inoculation tests with *Phytophthora* species

Once a *Phytophthora* species has been isolated, brought into pure culture and identified down to species level further research concerning the pathology often involves inoculation of the host plant. There are numerous reasons for inoculating host plants including:

- Determining the pathogenicity (ability to cause disease) of a particular *Phytophthora* species on a host. This is especially important in case the particular species of *Phytophthora* has not been associated with this host before. In such a case it is vital to prove Koch's postulates by showing that the strain causes the disease symptoms and can be isolated from the host again.
- Determining the aggressiveness of the *Phytophthora* isolates. Plant inoculation tests can determine whether different isolates of the same species or isolates representing different species cause the same level of disease on a particular host.
- Determining the virulence of particular isolates. These tests are performed in case there are different strains of *Phytophthora* belonging to the same species that can overcome particular disease resistance genes in the host plant. These tests are typically done with host specific *Phytophthora* species such as *P. sojae, P. infestans, P. vignae* etc.
- Screening germplasm for disease resistance using a particular strain or species of *Phytophthora*.

There are many ways in which plant inoculation tests can be performed depending on the question asked, the species of *Phytophthora*, the host plant species involved and the maturity of the host plant. In general the following methods are often used:

1. Inoculation of sporangia onto leaf or fruit tissue.
2. Insertion of mycelium into the stems of young seedlings.
3. Insertion of pieces of agar with mycelium into holes in the stem of woody adult plants.
4. Inoculation of soil and planting of young plants into this infected soil.

These methods are briefly discussed below to provide a starting point for plant inoculation tests. For each particular host-pathogen combination we strongly suggest you consult the literature for more detailed and specific protocols and procedures.

5.1 Inoculation of sporangia onto leaf or fruit tissue

These tests are often performed to assess resistance in different cultivars or lines of a host to different strains of a single *Phytophthora* species. Typically this is done with species causing foliar diseases, such as *P. infestans* on potato and tomato; *P. colocasiae* on taro and *P. palmivora* on leaves of tropical fruit crops such as durian. Sporangiospores of *Phytophthora* can be harvested from infected lesions taken from inoculated plants, or from culture plates by flooding the plates with distilled water and harvesting the spores a few hours after flooding. As a general guide, a small drop (10-50 µL) of water containing 10^4 sporangia/mL is placed on a detached leaf. It is best to use young just fully expanded leaves, which should be kept in a tray under moist conditions to favour development of disease but not too wet to favour general rot. Detached leaves can be placed on agar or stuck into foam to prevent them from drying out. It is best to place the leaves on a mesh, which is subsequently placed on wet paper towels in a tray with a lid. This gives a high

level of humidity without the leaves coming in direct contact with free water. After 24 hours the inoculum should be blotted with a tissue and any free water removed from the leaves by opening the trays to prevent rot. If the leaves senesce too quickly for symptoms to develop, a cytokinin plant hormone such as benzimidazole can be used to inhibit leaf senescence and maintain greeness. A concentration of up to 20 ppm may be used but we strongly suggest that you experiment with the precise concentration before inoculating the leaves. The leaves should then be incubated at 20-25°C with 16 hours light period for 5-7 days for symptoms to develop. Depending on the host and the *Phytophthora* species used, lesions will develop and sporulation can be observed, indicating susceptibility of the host. For other species, the growth rate of advancing lesion can be measured and used as an indicator of resistance.

5.2 Insertion of mycelium into the stems of young seedlings

This method is used for screening for resistance in the host and determining the virulence of particular strains of *Phytophthora*. This type of test is used for example in *P. sojae* on soybean (Ryley *et al.* 1991), *P. vignae* on cowpea etc. Typically around 10 seeds are sown into a pot and at the two-cotyledon stage a small slit is made into the stem with a scalpel and a weft of mycelium inserted. After all ten seedlings are inoculated a plastic bag is sprayed on the inside with distilled water and placed over the seedlings to create a moist and humid environment. The bag is removed 24-48 hours after inoculation. A reaction can typically be seen 4-6 days after inoculation. If the seedlings are susceptible, an advancing lesion on the stem will lead to collapse of the hypocotyl – the reaction is then rated as susceptible. Resistant seedlings will only show a wound and continue to grow.

5.3 Inoculation of woody adult plants

Phytophthora isolates can be grown on agar for this. Use a 10-mm corkborer to cut through the bark of the tree and a little in the wood approximately 1-1.5 meter above ground level. Place a mycelial disc of 10 mm into the hole and seal it with masking tape to prevent desiccation. Depending on the species of *Phytophthora* and the susceptibility of the host, lesion length observed as dark discolouration of the secondary phloem can be measured 6-8 weeks after inoculation.

5.4 Inoculation of soil

This method is routinely used for many *Phytophthora* species and there are numerous variations on this technique. Below is a general outline. Inoculation can either be done with agar containing mycelium, sporangiospores, or mycelium grown up on a natural substrate such as autoclaved wheat or millet. For species of *Phytophthora* such as *P. cinnamomi*, which do not readily sporulate, the use of natural substrate will give the best results. For production of a natural substrate mix 40 mL of wheat (or other grain such as barley, rye or millet) with 40 mL of distilled or deionised water, soak overnight; pour of excess water and autoclave the wheat in a glass flask at 121°C for 30 minutes on two consecutive days. Inoculate the flask with 5 –10 small plugs of agar containing mycelium and incubate at room temperature away from direct exposure to light. Shake the mixture daily to obtain an even spread of *Phytophthora* mycelium throughout the flask. After 2-3 weeks the wheat is fully colonised and can be ground in a coffee grinder or blender. Use about 1 gram of this mixture per litre of potting mix. We often mix it with sterile sand in order to obtain an even spread of this mixture throughout the potting mix. Then use this

potting mix to plant your seeds, or re-pot your seedlings into the pots containing the infected soil. In order to get good *Phytophthora* infection a series of wet and dry cycles is recommended. Place a saucer at the bottom of each pot and flood for 3 days in addition to watering from the top. For the next 4 days invert the saucer and water the plants only sparingly. This sequence of flooding for 3 days and drying for 4 days creates the ideal environment for sporangia and zoospore production and will provide a good environment for *Phytophthora* infection. If the pots are continuously waterlogged, you will get root rot and asphyxiation even in the uninoculated control pots. The degree of root infection can be assessed by counting the number of healthy root tips.

6 References

Bartnicki-Garcia, S., and M.C. Wang. 1983. Biochemical aspects of morphogenesis in *Phytophthora.* In *Phytophthora: its biology, taxonomy, ecology and pathology*, edited by D. C. Erwin, S. Bartnicki-Garcia, and P.H. Tsao. St. Paul, Minnesota, USA: American Phytopathological Society.

Bourke, P.M. 1964. Emergence of potato blight. *Nature* 203:805-808.

Brasier, C.M. 1983. Problems and prospects in *Phytophthora* research. In *Phytophthora: its biology, taxonomy, ecology and pathology*, edited by D. C. Erwin, S. Bartnicki-Garcia, and P.H. Tsao. St. Paul, Minnesota, USA: American Phytopathological Society.

Brasier, C.M. 1992. Evolutionary biology of *Phytophthora:* I. Genetic system, sexuality and the generation of variation. *Annual Review of Phytopathology* 30:153-171.

Campbell, W.A. 1949. A method of isolating *Phytophthora cinnamomi* directly from the soil. *Plant Disease Reporter* 33:134-135.

Cavalier-Smith, T. 1986. The kingdom Chromista: origin and systematics. In *Progress in phycological research*, edited by I. Round and D.J. Chapman, Bristol, England: Biopress.

Chee, K.H. and K.M. Foong. 1968. Use of cacao pod for recovering *Phytophthora* species pathogenic to *Hevea brasiliensis*. *Plant Disease Reporter* 52:5.

Chen, D.W. and G.A. Zentmyer. 1970. Production of sporangia by *Phytophthora cinnamomi* in axenic culture. *Mycologia* 62:397-402.

Desjardins, P.R., G.A. Zentmeyer, and D.A. Reynolds. 1969. Electron microscopic observations of the flagellar hairs of *Phytophthora palmivora* zoospores. *Canadian Journal of Botany* 47:1077-1079.

Drenth, A. 1994. Molecular genetic evidence for a new sexually reproducing population of *Phytophthora infestans* in Europe. PhD Thesis, Wageningen University, Wageningen, The Netherlands.

Eckert, J.W. and P.H. Tsao. 1960. A preliminary report on the use of pimaricin in the isolation of *Phytophthora* spp. from root tissues. *Plant Disease Reporter* 44:660-661.

Eckert, J.W. 1962. A selective antibiotic medium for isolation of *Phytophthora* and *Pythium* from plant roots. *Phytopathology* 52:771-777.

Elliott, C.G. 1983. Physiology of sexual reproduction in *Phytophthora*. In *Phytophthora: its biology, taxonomy, ecology and pathology*, edited by D. C. Erwin, S. Bartnicki-Garcia, and P.H. Tsao. St. Paul, Minnesota, USA: American Phytopathological Society.

Erwin, D.C. and O.K. Ribeiro. 1996. *Phytophthora diseases worldwide*. St. Paul, Minnesota: APS Press.

Galindo, A.J. and M.E. Gallegly. 1960. The nature of sexuality in *Phytophthora infestans*. *Phytopathology* 50:123-128.

Gerrettson-Cornell, L. 1974. A comparative test of isolation of *Phytophthora cinnamomi* Rands between the lupin baiting and a newly devised apple trap. *Phyton* 32:35-36.

Grimm, G.R. and A.F. Alexander. 1973. Citrus leaf pieces as traps for *Phytophthora parasitica* from soil slurries. *Phytopathology* 63:540-541.

Hawksworth, D. L., Kirk P. M., Sutton B. C., and Pegler D. N. 1995. *Ainsworth and Bisby's Dictionary of the Fungi*. 8th ed. Wallingford, UK: CAB International.

Hendrix, J.W. 1970. Sterols in growth and reproduction of fungi. *Annual Review of Phytopathology* 8:111-130.

Holliday, P. and W.P. Mowat. 1963. *Foot rot of Piper nigrum L. (Phytophthora palmivora), Phytopathological Papers No. 5*: Commonwealth Mycological Institute, Kew, Surrey, England.

Jeffers, S.N. and S.B. Martin. 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. *Plant Disease Reporter* 70:1038-1043.

Jenkins, S.F. 1962. Preliminary studies estimating the disease potential of *Phytophthora parasitica* var. *nicotianae* in infested tobacco soils. *Plant Disease Reporter* 46:825-826.

Kannwischer, M.E. and D.J. Mitchell. 1978. The influence of a fungicide on the epidemiology of black shank of tobacco. *Phytopathology* 68:1760-1765.

Kennedy, D.M. and J.M. Duncan. 1995. A papillate *Phytophthora* species with specificity to *Rubus*. *Mycological Research* 99:57-68.

Klotz, L.J. and T.A. DeWolfe. 1958. Techniques for isolating *Phytophthora* spp. which attack citrus. *Plant Disease Reporter* 42:675-676.

Ko, W.H. 1978. Heterothallic *Phytophthora:* evidence for hormonal regulation of sexual reproduction. *Journal of General Microbiology* 107:15-18.

Lee, B.S. and G. Varghese. 1974. Studies on the genus *Phytophthora* in Malaysia. I. Isolation techniques, comparative morphology and physiology and reaction to antibiotics. *Malaysian Agricultural Research* 3:13-21.

Masago, H., M. Yoshikawa, M. Fukada, and N. Nakanishi. 1977. Selective inhibition of *Pythium* spp. from soils and plants. *Phytopathology* 67:425-428.

Newhook, F.J. 1978. *Phytophthora cinnamomi* in native forests of Australia and New Zealand: indigenous or introduced? In *Microbial ecology*, edited by M. W. and. M. Loutit, J.A.R. Heidelberg, Germany: Springer-Verlag.

Newhook, F.J., and G.V. Jackson. 1977. *Phytophthora palmivora* in cocoa plantation soils in the Solomon Islands. *Transactions of the British Mycological Society* 69:31-68.

Orellana, R.G. 1954. Contribution to the study of survival, dissemination and control of *Phytophthora* of cacao. Paper read at 5th International-American Cacao Conference, at Turrialba, Costa Rica.

Papavizas, G.C., J.H. Bowers, and S.A. Johnston. 1981. Selective isolation of *Phytophthora capsici* from soils. *Phytopathology* 71:129-133.

Pegg, K.G. 1977. Soil application of elemental sulphur as a control of *Phytophthora cinnamomi* root and heart rot of pineapple. *Australian Journal of Experimental Agricultural and Animal Husbandry* 17:859-865.

Pratt, B.H. and W.A. Heather. 1972. Method for rapid differentiation of *Phytophthora cinnamomi* from other *Phytophthora* species isolated from soil by lupin baiting. *Transactions of the British Mycological Society* 59:87-96.

Ryley, M.J., N.R. Obst, and G.E. Stovold. 1991. A new race of *Phytophthora megasperma* f.sp. *glycinea* on soybean in Australia. *Australasian Plant Pathology* 20:97-100.

Satyprasad, K. and P. Ramaro. 1980. A simple technique for isolating *Phytophthora palmivora* from the soil. *Current Science* 49:360-361.

Schmitthenner, A.F. and R.G. Bhat. 1994. *Useful methods for studying Phytophthora in the laboratory*. Wooster, Ohio, USA: Department of Plant Pathology, Ohio Agricultural Research and Development Centre.

Stamps, D.J., G.M. Waterhouse, F.J. Newhook, and G.S. Hall. 1990. *Revised tabular key to the species of Phytophthora, Institute of Mycology Paper 162*: Agricultural Bureau of International Mycology Institute.

Thompson, J. N. and J. J. Burdon. 1992. Gene-for-gene evolution between plants and parasites. *Nature* 360:121-125.

Tsao, P.H. 1960. A serial end-point dilution method for estimating disease potentials of citrus *Phytophthoras* in soil. *Phytopathology* 50:717-724.

Tsao, P.H. 1990. Why many Phytophthora root rots and crown rots of tree and horticultural crops remain undetected. *OEPP/EPPO Bulletin 20:11-17*.

Tsao, P.H. and G. Ocana. 1969. Selective isolation of species of *Phytophthora* from natural soils on an improved antibiotic medium. *Nature* 223:636-638.

Turner, P.D. 1965. Behaviour of *Phytophthora palmivora* in soil. *Plant Disease Reporter* 49:135-137.

van de Peer, Y., G. van der Auwera, and R. De Wachter. 1996. The evolution of stramenopiles and alveolates as derived by substitution rate calibration of small ribosomal subunit RNA. *Journal of Molecular Evolution* 42: 201-210.

van der Plaats-Niterink, A.J. 1981. *Monograph of the genus Pythium, Studies in Mycology No. 21*. Baarn, Netherlands: Centraalbureau voor Schimmel cultures.

Wang, M. C. and S. Bartnicki-Garcia. 1973. Novel phosphoglucans from the cytoplasm of *Phytophthora palmivora* and their selective occurrence in certain life cycle stages. *Journal of Biological Chemistry* 248:4112-4118.

Waterhouse, G.M. 1963. *Key to the species of Phytophthora de Bary*, *Mycological Papers No. 92*: Commonwealth Mycological Institute, Kew, Surrey, England.

Zentmyer, G.A. 1980. *Phytophthora cinnamomi and the diseases it causes*, *Monograph No. 10*. St. Paul, Minnesota, USA: American Phytopathological Society.

Zentmyer, G.A., J.D. Gilpatrick, and W.A. Thorn. 1960. Methods of isolating *Phytophthora cinnamomi* from soil and from host tissue. *Phytopathology* 50:87.