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Organisation Européenne et Méditerranéenne pour la Protection des Plantes
European and Mediterranean Plant Protection Organization

Normes OEPP
EPPO Standards

Diagnostic protocols for regulated pests
Protocoles de diagnostic pour les organismes réglementés

PM 7/26

Organization Européenne et Méditerranéenne pour la Protection des Plantes
1, rue Le Nôtre, 75016 Paris, France

Approval

EPPO Standards are approved by EPPO Council. The date of approval appears in each individual standard. In the terms of Article II of the IPPC, EPPO Standards are Regional Standards for the members of EPPO.

Review

EPPO Standards are subject to periodic review and amendment. The next review date for this EPPO Standard is decided by the EPPO Working Party on Phytosanitary Regulations

Amendment record

Amendments will be issued as necessary, numbered and dated. The dates of amendment appear in each individual standard (as appropriate).

Distribution

EPPO Standards are distributed by the EPPO Secretariat to all EPPO member governments. Copies are available to any interested person under particular conditions upon request to the EPPO Secretariat.

Scope

EPPO Diagnostic Protocols for Regulated Pests are intended to be used by National Plant Protection Organizations, in their capacity as bodies responsible for the application of phytosanitary measures to detect and identify the regulated pests of the EPPO and/or European Union lists.

In 1998, EPPO started a new programme to prepare diagnostic protocols for the regulated pests of the EPPO region (including the EU). The work is conducted by the EPPO Panel on Diagnostics and other specialist Panels. The objective of the programme is to develop an internationally agreed diagnostic protocol for each regulated pest. The protocols are based on the many years of experience of EPPO experts. The first drafts are prepared by an assigned expert author(s). They are written according to a 'common format and content of a diagnostic protocol' agreed by the Panel on Diagnostics, modified as necessary to fit individual pests. As a general rule, the protocol recommends a particular means of detection or identification which is considered to have advantages (of reliability, ease of use, etc.) over other methods. Other methods may also be mentioned, giving their advantages/disadvantages. If a method not mentioned in the protocol is used, it should be justified.

The following general provisions apply to all diagnostic protocols:

- laboratory tests may involve the use of chemicals or apparatus which present a certain hazard. In all cases, local safety procedures should be strictly followed
- use of names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable
- laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated or that proper positive and negative controls are included.

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EU (2000) Council Directive 2000/29/EC of 8 May 2000 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. *Official Journal of the European Communities* L169, 1–112.

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Definitions

Regulated pest: a quarantine pest or regulated non-quarantine pest.
Quarantine pest : a pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled.

Outline of requirements

EPPO Diagnostic Protocols for Regulated Pests provide all the information necessary for a named pest to be detected and positively identified by an expert (i.e. a specialist in entomologist, mycology, virology, bacteriology, etc.). Each protocol begins with some short general information on the pest (its appearance, relationship with other organisms, host range, effects on host, geographical distribution and its identity) and then gives details on the detection, identification, comparison with similar species, requirements for a positive diagnosis, list of institutes or individuals where further information on that organism can be obtained, references (on the diagnosis, detection/extraction method, test methods).

Existing EPPO Standards in this series

Nineteen EPPO standards on diagnostic protocols have already been approved and published. Each standard is numbered in the style PM 7/4 (1), meaning an EPPO Standard on Phytosanitary Measures (PM), in series no. 7 (Diagnostic Protocols), in this case standard no. 4, first version. The existing standards are:

PM 7/1 (1) *Ceratocystis fagacearum*. *Bulletin OEPP/EPPO Bulletin* **31**, 41–44

PM 7/2 (1) *Tobacco ringspot nepovirus*. *Bulletin OEPP/EPPO Bulletin* **31**, 45–51

PM 7/3 (1) *Thrips palmi*. *Bulletin OEPP/EPPO Bulletin* **31**, 53–60

PM 7/4 (1) *Bursaphelenchus xylophilus*. *Bulletin OEPP/EPPO Bulletin* **31**, 61–69
PM 7/5 (1) *Nacobbus aberrans*. *Bulletin OEPP/EPPO Bulletin* **31**, 71–77
PM 7/6 (1) *Chrysanthemum stunt pospiviroid*. *Bulletin OEPP/EPPO Bulletin* **32**, 245–253
PM 7/7 (1) *Aleurocanthus spiniferus*. *Bulletin OEPP/EPPO Bulletin* **32**, 255–259
PM 7/8 (1) *Aleurocanthus woglumi*. *Bulletin OEPP/EPPO Bulletin* **32**, 261–265
PM 7/9 (1) *Cacoecimorpha pronubana*. *Bulletin OEPP/EPPO Bulletin* **32**, 267–275
PM 7/10 (1) *Cacyreus marshalli*. *Bulletin OEPP/EPPO Bulletin* **32**, 277–279
PM 7/11 (1) *Frankliniella occidentalis*. *Bulletin OEPP/EPPO Bulletin* **32**, 281–292
PM 7/12 (1) *Parasaissetia nigra*. *Bulletin OEPP/EPPO Bulletin* **32**, 293–298
PM 7/13 (1) *Trogoderma granarium*. *Bulletin OEPP/EPPO Bulletin* **32**, 299–310
PM 7/14 (1) *Ceratocystis fimbriata* f. sp. *platani*. *Bulletin OEPP/EPPO Bulletin* **33**, 249–256
PM 7/15 (1) *Ciborinia camelliae*. *Bulletin OEPP/EPPO Bulletin* **33**, 257–264
PM 7/16 (1) *Fusarium oxysporum* f. sp. *albedinis*. *Bulletin OEPP/EPPO Bulletin* **33**, 265–270
PM 7/17 (1) *Guignardia citricarpa*. *Bulletin OEPP/EPPO Bulletin* **33**, 271–280
PM 7/18 (1) *Monilinia fructicola*. *Bulletin OEPP/EPPO Bulletin* **33**, 281–288
PM 7/19 (1) *Helicoverpa armigera*. *Bulletin OEPP/EPPO Bulletin* **33**, 289–296

Several of the Standards of the present set result from a different drafting and consultation procedure. They are the output of the DIAGPRO Project of the Commission of the European Union (no. SMT 4-CT98-2252). This project involved four 'contractor' diagnostic laboratories (in England, Netherlands, Scotland, Spain) and 50 'intercomparison' laboratories in many European countries (within and outside the European Union), which were involved in ring-testing the draft protocols. The DIAGPRO project was set up in full knowledge of the parallel activity of the EPPO Working Party on Phytosanitary Regulations in drafting diagnostic protocols, and covered regulated pests which were for that reason not included in the EPPO programme. The DIAGPRO protocols have been approved by the Council of EPPO as EPPO Standards in series PM7. They will in future be subject to review by EPPO procedures, on the same terms as other members of the series.

Diagnostic protocols for regulated pests[1]
Protocoles de diagnostic pour les organismes réglementés

Phytophthora cinnamomi

Specific scope

This standard describes a diagnostic protocol for *Phytophthora cinnamomi*.

Specific approval and amendment

Approved in 2003-09.

Introduction

Phytophthora cinnamomi is a soil-borne pathogen that causes crown and root rot of many horticultural, ornamental and forestry crops. It preferentially attacks 'feeder' roots. The geographical origin of *P. cinnamomi* is not clearly established. It was first described on *Cinnamomum burmannii* (*Lauraceae*) in Sumatra (ID) in 1922, but now has a nearly worldwide distribution, including most of Europe (CABI, 1991). The pathogen is found in tropical and subtropical countries and in the Mediterranean and some mild, temperate regions where it has almost certainly been introduced (EPPO/CABI, 1998).

P. cinnamomi is the most widely distributed *Phytophthora* species, with over 1000 host species (Zentmyer, 1983). The most significant food-crop losses due to *P. cinnamomi* root rot occur in avocado but the pathogen also attacks *Ananas comosus*, *Castanea dentata* and *C. sativa*, *Cinchona* spp., *Chamaecyparis lawsoniana*, *Cinnamomum* spp., conifers, *Ericaceae* (including *Rhododendron* spp.), *Eucalyptus* spp., especially *E. marginata*, *Fagus* spp., *Juglans* spp., *Pinus* spp., *Prunus* spp., *Quercus* spp. and many ornamental trees and shrubs, including *Vaccinium macrocarpon*. It has caused extensive damage to natural *Eucalyptus* forest in Western Australia.

The recorded host range includes most of the temperate fruit trees, but these are not relevant hosts in practice. In the EPPO region, the most significant hosts are nursery stock of ornamental and amenity trees and avocados, in the limited areas where they are grown. It has been reported to be the main causal agent of ink disease of *C. sativa* in southern France and has been indicated as a possible causal agent of oak decline in the Iberian peninsula.

Propagules are spread by soil movement, including wind-blow or debris, or by water flow and run-off in drainage/irrigation ditches. Control is complicated by the very wide host range as well as by the longevity (often many years) of propagules (mainly sporangia and encysted zoospores) in nonsterile moist soil and root debris at depths at which soil fumigation is not always effective (Munnecke, 1984). Symptomless plants are a major means of spreading the pathogen to disease-free areas and this is the main problem for intensive production systems in the nursery industry. The first line of control is therefore planting disease-free stock. Imported plants for planting should be kept well separated in nurseries and preferably grown in containers for several months until their phytosanitary status has been checked. Strict hygiene should be observed at all times.

Identity

Name: *Phytophthora cinnamomi* Rands
Taxonomic position: Chromista: Oomycota, Oomycetes, Pythiales, Pythiaceae
Bayer computer code: PHYTCN
Phytosanitary categorization: this pathogen was formerly regulated by the EU in relation to avocado. It retains its importance as a serious pest mainly transmitted by plants for planting (potentially a regulated non-quarantine pest)

Detection

Symptoms

P. cinnamomi is primarily a root pathogen of woody species and causes rot of fine feeder roots leading to death of host plants. Larger roots are only occasionally attacked. Rot may extend into the base of the stem with brown lesions forming in the wood, a symptom that can be seen by peeling off the bark. Foliage becomes chlorotic and wilted and, depending on the severity of the root rot, dies back (Web Fig. 1A). *P. cinnamomi* causes also stem cankers which often result in sudden death of

[1]The Figures in this Standard marked 'Web Fig.' are published on the EPPO website www.eppo.org.

trees (Web Fig. 1B). Other symptoms include decline in yield, decreased fruit size, gum exudation, heart rot (e.g. pineapple), and collar rot, particularly when the plant is infected through grafts near soil level. Infected plants can collapse suddenly but in other cases can survive for several years, mainly in areas with cool, damp climates. *P. cinnamomi* infection can also occur together with other *Phytophthora* species, mainly *P. cambivora*, *P. cryptogea*, *P. citricola* and *P. cactorum*.

Morphology

P. cinnamomi, listed in Waterhouse's group VI (Waterhouse & Waterston, 1966; Stamps *et al*., 1990), produces coralloid hyphae and botryose swellings (Web Fig. 5A,B,C) on malt or V8 agar. It also forms globose thin-walled, mainly terminal chlamydospores (Web Fig. 2B), often in characteristically grape-like clusters of 3–10 (Stamps *et al*., 1990; Erwin & Ribeiro, 1996). The usual temperature range for growth is 5–34 °C; its cardinal temperatures are: minimum 5–15 °C; optimum 20–32.5 °C; maximum 30–36 °C. In soil, mycelium grows optimally at 24–28 °C. *P. cinnamomi* produces large, ovoid, obpyriform or ellipsoid non-papillate, non-deciduous sporangia in certain liquid media (see Appendix 4); non-sterile soil leachate is usually highly stimulatory to sporangium production. New sporangia are produced by internal or external proliferation (Web Fig. 2A) or by sympodial development of the sporangiophore immediately below empty sporangia. Sporangia average 75×40 µm (length × breadth) with a length-breadth ratio of 1.54. They are produced at soil matric potentials between −10 mb/and 2500 mb at 15–35 °C (with an optimum at 24 °C, pH 5.5). The temperature, matric potential and pH of the soil also influence whether sporangia germinate directly or release 10–30 motile zoospores. *P. cinnamomi* is a heterothallic species forming oospores either by pairing of *A1* and *A2* types or by interspecific crosses with the opposite mating type of, e.g. *P. cryptogea*. The A2 type can be functionally homothallic when stimulated by various substances, such as volatiles produced by *Trichoderma viride*, or extracts of avocado root. Antheridia (19×17 µm) are amphigynous (Web Fig. 3A), some are bicellular. Oogonia (21–58 µm), which are round, hyaline to yellow brown, and smooth-walled, easily distinguish *P. cinnamomi* from *P. cambivora*; oogonia of the latter are bullate (Web Fig. 3B). Oospores, 19–54 µm in diameter, are round, hyaline to yellow brown, and plerotic. The morphological features of *P. cinnamomi* are illustrated in Web Figs 2–5.

Identification

Isolation and identification in culture

Plants with early external symptoms should be uprooted, cut longitudinally just above the rotted roots or crowns and inspected for the browning of the woody and cortical tissues. Feeder roots can be observed under the compound microscope for the presence of propagules (mainly chlamydospores, and occasionally sporangia) of *P. cinnamomi* using non-fixed or fixed freehand or paraffin sections or whole mounts (Appendix 1). Oospores are seldom found in infected rootlets as, worldwide, the main mating type is A2. The disease can be positively diagnosed by baiting soil with susceptible plant parts (Appendix 2), such as susceptible fruits (avocado) or leaves of rhododendron, or by plating soil and/or roots on a selective medium (Appendixes 2 and 3). Isolation of *P. cinnamomi* from feeder roots on selective medium is performed very easily and quite successfully (Web Fig. 1C). However, direct isolation from soil is often unsuccessful, even in severely infected plantations, and baiting gives better results. For direct isolation from feeder roots, rootlets are washed free from soil in tap water, cut in small segments (5–8 mm) and plated directly in the medium in Petri plates (6–10 segments per plate). Plates are incubated in the dark for 3–6 days at 22–24 °C. After isolation, axenic colonies suspected to be *Phytophthora* are transferred to corn meal agar (CMA) and potato dextrose agar (PDA) for observations by both dissecting and compound microscope. Samples should be kept in plastic bags at room temperature and processed within a few days of collection to avoid desiccation. Isolation onto selective medium is time-consuming (it requires at least 6 days), but it is easy to perform and the pathogen can be identified with certainty.

Colonies on PDA generally have a pattern resembling the petals of a rose or camellia flower. *P. cinnamomi* is easy to identify because of its unique morphological features: distinctive coralloid mycelium with abundant hyphal swellings, swollen vesicles, and sessile terminal or lateral protuberances, produced singly or in clusters. Chlamydospores are formed abundantly in culture and infected tissues and are borne on the parent hyphae or on new hyphal branches. Hyphal swellings are also formed more profusely than in other species. Sporangia are not produced readily in axenic culture, but incubation of mycelial discs in nonsterilized soil extract (10 g of soil per L) (Mehrlich, 1935) or frequent washing of mycelial-agar discs in a salt solution (Chen & Zentmyer, 1970) usually induce sporangium differentiation (Appendix 4).

Molecular methods

Molecular methods such as isozyme analysis and analysis of mitochondrial DNA can provide confirmatory evidence for identification (Forster & Coffey, 1991). In both total protein and isozyme analysis, *P. cinnamomi* is homogeneous and distinct from *P. cambivora* or other *Phytophthora* species causing root rot of trees such as *P. cactorum*. A1 isolates can be distinguished from A2 on the zymogram of isocitrate dehydrogenase (Oudemans & Coffey, 1991). Protocols for polyacrylamide gel electrophoresis of mycelial proteins are described in Appendix 4.

The DNA sequence of the internally transcribed spacers, ITS1 and ITS2, and the 5.8S subunit, which they flank, of the ribosomal RNA gene repeat (rDNA) of *P. cinnamomi* has been determined, and compared with the equivalent sequences of nearly all extant species of *Phytophthora* (Cooke *et al*. 2000). The sequence is quite unique and characteristic of *P. cinnamomi*,

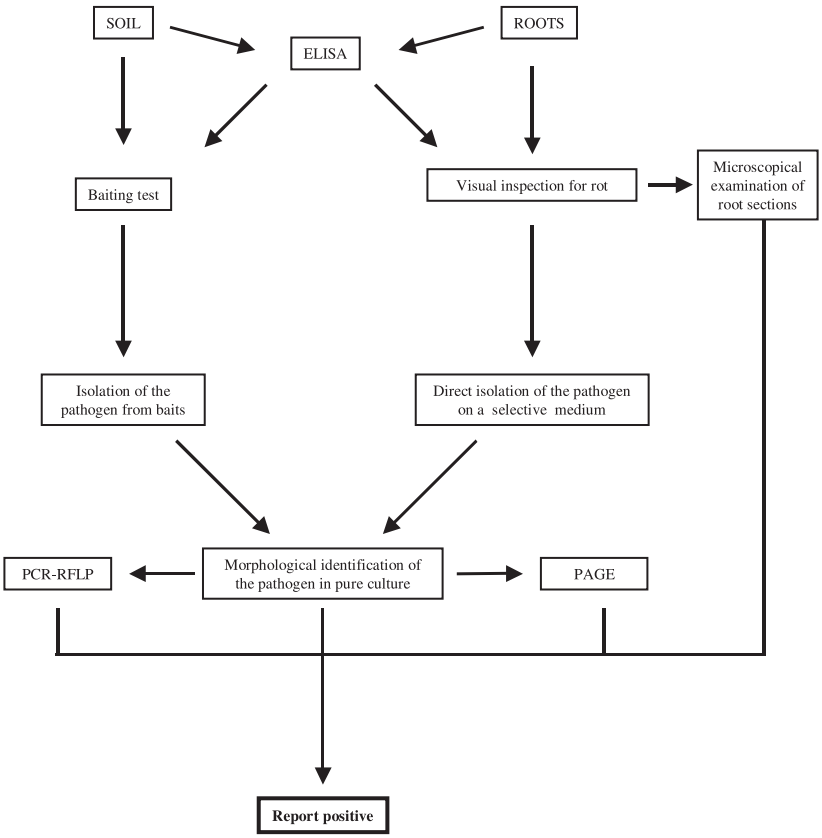

Fig. 6 Decision scheme for the detection and identification of *Phytophthora cinnamomi*.

and has been used to confirm the identity of unknown cultures. Usually, RFLP analysis of the PCR product is good enough for this purpose (giving results in a few hours), but increasingly the actual sequence is being used. Full details of how to identify *P. cinnamomi* (and other species) by PCR and RFLP are given in Appendix 6. They can be also found at http://www.phytid.org.

Immunological methods

P. cinnamomi can be detected by serological methods. These allow rapid and accurate detection but should not replace direct isolation totally.

ELISA can be used for detection. Immunoassays based on DAS-ELISA are specific to the genus *Phytophthora* and have been validated for *P. cinnamomi*. Kits are available as laboratory and on-site versions. These assays are sensitive at very low levels of the target pathogen and can also be used to give a quantitative assessment of the pathogen in soil or plant tissue samples. The method requires experience and specialized laboratory equipment as well as careful sampling of infected tissues. Possible additional disadvantages of the ELISA test are: detection of dead as well as living tissue, possible cross reaction with soil particles and certain species of *Pythium*.

Cahill & Hardham (1994) report specific identification of *P. cinnamomi* by a dipstick immunoassay with monoclonal antibody, Cpa-3, that recognizes an antigen located on the cyst periphery. This could be used as a reliable diagnostic tool to replace or enhance classical isolation and detection methods.

Use of fluorescent antibodies, observed with an ultraviolet light microscope, has been developed for the detection of *P. cinnamomi* (MacDonald & Duniway, 1979). This technique is elegant and highly specific, but requires laboratory personnel with expertise in serological methodology.

Generally, immunological methods should be regarded as being complementary to other diagnostic techniques. They are useful for preliminary rapid screening when large numbers of samples have to be tested. However, they are not necessarily species-specific and require to be confirmed by morphological or molecular methods.

Reference materials

Reference cultures can be obtained from most major mycological collections but in particular from the American Type Culture Collection (ATCC) in USA, the International Mycological Institute (IMI) in England, the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands. The type culture of *P. cinnamomi* is held under the following designations: ATCC 1407; IMI 022938; CBS 144.22.

Possible confusion with similar species

Generally, the unique morphological features described above and the robust tough hyphae of *P. cinnamomi* make it relatively

easy to identify. It is most closely related to *P. cambivora* and *P. fragariae* but can be distinguished simply from both: *P. cambivora* forms very distinctive bullate oogonia (Web Fig. 3) in paired cultures and it seldom differentiates the lightly pigmented, botryose, thin-walled hyphal swellings and chlamydospores of *P. cinnamomi*; *P. fragariae* has a very limited host range (strawberry or raspberry only, depending on the variety) and is slow-growing. Both form fluffy white colonies without pattern.

Requirements for positive diagnosis

The procedures for the detection and identification described in this protocol, and the decision scheme in Fig. 6, should have been followed. Diagnosis is considered positive when the pathogen is isolated by baiting soil with susceptible plant parts, or by plating feeder roots on selective agar media. Axenic cultures, with rose or camellia colony pattern, are observed under the microscope for typical structures of *P. cinnamomi*. However, *P. cinnamomi* has a unique ITS-RFLP profile that can be used to confirm its identity (see above).

Report on the diagnosis

A report on the execution of the protocol should include:

- information and documentation on the origin of the infected material and plant species
- measurements and photographs of cultures with coralloid-type mycelium, numerous hyphal swellings, swollen vesicles, and sessile terminal or lateral protuberances, chlamydospores
- polyacrylamide slab gels of native total mycelial proteins and isozymes (photographs or 'exsiccata'), only if a reference strain or reference 'exsiccata' with electrophoretic profiles obtained using standard procedures are available
- comments as appropriate on the certainty or uncertainty of the identification.

A culture of the pathogen may be required.

Further information

Further information on this organism can be obtained from: C.M. Brasier, Forestry Commission Research Station, Alice Holt Lodge, Farnham, Surrey GU10 4LH (UK); M.D. Coffey, Department of Plant Pathology, University of California, Riverside, California 95521-0122 (USA); G.E.St.J. Hardy, School of Biological Science, Murdoch University, Murdoch, WA 6150 (Australia); S. Werres, Federal Biological Research Centre for Agriculture and Forestry, Institute for Plant Protection in Horticulture, Messeweg 11/12D – 38104, Braunschweig (Germany).

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Appendix 1. Examination of roots using light microscopy

The methods follow Dhingra & Sinclair (1985).

Staining of freehand sections

Place non-fixed sections in a dish containing Ruthenium red (0.01% aqueous solution). After sufficient staining, remove sections and wash for 1–2 min in water. Mount in glycerine.

Cover fixed sections on a slide with 0.5% lacto-acid fuchsin and heat over flame until the liquid steams for several minutes (do not boil). Drain off the excess stain and place a drop of 0.25% cotton blue in lactophenol over each section. Heat until steaming (do not boil). Wash off the excess stain and mount in glycerine.

Staining of paraffin sections

Dewax the sections and place in 95% ethanol. Stain for 30 min in silver nitrate-bromophenol (100 mg bromophenol, 3 g silver nitrate, and 50 mL 95% ethanol). Wash in water and then pass through 95% ethanol for 5 s. Stain for 1 s in methylene blue (saturated solution in 95% ethanol). Rinse immediately with water. Oospores stain blue to black.

Preparation of whole mounts

Autoclave whole roots or rootlets, either fresh or fixed, for 10 min at 110 °C in KOH or boil in two changes of KOH. Cool roots, wash in 2–3 changes of distilled water, transfer them to 3% aqueous NaOCl acidified with a few drops of concentrated HCl for 3–10 min. Clear roots to a pale straw colour (do not bleach roots in hypochlorite until they are colourless as this results in tissue degradation). Wash the roots in distilled water and stain by boiling in alcoholic lactophenol or cotton blue. The structures of *Phytophthora* spp. appear dense blue.

Appendix 2. Baiting techniques for isolation from soil

The methods follow Ribeiro (1978), Shew *et al*. (1979), Werres *et al*. (1997), Zentmyer (1980).

Fruit trap

Half embed a firm avocado fruit (e.g. cv. Fuerte) in soil flooded with water. Leave for 48–96 h (optimum temperature 27 °C). Infections appear as brown, firm, circular spots at the water line. Other organisms rarely invade firm unwounded fruit. Transfer a small portion of the infected tissue at the margin of the lesion to an agar medium to obtain a pure culture of *P. cinnamomi*. Pear fruits can also be used as baits (lesion develops in 5–7 days).

Avocado roots

Dip small, washed, infected roots in 70% alcohol for 15 s and blot dry. Place on CMA plates and incubate for 48 h at 20–24 °C. *P. cinnamomi* can readily be identified at this stage, but may later be overgrown by other organisms. Roots can also be directly plated on BNPRAH selective medium (Appendix 3).

Rhododendron leaves

Mix 200 mL of test soil and 400 mL of deionized water within a plastic container (e.g. $11.5 \times 18.5 \times 5$ cm, depth of water above soil sample should be standardized). Wash freshly picked well developed leaves of *Rhododendron catawbiense* (e.g. cv. Cunningham's White) with tap water and dry them carefully with filter paper. Place 5 leaves per container and let them float on the water surface. Incubate at room temperature (about 20 °C) in natural light for 2–8 days. Infections by *P. cinnamomi* appear as water-soaked spots on the leaves. It is advisable to prepare both a positive control (a pure culture of *P. cinnamomi* in a Petri dish) and a negative control (a Petri dish containing carrot piece agar) for the baiting test. Mix the agar of the Petri dish with 100 mL of sterile sand-soil mixture (1 + 3 v/v) and add 500 mL of sterile deionized water.

The pathogen can be isolated from leaf spots. Wash symptomatic leaves with running tap water and surface-disinfect with 0.037% active chlorine for 1–2 min. Wash with sterilized deionized water and blot dry. Plate tissue pieces (3×3 mm) on carrot agar (15 g carrot pieces, 15 g agar, 1000 mL deionized water) in Petri dishes. Incubate at 20 °C in the dark. Prepare hyphal tip subcultures for species identification.

These three baiting methods are equally effective in detecting *P. cinnamomi* in infested soil. Other methods include the use of cedar needles, fir seedlings and lupin radicles as baits. Baits are flooded on or immersed in 100 mL of water added to 5–50 g of soil; they are incubated at 20 °C for 1–2 days.

Appendix 3. BNPRAH selective medium for isolation from infected rootlets or soil

For this method (Masago *et al*., 1977), potato dextrose agar (1% agar) is amended (μg mL^{-1}) with benomyl (10), pentachloronitrobenzene (25), nystatin (25), ampicillin (500), rifampicin (10), and hymexazol (50). Hymexazol suppresses many but not all *Pythium* species. Most *Phytophthora* species are tolerant, though some, like *P. fragariae*, are very sensitive. This method is very effective for *P. cinnamomi*.

Appendix 4. Techniques for producing sporangia

Saline solution

This method is modified after Chen & Zentmyer (1970). Prepare V-8 juice broth, by thoroughly mixing 200 mL V-8 juice with 2 g of $CaCO_3$, filtering the mixture with Whatman

no. 1, and diluting with deionized water to 1 L. Dilute this broth 1/10 with water. Transfer inoculum pieces (about 200–400, 2–3 mm^3 in size) from 3 to 4 day-old colonies to a Petri dish containing diluted broth. 18–24 h later, wash the mycelial mats with a mineral salt solution composed as follows: 100 mL deionized water with 0.01 M $Ca(NO_3)_2$, 0.005 M KNO_3, 0.004 M $MgSO_4$ and 1 mL of chelated iron solution (13.05 g EDTA, 7.5 g KOH, 24.9 g $FeSO_4 \cdot 7H_2O$, 1000 mL deionized water), sterilized by filtration on a 0.22-µm filter. Wash the mycelial mats with 15–20 mL of the solution at least 4 times, at 15–60 min intervals. Drain solution thoroughly from the dish. Incubate dishes under a 40-W fluorescent cold daylight lamp suspended 40 cm above the colonies at room temperature or 24 °C. Sporulation begins within 8 h from time of first washing and reaches a maximum in 24–36 h. This has proved to be a reliable method for most isolates of *P. cinnamomi* tested.

Non-sterile soil extract

This method follows Zentmyer & Marshall (1959), as modified by C. Olsson, Göteborg (SE). Prepare soil leachate as follows: 20 g garden soil (free from pesticides), 1000 mL distilled water. Mix for 12–24 h on a magnetic stirrer and then centrifuge for 10 min at 5000 rev min^{-1} Do not autoclave (if sterile leachate is required, use a 0.45-µm membrane filter). Store the leachate in the refrigerator (1–3 weeks, but the leachate should preferably be fresh).

To produce sporangia, pour 7 mL of soil leachate into 65-mm Petri dishes and add 6 agar discs (about 5 mm diameter) taken from the edge of a young colony (7–10 days old) of *P. cinnamomi*. Incubate at 14–15 °C. On the second day, change soil leachate. Sporangia differentiate on the 3rd day. To produce zoospores, transfer Petri dishes from the cool incubator to room temperature in the light.

Appendix 5. Polyacrylamide gel electrophoresis (PAGE) of mycelial proteins

Extraction of proteins

Mycelium from 8 to 9-day-old cultures, grown on carrot broth (carrot 400 g, distilled water 1000 mL) and incubated in the dark at 25 °C, is harvested by filtration under vacuum onto Buchner funnel with filter paper (Whatman n°1) and washed several times with sterile distilled water and then with the extraction buffer (0.5 M Tris-HCl, pH 6.8, 0.001 M EDTA, 0.001 DTT and 0.001 M PMSF). The mycelium could also be frozen at –20 °C and processed later. The buffer-soluble proteins are extracted by grinding blotted dry mycelium with a pestle in a mortar containing either quartz sand or liquid nitrogen. Add about 1 mL of extraction buffer per g of mycelium to the powdered mycelium. Mycelial fragments are removed by centrifugation at 40 000 *g* for 40 min, and the resulting supernatant is used to load the wells of the gel. About 10 µL of staining solution (10% glycerol and 0.002% bromophenol blue) is added to each sample. All the above steps are carried out at 4 °C.

Electrophoresis

Extracted proteins are analysed by PAGE either on a linear gradient (5–20% polyacrylamide) or on 7.5% polyacrylamide slab gels in a non-dissociating discontinuous buffer system. Tris-glycine buffer (pH 8.3) is routinely used for the electrophoretic run. Each sample, containing 100–400 µg of proteins as determined by the method of Bradford (Coomassie Protein Assay Reagent, Pierce, Rockford, US), is pipetted onto the polyacrylamide stacking gel (3.5%). Electrophoresis is carried out at 4 °C.

Staining protocol for total proteins

Coomassie blue is a general stain for proteins in gels. Gels are stained for 20–30 min at 60 °C in staining solution (Coomassie Brilliant Blue R 250 1.25 g, methanol 227 mL, glacial acetic acid 46 mL, water to 500 mL) or at room temperature, then removed from the stain and destained (methanol 7 mL), glacial acetic acid 7 mL, water to 100 mL) for 48 h. Protein bands appear as dark blue on a light blue background.

Staining protocol for isozymes

Esterase (EC 3.1.1.1 or EC 3.1.1.2) and glucose phosphate isomerase (EC 5.3.1.9) are suggested among the isoenzymes that can be tested. Freshly prepared solutions should be used.

For esterase (Shaw & Prasad, 1970), the gel after electrophoresis is washed with 100 mL of buffer (Tris-HCl 0.5 M, pH 7.1) and then incubated for 30 min or until the appearance of the bands, at 30 °C in the dark, with the following staining solution: 0.2 g Fast blue RR salt, 6 mL of substrate (0.1 g α-naphthyl acetate, 0.1 g β-naphthyl acetate, 5 mL acetone and 5 mL distilled water), 20 mL of buffer and water to a final volume of 200 mL. All above described steps are carried out in the dark as Fast blue RR salt is highly light-sensitive.

For glucose phosphate isomerase (Manchenko, 1994), the gel after electrophoresis is washed with 100 mL of buffer (0.1 M Tris-HCl, pH 8.5) and then incubated at 37 °C in the dark until dark blue bands appear with the following staining solution: 100 mL of buffer, 10 mg of $NADP^+$, 80 mg of $MgCl_2$, 1 mg of PMS, 10 mg MTT, 10 units of glucose-6-phosphate dehydrogenase and 20 mg of fructose 6-phosphate. Fix stained gel in 50% ethanol.

Images of the digest patterns of 18 *Phytophthora* spp., including *P. cinnamomi*, can be seen in Cooke & Duncan (1997).

Appendix 6. ITS fingerprinting

These protocols are designed for identification of a pure *Phytophthora* culture. To hasten the identification process, PCR is carried out on mycelial fragments picked directly from the agar plate. A tiny amount of mycelium (the smaller the better) should be taken from the periphery of the colony using a sterile needle (avoiding any agar). While successful in the majority of

cases, this sometimes fails and then mycelium should be grown in liquid medium, washed and used in the DNA extraction process below.

DNA extraction from mycelium

Place about 0.1 g of fresh mycelium (size of match head) into a sterile 1.5-mL Eppendorf. Add a small amount of sterile sand (about 50 mg) and polyvinylpolypyrrolidone (PVPP) (Sigma Catalogue number 81385) (about 10 mg) and 750 µL of extraction buffer (200 mM Tris HCl, pH 7.5; 250 mM NaCl; 25 mM EDTA, 0.5% SDS). Grind material using a plastic Treff Eppendorf homogenizer. Centrifuge at 13 000 rev min^{-1} for 5 min and remove supernatant to a clean Eppendorf. Add 500 µL of phenol/chloroform/iso amyl alcohol (25 : 24: 1) and invert gently. Centrifuge at 13 000 rev min^{-1} for 5 min and remove aqueous layer to a fresh tube. Fill tube to the top with isopropanol (–20 °C) and invert gently. Centrifuge at 13 000 rev min^{-1} for 10 min, then carefully pour off the isopropanol. Wash the pellet in 70% ethanol (1 mL) by gentle inversion. Spin at 13 000 rev min^{-1} for 2 min then pour off excess. Air-dry the pellet, and re-suspend in 100 µL SDW with RNase (5 mg mL^{-1}). Store at – 20 °C.

PCR assay

This protocol is based on 25-µL reaction mixtures in 0.2-mL PCR tubes and a PCR machine with a hot lid. If using instead 0.5-mL tubes, on a machine without a hot lid, 50-µL reaction mixtures and a mineral oil layer are recommended. The reaction mixture has the following composition: 14.5 µL sterile distilled water, 2.5 µL 10X PCR buffer supplied with kit (Tris-HCl); 2.5 µL bovine serum albumin (Sigma Catalogue Number A-7030; filter-sterilize and dilute to 10 mg mL^{-1} before use), 2.5 µL DNTPs (1 mM mix), 0.75 µL $MgCl_2$ (1.5 mM), 0.5 µL forward primer (ITS6) to a final concentation of 0.5 µM; 0.5 µL reverse primer (ITS4) to a final concentration of 0.5 µM, 1 Unit Taq polymerase, 1 µL template DNA (or add 1 µL of extra water if adding mycelium directly to the reaction). For 25-µL thin-walled PCR tube reactions, a single step at 94 °C for 3 min is followed by 35 cycles of 55 °C for 30 s, 72 °C for 60 s and 94 °C for 30 s, and a final single step at 72 °C for 10 min. It is important to run positive (DNA from authentic *Phytophthora cinnamomi*) and negative (water instead of DNA) controls for each reaction.

The primers ITS6 (5′-GAAGGTGAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) which are used amplify the ITS regions of many eukaryotes, which can be distinguished by product size. Fungi and plant species, for example, typically yield a fragment of about 600 bp. *Phytophthora* spp. yields a PCR product in the range 862–941 bp (according to species). The yield and size of the product should be tested by agarose electrophoresis prior to digestion. If the PCR product is not within the above size range, then the test organism is unlikely to be a *Phytophthora*. It is important to have a good product yield to ensure that the digestion product can be clearly resolved.

Digest conditions

A 10-µL sample of the PCR product is digested with the restriction enzymes AluI, MspI or TaqI according to the enzymes manufacturer's instructions. In many cases, digestion with just AluI and MspI will be adequate for an accurate identification. If resources are limited, the TaqI digest may be omitted. The fragments from TaqI are typically small (< 300 bp) and therefore more difficult to resolve accurately. The digested ITS fragments can be run on standard agarose or, ideally, on a low melting temperature agarose such as NuSieve 3.1 (FMC BioProducts). If running on standard agarose (which is considerably cheaper) a high percentage (2.5% +) gel will be needed to obtain sufficient resolution. Resolution, especially of the smaller fragments, will be improved by running the gel slowly and in TAE buffer. We recommend running the samples on 15–20 cm 2.5% NuSieve agarose gels over several (4 +) hours. *P. cinnamomi* is identified by its electrophoretic pattern of digested ITS fragments compared with the positive control.

Fig. 1 Symptoms of *Phytophthora cinnamomi.*

A. An avocado tree affected by *Phytophthora cinnamomi* root rot showing secondary symptoms of decline. **B.** Collar rot of an avocado tree caused by *P. cinnamomi*. This symptom occurs only on very susceptible rootstocks. The bark has been peeled off to show the brown lesion in the wood. **C.** Colonies of *P. cinnamomi* growing from avocado root segments plated on BNPRAH selective medium.

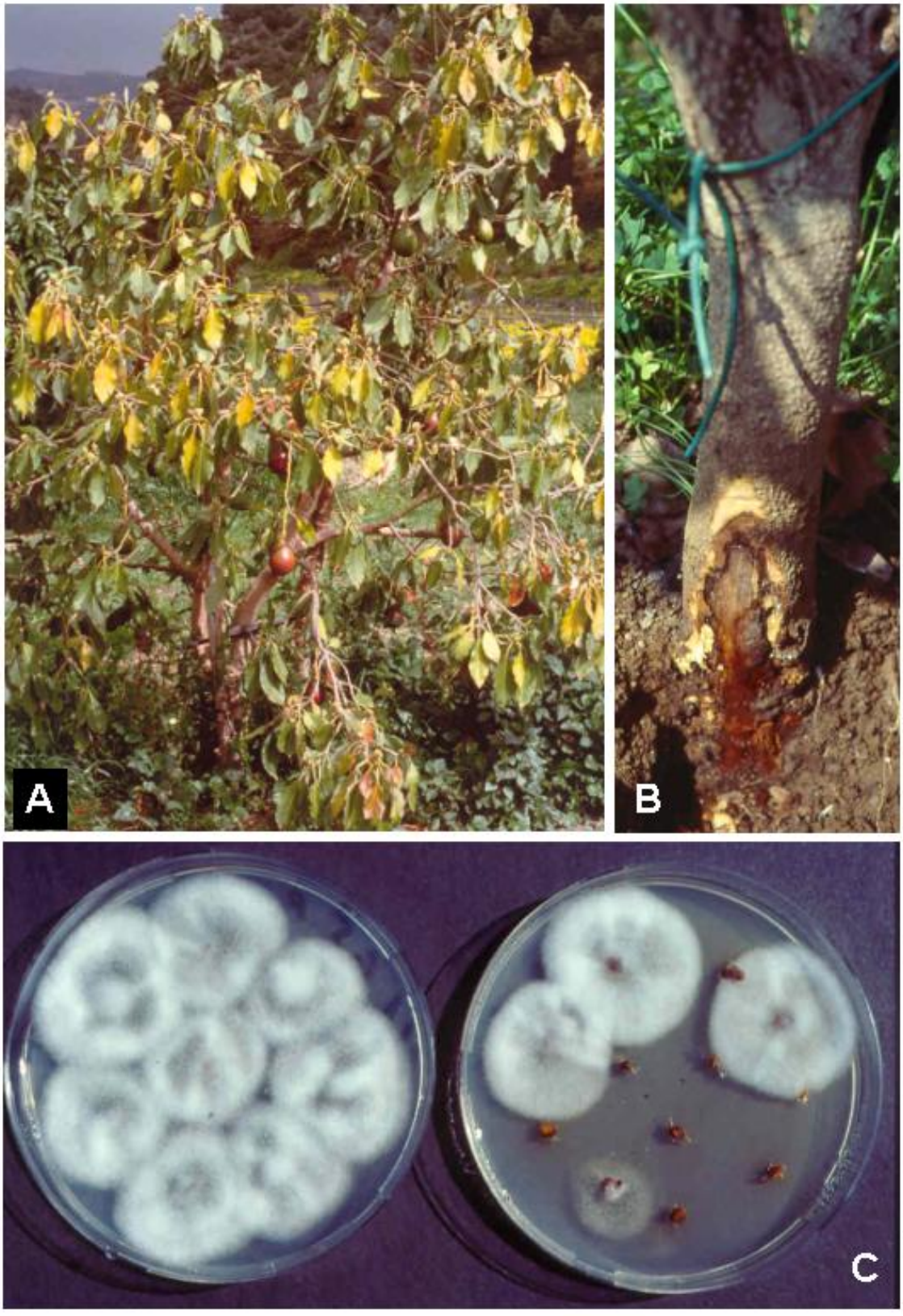

Fig. 2 A. Internally proliferating sporangium. **B.** Globose apical chlamydospore of *Phytophthora cinnamomi*.

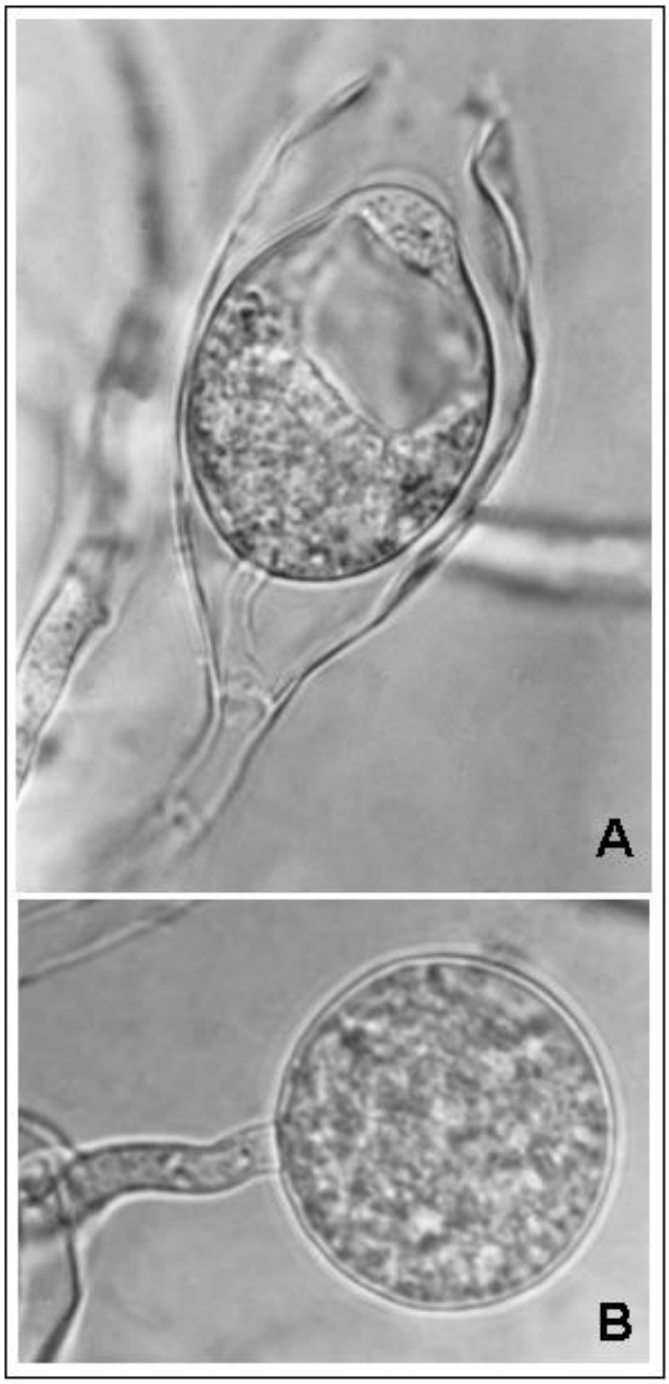

Fig. 3 Comparison between sexual structures of *Phytophthora cinnamomi* and *P. cambivora*. **A.** Oogonium, oospore and antheridium (amphigynous) of *P. cinnamomi*. **B.** Oogonium, oospore and antheridium (amphigynous) of *P. cambivora*. Note the bullate wall of the oogonium, which is a unique morphological feature of this species.

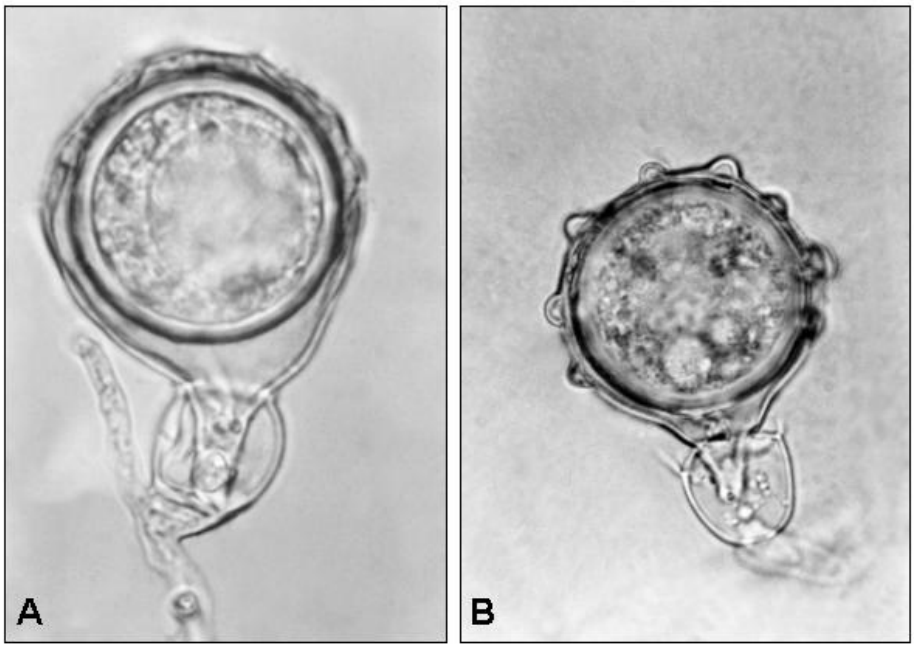

Fig. 4 Morphology of *Phytophthora cinnamomi*. Non-papillate sporangia, proliferation of sporangia, globose oogonia and oospores, globose chlamydospores, and numerous hyphal swellings. (Drawing by A. Vaziri; after Erwin and Ribeiro, 1996)

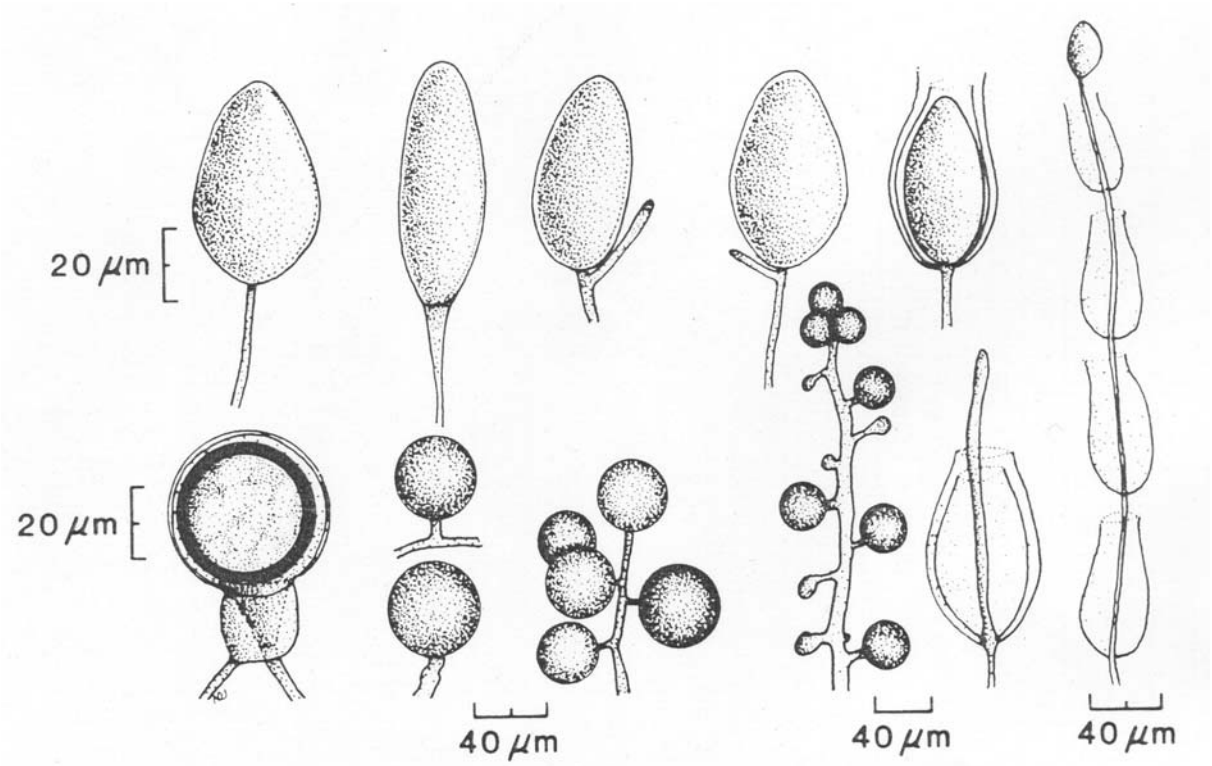

Fig. 5. **A**. Coralloid-type mycelium with a hyphal swelling. **B**. Cluster of hyphal swellings. **C**. Hyphal swelling with a bizarre shape.