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ISPM 27
Diagnostic protocols for regulated pests

DP 26: *Austropuccinia psidii*

Adopted 2018; published 2018

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1. Pest Information

Austropuccinia psidii (G. Winter) Beenken (2017) (formerly *Puccinia psidii*, Beenken, 2017), a rust pathogen first described from *Psidium guajava* in Brazil (Winter, 1884), is considered to be native to South and Central America and possibly the Caribbean (Coutinho *et al.*, 1998). It was first reported outside its native range in the late 1970s, in the United States of America, and has subsequently been reported in Australia, China, Japan, New Caledonia, South Africa (da S. Machado *et al.*, 2015) and Indonesia (McTaggart *et al.*, 2016). The fungus infects young actively growing tissues of plants within the family Myrtaceae (Morin *et al.*, 2012). The most economically important hosts are *Eucalyptus* species. The impact in susceptible species is branch death and dieback, a significant reduction of canopy density, and tree death (Pegg *et al.*, 2014).

A. psidii is considered to be a threat to plants of the family Myrtaceae worldwide (Coutinho *et al.*, 1998). Since the rust spread out of its native region, its host range has expanded rapidly (Maier *et al.*, 2016). As of September 2014, the global host list comprised more than 300 species from 73 genera in this family (Giblin and Carnegie, 2014), but it is likely that the majority of the thousands of Myrtaceae species have the potential to be infected (Carnegie and Lidbetter, 2012; Morin *et al.*, 2012). *A. psidii* is not known to infect host plants that are not Myrtaceae. There is evidence of physiological specialization within *A. psidii* (Graça *et al.*, 2013), which may have quarantine implications (Roux *et al.*, 2016).

A. psidii is an obligate biotroph with an autoecious, but incomplete, life cycle, producing urediniospores, teliospores and basidiospores on an infected host (Glen *et al.*, 2007). Under natural conditions, *A. psidii* can reproduce quickly and simply through asexual reproduction whereby urediniospores are produced in pustules known as uredinia. These spores are dispersed to leaves on the same plant or to other hosts, which in turn are infected and on which the pathogen produces pustules with more urediniospores. In some circumstances, the uredinia may switch to producing teliospores, which can germinate *in situ* to produce basidiospores. Teliospores may also be produced by another type of spore producing body, telia. Teliospore and basidiospore production were initially considered rare stages of the life cycle, but in some regions are often observed along with urediniospore production within a single sorus (Pegg *et al.*, 2014). While the production of all three types of spores in a host is considered to be a strategy for survival in adverse conditions, the role of teliospores and basidiospores in the life cycle of *A. psidii* has not been understood (Morin *et al.*, 2012; Giblin, 2013). Spermagonia and aecia have never been observed.

A. psidii prefers wet tropical and subtropical regions where moist conditions and warm temperatures prevail, but a spread to cool regions has been reported (Kriticos *et al.*, 2013) and the optimum temperature for survival of the fungus is unknown. Disease development is favoured following periods of rainfall or in high humidity or fog. Extended periods of leaf wetness promote urediniospore germination and infection of the host. For infection to occur, urediniospores must encounter a host plant during stages of active growth or flush, which can occur throughout the year depending on the host species and climatic conditions (Pegg *et al.*, 2014).

Although urediniospores of *A. psidii* can be dispersed over long distances by wind, far reaching dispersal into new geographical regions is believed to result from human activities (Giblin, 2013). Modes of spread include: infected or contaminated planting material, nursery stock, plant cuttings, flowers and germplasm; animals and insects such as bees, birds, bats and possums that have been in contact with urediniospores; contaminated plant waste, timber, and wood packaging material; contaminated equipment and tools used on or around plants (e.g. chainsaws, secateurs); and contaminated clothing, shoes and other personal effects (Giblin, 2013). Once an initial infection occurs, urediniospores are readily dispersed naturally to nearby susceptible hosts.

2. Taxonomic Information

Name: *Austropuccinia psidii* (G. Winter) Beenken (2017)

Basionym: *Puccinia psidii* G. Winter, 1884

Synonyms: *Caeoma eugeniarum* Link, 1825

Uredo neurophila Speg., 1884

Uredo subneurophila Speg., 1884

Uredo flavidula G. Winter, 1885

Uredo myrtacearum Pazschke, 1890

Uredo eugeniarum Henn., 1895

Aecidium glaziovii Henn., 1897

Dicaeoma psidii (G. Winter) Kuntze, 1898

Uredo pitangae Speg., 1899 (published as "*pitanga*")

Puccinia jambosae Henn., 1902

Uredo puttemansii Henn., 1902

Uredo goeldiana Henn., 1903

Uredo rochaei Puttemans, 1906

Puccinia rompelii Magnus, 1907

Puccinia jambolani Rangel, 1912

Uredo myrciae Mayor, 1913

Puccinia barbacensis Rangel, 1916

Puccinia brittoi Rangel, 1916

Puccinia cambucae Puttemans, 1916

Puccinia eugeniae Rangel, 1916

Puccinia grumixamae Rangel, 1917

Bullaria psidii (G. Winter) Arthur and Mains, 1922

Puccinia camargoi Puttemans, 1930

Puccinia actinostemonis H.S. Jacks. and Holw., 1931

Uredo rangelii Simpson *et al.*, 2006

Taxonomic position: Eukaryota, Fungi, Basidiomycota, Pucciniomycotina, Pucciniomycetes, Pucciniales, Sphaerophragmiaceae, *Austropuccinia*

Common names: Eucalyptus rust, guava rust, myrtle rust, ohia rust

Reference: Mycobank MB#819171 (*P. psidii* MB#213865)

Because of the age and poor quality of the original type specimen, this species has been epitypified for precise application of this taxon name (da S. Machado *et al.*, 2015).

3. Detection

All plants in the family Myrtaceae should be considered potentially susceptible to infection by *A. psidii*, and rust infecting any plants in this family should be investigated to rule out *A. psidii* infection.

Site characteristics and climatic conditions that favour lengthy periods of leaf wetness, combined with susceptible new growth on the myrtaceous host plant, provide ideal conditions for sign manifestation and detection. The use of a hand lens (10× magnification) is recommended for examining suspect lesions.

This diagnostic protocol describes established methods for the detection and identification of *A. psidii*. It is not a comprehensive review of all methods available for the diagnosis of *A. psidii*. Detection of *A. psidii* can be achieved using the biological and molecular methods shown in the flow chart in Figure 1. It is important to first determine whether the host of the sample belongs to the family Myrtaceae. If it does, the diagnosis may start with morphological observation of rust structures on the plant host material. Observation may lead to a diagnosis or highlight the need for a further study with molecular methods. In the case of a first detection in a country, confirmation with DNA sequencing is recommended. When plant samples are received with signs or symptoms of a suspected rust, and the host is unknown, conclusive identification of *A. psidii* can be achieved only with DNA sequencing.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and reproducibility achieved. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

3.1 Signs and symptoms of infection

Symptoms of infection by *A. psidii* range from minor leaf spots to severe foliage and stem blight, and in some species, flowers and fruits are infected (Figures 2–4). Since it established in Australia in 2010, the rapid expansion of the fungus' host range has resulted in a wide range of symptoms being observed which vary depending on the host species, the level of host susceptibility within a host species and the age of the host tissue. Symptoms become more obvious during rust epidemics.

The primary sign is the appearance of yellow pustules (uredinia) on the upper and lower leaf surfaces of Myrtaceae hosts, with a higher prevalence on the lower leaf surfaces (abaxial). Pustules can also be found on stems, fruits and flowers.

The first symptoms of infection are often chlorotic flecks and young sori on leaves, shoots and fruits, which appear two to four days after infection. These early signs and symptoms are similar to those caused by many other pests and disorders. Hence, identification based on signs and symptoms alone may not be sufficient for diagnosis. Further testing to confirm *A. psidii* infection can be carried out using molecular diagnostic methods.

The early symptoms are followed by the production of masses of bright yellow urediniospores. Teliospores, which are brown, can be produced in the same sori intermingled with urediniospores. The infected area on the host spreads radially outwards and with age, multiple pustules eventually merge and coalesce. From this stage onwards, it is not difficult to distinguish rust lesions from insect damage or necrosis from various causes. Lesions tend to be angular in shape, extending through the leaf (Coutinho *et al.*, 1998; Pegg *et al.*, 2014). Lesions can turn reddish-purple then grey with age, and often have a purple or dark-brown margin. For example, older symptoms on leaves and shoots of many *Eucalyptus*, *Melaleuca* and *Callistemon* show purpling on their margins. Lesions on fleshy fruits of *Eugenia*, *Psidium* and *Syzygium* may not have obvious margins because the symptoms are covered with heavy spore masses in an early stage of disease development or because they develop fruit rot, caused by secondary pathogens, as the fruits ripen.

Secondary infections can occur within days but are confined to new young tissue, shoots and expanding foliage. Prolonged infection can cause deformed leaves, heavy defoliation of branches, dieback, stunted

growth and even plant death. Severe rust infections in young trees may kill shoot tips, causing loss of leaders and a bushy habit. Prolific branching (witches' brooms), galling, persistent localized lesions, and stem swellings may be a sign of previous rust infection; however, these symptoms may also result from other causes.

On resistant hosts, *A. psidii* may induce a hypersensitive reaction expressed as flecks, chlorotic halos or brown spots but with no urediniospores or teliospores produced (Junghans *et al.*, 2003). However, depending on the level of resistance, punctiform pustules may be formed over the brown necrotic lesions (Glen *et al.*, 2007).

3.2 Sampling and sample preparation

Samples of suspected rust or suspicious lesions on plant stems, flowers, fruits, shoots or leaves should be collected. Where possible, triplicate samples from each infected host species should be collected, with each sample comprising several lesions. The likelihood of obtaining enough material for DNA extraction and of finding features for a robust morphological diagnosis increases with increasing number of collected lesions. In the event of an older infection, woody twigs and branches with swellings or galls or other evidence of infection should be sampled.

Direct handling of the plant material should be avoided to prevent spread of the disease, as rust spores are readily dispersed by wind and carried on clothing and other equipment, and can survive for up to three months. A new pair of disposable gloves should be worn for each sampling. A paper bag should be placed around the sample before snipping the infected part from the plant. Collecting tools should be thoroughly dipped and lightly shaken in bleach (a solution of sodium hypochlorite (NaOCl) diluted to at least 5% active chlorine) for about 1 min, followed by draining and drying with paper towelling before and after each sampling. Each sample should be sealed in a single paper bag and labelled.

The rust samples need to be transported on the collected plant material (stems, flowers, fruits, shoots or leaves) and the spores should remain *in situ* in the pustules for all pertinent diagnostic information to be retained.

Samples should be sent to the diagnostic laboratory in sealed plastic containers to avoid damage to the paper bags and contamination among samples. Storage and transport at cool temperatures (e.g. 4 °C) is recommended to prolong sample life, but even at this temperature samples can deteriorate if stored for more than seven days.

In the diagnostic laboratory, the sample should be allowed to dry inside the paper bag at room temperature (20–25 °C under low humidity conditions). All plant material should be preserved as air-dried samples in sealed paper bags to minimize the growth of saprophytic organisms.

3.3 Morphological detection

Because rust spores have a high potential for dispersal, samples should be handled with care during the diagnostic process. A large sheet of paper can be placed under the work area and changed between samples. Use of a laminar flow cabinet is not advisable as this may disperse the spores. Gloves should be changed and equipment (scalpels, forceps, etc.) disinfected between samples. At the end of the work period, all packaging, gloves, benchcoating, paper, tissues, etc. should be considered contaminated, double-bagged and disposed of as required for quarantineable waste (e.g. by autoclaving or incineration). Equipment should be surface sterilized with 70% ethanol or a similar disinfectant known to be effective against rust spores.

A. psidii, like other rust pathogens, cannot be cultured *in vitro*; therefore, morphological identification is based on the symptoms of infected host material. The lesions should be investigated for rust sori and spores using appropriate magnification without destroying the sori or contaminating them with histological fluids.

The samples should be examined under a dissecting microscope for rust sori. Rust spores should be picked up carefully with a disposable scalpel or needle, mounted in a drop of clear mountant such as

lactic acid on a microscope slide and covered with a coverslip. The slide should be heated gently to remove air bubbles, then observed under a compound light microscope fitted with high quality optics. Urediniospores and teliospores of *A. psidii* are readily distinguishable by light microscopy (Figure 5).

Examination of cross-sections or squash preparations of uredinia is necessary to confirm the absence of paraphyses (sterile accessory hymenial structures), as well as to confirm urediniospore morphological characters (see section 4.1).

Samples of early infections may not show the morphological characters required for the identification of *A. psidii*. Incubation for 10–14 days at 25°C in 80% relative humidity (in a humid chamber) may elicit these characters. For a faster diagnosis or to avoid the potential increase of inoculum, the pathogen may be identified using polymerase chain reaction (PCR) (section 3.4).

3.4 Molecular detection

Molecular methods have been developed to detect *A. psidii* directly from different types of infected plant material. These methods are a nested PCR (Langrell *et al.*, 2008) and a real-time PCR (Baskarathevan *et al.*, 2016). The real-time PCR is faster and more sensitive than the nested PCR as well as having a reduced risk of the cross-contamination that is inherent in nested PCR (Baskarathevan *et al.*, 2016). These methods may be useful for the rapid screening of samples after the first detection has been confirmed. Definitive identification requires comparison of fungal barcoding regions with those published for the epitype (da S. Machado *et al.*, 2015; Rodas *et al.*, 2015). This requires DNA extracted from fungal spores (section 4.2).

3.4.1 Preparation of plant material

DNA for PCR analysis can be extracted from individual sori or, if sori are not yet erumpent, from small pieces (10–100 mm^2) of infected plant tissue excised from the sample. If spores are abundant, they should be used in preference to other plant material, and should be placed into a microcentrifuge tube using a clean brush for each sample.

The sample is placed into a tube or clean sterile mortar bowl. Different grinding methods can be used such as mortar and pestle with liquid nitrogen, plastic pestles and microcentrifuge tubes with liquid nitrogen, or a TissueLyser (Qiagen[1]). New tubes or clean mortars and pestles must be used for each sample. Mortars and pestles should be washed in soapy water, rinsed with ethanol and autoclaved prior to and after use. The spore wall is very strong and all methods for DNA extraction and purification depend on its adequate disruption. For the TissueLyser, the addition of two 3 mm tungsten carbide beads, the pre-freezing of tubes in liquid nitrogen for 1 min, and two 2 min sessions in the TissueLyser at 30 Hz is sufficient for adequate grinding. For other methods, adequate grinding can be checked by microscopic examination of the ground material: if >50% of the urediniospores have lost their contents and are hyaline rather than yellow, grinding is sufficient.

3.4.2 Nucleic acid extraction

Various commercially available kits, such as the DNeasy Plant Mini Kit (Qiagen[1]) or the Wizard Genomic DNA Purification Kit (Promega[1]), are suitable for DNA extraction and purification (following the manufacturer's instructions), as are standard manual DNA extraction procedures usually used for the extraction of DNA from plants or fungi. Quantification of DNA is inexact, varying with the procedure used, and the DNA obtained may be a mixture of plant and fungal DNA. Therefore, a range of DNA concentrations should be tested using the fungal-specific primers ITS1-F and ITS4 (section 3.4.3) to confirm the quality of the DNA and whether sufficient fungal DNA can be amplified. The DNA obtained should be stored at −20 °C.

[1] The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable.

3.4.3 Conventional PCR and sequencing

This method does not require species-specific primers or probes and many diagnostic laboratories may have the required primers and other reagents in stock. The ribosomal (r)DNA internal transcribed spacer (ITS) region should be amplified using the primer combination ITS1-F/ITS-Rust1 (Kropp *et al.*, 1995). The primer combination ITS1-F/ITS4 (Gardes and Bruns, 1993) can also be used, but with these primers there is the possibility that contaminating fungi may be co-amplified, resulting in an illegible chromatogram, or preferentially amplified, resulting in a legible chromatogram that gives no information about the rust species and may cause some confusion. Primers have also been designed to amplify fragments of the *β-tubulin* (primer combination Ppsi-BtubF/Ppsi-BtubR) and *elongation factor 1α* (primer combination PPEFF/PPEFR) genes in *A. psidii* (da S. Machado *et al.*, 2015), but the specificity of these primers has not been evaluated against other rust fungi. The PCR products should be sequenced (in-house or sent to a sequencing facility) and the sequence data compared with the reference data described in section 4.2.

Table 1 lists the primer sequences and Table 2 outlines the PCR.

Table 1. Primers for conventional PCR

Gene region	Primer name	Primer sequence (5′-3′)	Reference
rDNA ITS including 5.8S rDNA	ITS1-F	CTT GGT CAT TTA GAG GAA GTA A	Gardes and Bruns (1993)
	ITS4	TCC TCC GCT TAT TGA TAT GC	White *et al.* (1990)
rDNA ITS including 5.8S rDNA and ~500 bp LSU rDNA	ITS1-F	CTT GGT CAT TTA GAG GAA GTA A	Gardes and Bruns (1993)
	ITS-Rust1	GCT TAC TGC CTT CCT CAATC	Kropp *et al.* (1995)
β-tubulin	Ppsi-BtubF	CTT TTG GTT CAC TCT TCA GAC C	da S. Machado *et al.* (2015)
	Ppsi-BtubR	AGA TGA TAA AAG ACT ACT GAC TCC	
elongation factor 1α	PPEFF	AAG GAT GCT GCT GAC ATG GGC	da S. Machado *et al.* (2015)
	PPEFR	ATC CCG AAA TGG GGA CAA AAG G	

bp, base pairs; ITS, internal transcribed spacer; LSU, large subunit.

Table 2. Conventional PCR master mix composition, cycling parameters and amplicons

Reagent	Final concentration
PCR-grade water	–[†]
PCR buffer	1×
$MgCl_2$	2.0 mM
dNTPs (each)	200 µM
BSA[‡]	0.2 mg/ml
Forward primer	0.25 µM
Reverse primer	0.25 µM
DNA polymerase	1 U
DNA (volume)	5 µl
Cycling parameters	
For ITS1-F/ITS4, Ppsi-BtubF/Ppsi-BtubR, PPEFF/PPEFR	
Initial denaturation	94 °C for 2 min
Number of cycles	30
- Denaturation	94 °C for 30 s
- Annealing	55 °C for 30 s
- Extension	72 °C for 30 s
Final extension	72 °C for 10 min
For ITS1-F/ITS-Rust1	
Initial denaturation	95 °C for 3 min
Number of cycles	35
- Denaturation	94 °C for 30 s
- Annealing	44 °C for 30 s
- Extension	72 °C for 2 min
Final extension	72 °C for 10 min
Expected amplicons	
ITS1-F/ITS4	700 bp
Ppsi-BtubF/Ppsi-BtubR	816 bp
PPEFF/PPEFR	635 bp
ITS1-F/ITS-Rust1	1 240 bp

[†] For a final reaction volume of 25 µl.

[‡] Addition of bovine serum albumin is recommended but not essential.

bp, base pairs; BSA, bovine serum albumin; PCR, polymerase chain reaction.

3.4.4 Species-specific real-time PCR of Baskarathevan *et al.* (2016)

This method is fast, sensitive and is suitable for high throughput and for detection of the pathogen in its early infection stages, but it requires species-specific primers and a dual-labelled probe. Species specificity has been demonstrated against *Puccinia* species including *P. coronata*, *P. graminis*, *P. hemerocallidis*, *P. hordei*, *P. myrsiphylla*, *P. oxalidis* and *P. striiformis* (Baskarathevan *et al.*, 2016). *A. psidii* is so phylogenetically remote from other rust species that infect Myrtaceae that cross-amplification is considered unlikely. *In silico* analysis using the Standard Nucleotide Basic Local Alignment Search Tool (BLASTN) confirmed that these primers do not bind to DNA sequences of other rust species, including those found on Myrtaceae. The TaqMan real-time PCR can detect as little as 0.011 pg of *A. psidii* genomic DNA (Baskarathevan *et al.*, 2016). An internal control based on

amplification of the host cytochrome oxidase (*COX1*) gene (Weller *et al.*, 2000) should be included unless the PCR is performed on fungal material. When the internal control is included, an additional PCR reagent "COX Block" (dNature Diagnostics & Research Ltd[1]) is required in the reaction to delay the amplification of the *COX* gene, so that the internal control does not outcompete the *A. psidii* DNA and prevent it from being detected. This PCR can be run as a duplex reaction, provided the spectrum of the probe dye for COX does not overlap with that of the target probe (Table 3).

The method is repeatable and reproducible with a coefficient of variation when repeated (for cycle threshold (Ct)) between 0.8 and 1.6 (Baskarathevan *et al*., 2016). Three combinations of primers/probes have been developed – two targeting the rDNA ITS and one targeting *β-tubulin* – but only the most sensitive primer combination is outlined here.

Table 3 lists the primer sequences and Table 4 outlines the PCR.

Table 3. Primers and probe for real-time PCR

Gene region	Primer or probe name	Primer or probe sequence (5′-3′)	Reference
A. psidii rDNA ITS1	PpsiITS1F	GTA GCT TTA TTG AAA CAT AGT AA	Baskarathevan *et al.* (2016)
	PpsiITS1R	TGA TTT TAG ACA ATA ATA ATA AGG G	
	PpsiITS1P	FAM-AGA TTA ATA TCT TTG CCA CGT ATA CCA-BHQ1	
Host cytochrome oxidase†	COX-F	CGT CGC ATT CCA GAT TAT CCA	Weller *et al.* (2000)
	COX-R	CAA CTA CGG ATA TAT AAG AGC CAA AAC TG	
	COX-P	CAL Fluor Red 610-TGC TTA CGC TGG ATG GAA TGC CCT-BHQ2	Amended from Weller *et al.* (2000)

† Optional positive control.

ITS, internal transcribed spacer; P, probe; PCR, polymerase chain reaction.

Table 4. Real-time PCR master mix composition, cycling parameters and amplicon

Reagent	Final concentration
PCR-grade water	–[†]
PCR buffer	1×
$MgCl_2$	4.2 mM
dNTPs (each)	200 µM
BSA	0.5 mg/ml
Primer PpsiITS1F	0.30 µM
Primer PpsiITS1R	0.30 µM
Probe PpsiITS1P	0.12 µM
Primer COX-F[‡]	0.30 µM
Primer COX-R[‡]	0.30 µM
Probe COX-P[‡]	0.10 µM
COX Block[‡]	750 nM
DNA polymerase	1 U
DNA (volume)	5 µl
Cycling parameters	
Initial denaturation	95 °C for 3 min
Number of cycles	40
- Denaturation	95 °C for 5 s
- Annealing and extension	59 °C for 30 s
Expected amplicon (size)	
PpsiITS1F/PpsiITS1R	91 bp

[†] For a final reaction volume of 20 µl.

[‡] For internal control (host material).

bp, base pairs; BSA, bovine serum albumin; PCR, polymerase chain reaction.

3.4.5 Species-specific nested PCR of Langrell *et al.* (2008)

A species-specific nested PCR targeting the rDNA ITS region is available for laboratories that lack the facilities to perform the TaqMan real-time PCR (section 3.4.4). The nested PCR is sensitive, detecting as little as one or two urediniospores, but it is not recommended for laboratories that lack extensive experience with nested PCR as it is prone to cross-contamination and care must be taken to avoid this.

The first round of PCR is carried out with the primer combination Ppsi1/Ppsi6 and the product of this amplification is diluted 1:5 in Tris-ethylenediaminetetraacetic acid (EDTA) (TE) buffer and used as a template in the second round of PCR, with internal primers Ppsi2/Ppsi4. The cycling parameters in Table 6 have been amended from Langrell *et al.* (2008).

PCR products can be visualized on a transilluminator after electrophoresis on agarose gel and staining with a compatible DNA-binding dye such as ethidium bromide or gel red.

Table 5 lists the primer sequences and Table 6 outlines the PCR.

Table 5. Primers for nested PCR

Gene region†	Primer name	Primer sequence (5′-3′)
First round	Ppsi1	TTC TAC CTT ATT ACA TGT AGC T
	Ppsi6	GTC ATA TTG ACA GGT TAG AAG C
Second round	Ppsi2	ATA GTA ATT TGG TAT ACG TGG C
	Ppsi4	GTC AAT CCA AAT CAA AGT ATG

Source: Langrell *et al.* (2008).

† Ribosomal (r)DNA internal transcribed spacer (ITS) including 5.8S rDNA.

PCR, polymerase chain reaction.

Table 6. Nested PCR master mix composition, cycling parameters and amplicons

Reagent	Final concentration
PCR-grade water	–†
PCR buffer	1×
$MgCl_2$	1.5 mM
dNTPs (each)	100 µM
BSA	0.2 mg/ml
Forward primer	0.10 µM
Reverse primer	0.10 µM
DNA polymerase	1.25 U
DNA (volume)	5 µl
Cycling parameters‡	
Initial denaturation	95 °C for 3 min
Number of cycles	30
- Denaturation	95 °C for 1 min
- Annealing	57 °C for 1 min
- Extension	72 °C for 1 min
Final extension	72 °C for 7 min
Expected amplicons (size)	
Ppsi1/Ppsi6	508 bp
Ppsi2/Ppsi4	379 bp

† For a final reaction volume of 25 µl.

‡ For both rounds of the nested PCR.

bp, base pairs; BSA, bovine serum albumin; PCR, polymerase chain reaction.

3.4.6 Controls for molecular tests

For the test result to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the DNA sequence of the target pest. For PCR, a positive nucleic acid control and a negative amplification control (no template control) are the minimum controls that should be used.

Positive nucleic acid control. This control is used to monitor the efficiency of the test method (apart from the extraction), and specifically the amplification. Pre-prepared (stored) genomic DNA, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product) may be used. Any fungal DNA will be a suitable positive control for the ITS1-F/ITS4 primers, and any rust DNA for the ITS1-F/Rust1 primers. The other primer pairs (PpsiBtubF/PpsiBtubR, PPEFF/PPEFR, PpsiITS1F/PpsiITS1R,

Ppsi1/Ppsi6 and Ppsi2/Ppsi4) require *A. psidii* DNA (genomic DNA or suitable plasmid or amplicon) as a positive control. In the absence of a positive control, it may be possible to confirm the presence of *A. psidii*, but not its absence. To obtain *A. psidii* genomic DNA to use as a positive control, a request should be made to countries that have *A. psidii*, such as Brazil and Australia.

Internal control. The efficiency of the extraction method is confirmed with amplification of the rDNA ITS using the primers ITS1-F/ITS4.

Negative amplification control (no template control). This control is necessary to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added in place of template DNA at the amplification stage.

Negative extraction control. This control is used to monitor both contamination during nucleic acid extraction and cross-reaction with the host tissue. The control comprises nucleic acid that is extracted from uninfected host tissue and subsequently amplified. Alternatively, extraction blanks may be processed with the samples to be tested if sufficient uninfected host tissue is not available.

3.4.7 Interpretation of results

3.4.7.1 Species-specific real-time PCR

The real-time PCR will be considered valid only if both these criteria are met:

- the positive control produces an amplification curve with the pathogen-specific primers and probe
- no amplification curve is seen with the negative extraction control and the negative amplification control.

If the *COX* internal control primers are also used, then the negative control (if used), the positive control, and each of the test samples must produce an amplification curve. Failure of the samples to produce an amplification curve with the internal control primers suggests, for example, that the DNA extraction has failed, the DNA has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract, or the nucleic acid has degraded.

A sample will be considered positive if it produces a typical amplification curve. The cycle cut-off value needs to be verified in each laboratory when implementing the method for the first time. Guidance on how to determine the cycle cut-off value can be found in Chandelier *et al.* (2010).

Baskarathevan *et al.* (2016) were able to detect 0.011 pg of *A. psidii* DNA at a Ct of 35, which represents less than one genome copy for an expected genome size of 100–150 mega base pairs. The infected plant samples had a Ct ranging from 17 to 35, depending to some extent on the severity of infection.

3.4.7.2 Species-specific nested PCR

The species-specific nested PCR will be considered valid only if both these criteria are met:

- the positive control produces the correct size amplicon (379 base pair (bp)) after the second round of PCR for *A. psidii*.
- no amplicons of the correct size are produced in the negative extraction control and the negative amplification control.

4. Identification

Any rusts detected on Myrtaceae species should be regarded as suspect and reported for prompt identification. In most cases, *A. psidii* can be identified by morphological characters (section 4.1). A final morphological diagnosis is based on the absence of marginal paraphyses and on urediniospore characters. However, given that the newly described *Phakopsora myrtacearum* from *Eucalyptus* in Africa has morphological characters that overlap with *A. psidii* (Maier *et al.*, 2016), the identity of the pathogen on *Eucalyptus* must be confirmed by using molecular methods (Figure 1). All detections that comprise a new record for a country should be confirmed by a second official laboratory. In these cases,

it is also recommended that identification be confirmed by DNA sequencing to support the morphological identification.

Although more than 40 species of rust fungi have been reported from Myrtaceae species, most have since been synonymized with *A. psidii* (Maier *et al.*, 2016). There are six currently accepted rust species on Myrtaceae (Maier *et al.*, 2016) (Table 7). These are *A. psidii*, *Puccinia cygnorum*, *Phakopsora juelii*, *Ph. myrtacearum*, *Uredo seclusa* and *Uredo xanthostemonis*. *Ph. myrtacearum* is phylogenetically distinct from *A. psidii* (Maier *et al.*, 2016) and while no sequence data are available for *Ph. juelii* (=*Ph. rossmaniae*), it is also expected to be phylogenetically distinct. *P. cygnorum* is phylogenetically distinct from *A. psidii* (Carnegie *et al.*, 2010) and not expected to amplify with the species-specific primers for *A. psidii*. No sequence data are available for *U. seclusa* or *U. xanthostemonis.* The former species is known only from the type collection, and the latter has only been recorded from *Xanthostemon* spp. and is a member of the Phakopsoraceae so should be quite distinct from *A. psidii*.

4.1 Morphological identification

Microscopic examination of suspect rust samples can be used to look for key morphological characters of *A. psidii* (Table 7).

Table 7. Morphological characters of the six rust species currently accepted as infecting Myrtaceae

Rust species	Urediniospores (µm)	Teliospores (µm)	Paraphyses	Comment
Austropuccinia psidii	15–26 × 14–22	23–50 × 14–28 Pedicel fragile	Absent	Wide host range
Puccinia cygnorum	Unknown	35–60 × 12–20 Pedicel persistent	n/a	Known only on *Kunzea ericifolia* from near Perth, Western Australia
Phakopsora juelii	14–23 × 12–18	10–14 × 6–9 Subepidermal, aseptate	Present	Known on *Campomanesia* spp. from Brazil
Phakopsora myrtacearum	20–30 × 14–20	Unknown	Absent	Known only on *Eucalyptus* spp. from southern and eastern Africa
Uredo seclusa	24–32 × 15–20	Unknown	Absent	Known only from type specimen from Brazil
Uredo xanthostemonis	17–28 × 15–20	Unknown	Present	Known only on *Xanthostemon* spp. from Australia

Source: Maier *et al.* (2016).
n/a, not available.

The following key (Maier *et al.*, 2016) can be used to distinguish the two described rust fungi on eucalyptus:

1. Uredinia forming bright yellow–orange pustules on leaves, petioles, shoots, flowers or fruits. Urediniospores mainly globose to subpyriform, 15–26 × 14–22 µm ..... ***Austropuccinia psidii***

– Uredinia pale yellow to light brown, on discoloured areas of leaves. Urediniospores mainly pyriform to ellipsoid, 20–30 × 14–20 µm ..... ***Phakopsora myrtacearum***

4.1.1 Morphological characters of *A. psidii*

Sori are scattered on green tissue, leaf spots, stems, flowers and fruits, and are mainly hypophyllous. **Uredinia**, which are bright yellow to orange yellow, are usually more common than **telia**, which are dark brown; both are aparaphysate. Both teliospores and urediniospores may occur together in the same sorus (Pegg *et al.*, 2014).

The following descriptions are taken from Pegg *et al.* (2014) and are a composite morphological description based on samples from 11 host genera.

Uredinia on chlorotic, red–purple or greyish leaf spots with a darker margin up to 1 mm diameter, amphigenous, mostly abaxial, subepidermal, erumpent, round, up to 500 µm, bright yellow to orange to yellowish brown (depending on host genus).

Urediniospores globose to subglobose or ellipsoidal to ovoid or obpyriform, yellowish brown, 15−26 × 14–22 µm; wall 1.0–3.0 µm thick, finely echinulate, germ pore absent or inconspicuous (Figures 5(a), (b), (d)). The presence of a tonsure (smooth patch) on urediniospores is often observed, but its presence or absence is not consistent even in the same sorus.

Telia on fruit, leaves or stems, up to 0.5 mm diameter, abaxial, erumpent, pulvinate, yellow to yellowish brown.

Teliospores cylindrical to ellipsoidal, apex rounded, pale yellowish brown, 23–50 × 14–28 µm; wall 1.0–2.0 µm thick, smooth, two-celled, pedicel up to 15 µm long (Figures 5(c), (d), (e)).

Basidia cylindrical, up to 110 µm long and 6–8 µm wide, hyaline, four-celled, produced from each cell of the teliospores, apically in upper cell and laterally in lower cell.

Basidiospores globose to pyriform, 8–11 µm diameter, hyaline, smooth, germinate *in situ* without dormancy from an apical pore (Figure 5(f)).

4.2 Molecular identification

Morphological variation has in the past led to erroneous identification and classification of *A. psidii*. In 2015, an epitype was designated for *A. psidii* for the precise application of the taxon name, providing DNA characterization for stability and comparison. The epitype and 17 *A. psidii* specimens from collections from Australia, Hawaii, New Caledonia and South America had identical DNA sequences for three genetic regions: ITS, *β-tubulin* and *elongation factor 1α* (da S. Machado *et al.*, 2015). The epitype sequences are deposited in GenBank as KM282154 (ITS), KM282123 (*β-tubulin*) and KM282143 (*elongation factor 1α*). They should be referred to for comparison when identifying samples.

For a definitive identification, the preferred method is to extract DNA from rust spores (section 3.4.1 and 3.4.2), amplify the selected region or regions (section 3.4.3), and compare the sequence data of the fungal barcoding region, the rDNA ITS region, with GenBank KM282154. Sequence similarity should be ≥99%. DNA sequencing of secondary regions such as *β-tubulin* and *elongation factor 1α* genes and the rDNA large sub-unit (LSU) region provides support for initial diagnoses. All regions have very low intraspecific variation (<1%), and they have barcode gaps of 10% (ITS), 17% (*β-tubulin*) or 20% (*elongation factor 1α*).

DNA sequencing of the primary fungal barcode region, rDNA ITS, supported by one or more secondary barcode regions, provides the most robust form of molecular identification (Schoch *et al.*, 2012). Any combination of the rDNA ITS plus *β-tubulin* and *elongation factor 1α* regions described in section 3.4 is suitable for identification. The DNA sequences of species-specific PCR products are acceptable if they meet the minimum length of 400 bp indicated as desirable for DNA barcodes (Kress and Erickson, 2008). Chromatograms should be edited to trim the background or "noise" peaks and the sequence used to search the International Nucleotide Sequence Databases (GenBank, European Molecular Biology Laboratory, and DNA Data Bank of Japan) using the BLAST algorithm (Altschul *et al.*, 1997).

4.2.1 Interpretation of results

Appropriate reference sequences from the *A. psidii* epitype are available – GenBank accession numbers KM282154 (ITS), KM282123 (*β-tubulin*) and KM282143 (*elongation factor 1α*). These regions all have less than 1% intraspecific variation and >10% interspecific variation, so a sequence similarity of >98% to any of these *A. psidii* reference sequences over an alignment longer than 400 bp can be taken as confirmation of identification as *A. psidii* (Rodas *et al.*, 2015).

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance (ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*)) and where *A. psidii* is found in an area for the first time, the records and evidence and additional material should be kept for at least one year in a manner that ensures traceability:

- After samples have been detected as positive, the DNA should be labelled and stored frozen at −20 or −80 °C.
- The remaining infected plant material should be pressed and dried, packaged and labelled, and stored as appropriate for herbarium specimens.
- Microscopic slides should be sealed and stored with the plant specimens.

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

Agriculture Victoria, Department of Economic Development, Jobs, Transport and Resources, Australia, AgriBio Centre for AgriBioscience, La Trobe University, 5 Ring Road, Bundoora, Victoria 3083, Australia (Jacqueline Edwards; email: jacky.edwards@ecodev.vic.gov.au).

School of Land and Food, University of Tasmania, Private Bag 98, Hobart, Tasmania 7001, Australia (Morag Glen; email: Morag.Glen@utas.edu.au).

Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES), Laboratoire de la Santé des Végétaux [Plant Health Laboratory], Unité de mycologie [Mycology Unit], Domaine de Pixérécourt – Bâtiment E, C.S. 40009, 54220 Malzéville, France (Jacqueline Hubert; email: jacqueline.hubert@anses.fr).

General Research and Biotechnology Unit, Nigeria Agriculture Quarantine Service, Post-Entry Quarantine Station, Moor Plantation (NCRI Compound), Apata, Ibadan, Oyo State, Nigeria (Kazeem Shakiru Adewale; email: kazeems2001@yahoo.com).

United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Regulations, Permits and Manuals, 4700 River Rd. Unit 133, Riverdale, MD 20737, United States of America (José R. Hernández; email: Jose.R.Hernandez@aphis.usda.gov).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

The first draft of this protocol was written by J. Edwards (Agriculture Victoria, Department of Economic Development, Jobs, Transport and Resources, Australia (see preceding section)), M. Glen (School of Land and Food, University of Tasmania, Australia (see preceding section)), J. Hubert (Mycology Unit, ANSES, France (see preceding section)), J. Hernandez (USDA-APHIS, United States of America (see preceding section)) and K. Shakiru Adewale (General Research and Biotechnology Unit, Nigeria Agricultural Quarantine Service, Nigeria (see preceding section)). In addition, the following experts were significantly involved in the development of this protocol: M. Piepenbring (Department of Mycology, Goethe University Frankfurt am Main, Germany), C. Rodriguez-Delgado (Department of Agriculture and Water Resources, Australia), F. Sorgoni (Ministero delle Politiche Agricole Alimentari e Forestali and Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Italy) and H. Shirato (Ministry of Agriculture, Forestry and Fisheries, Yokohama Plant Protection Station, Japan).

8. References

The present annex may refer to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

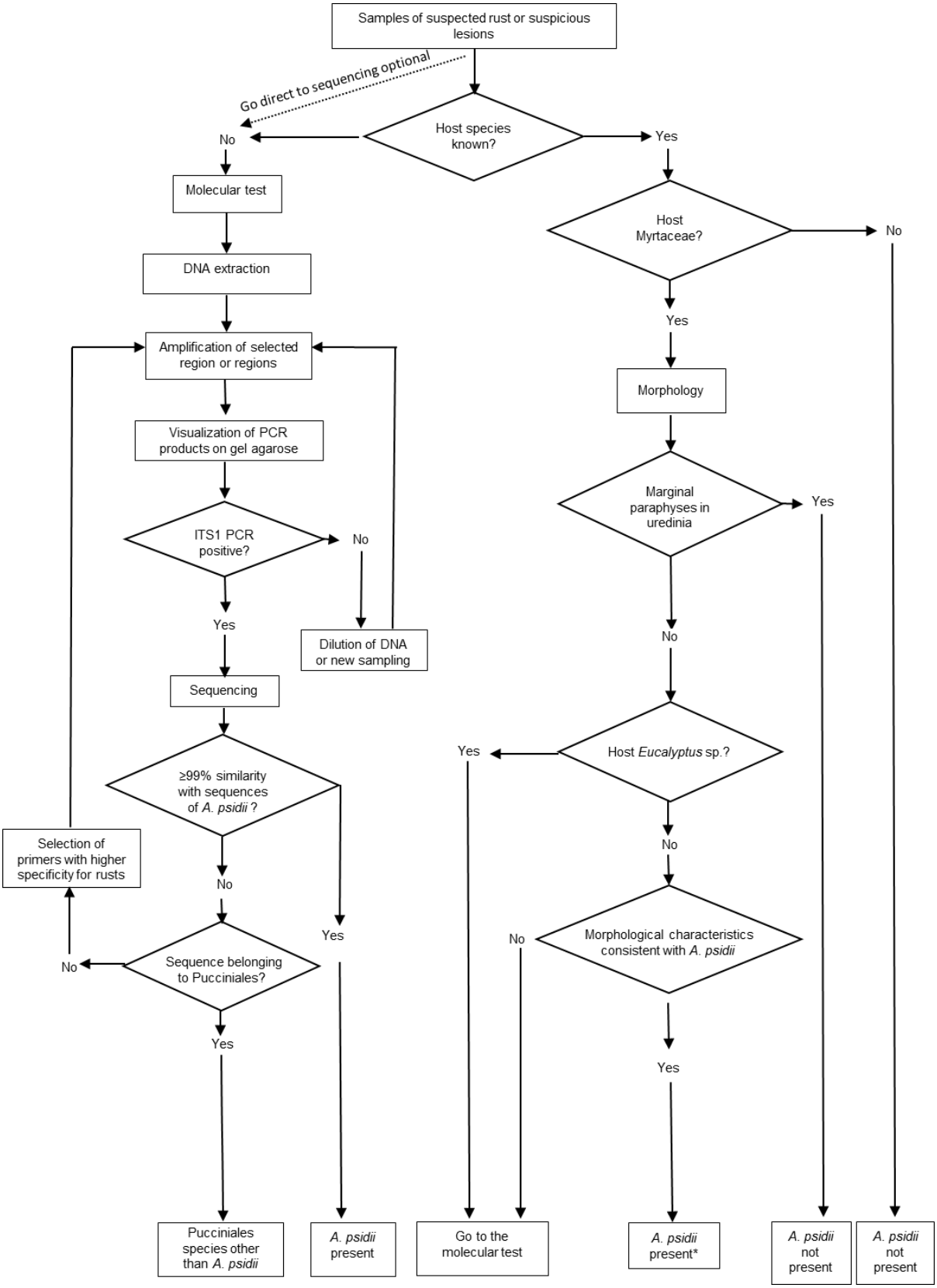

Figure 1. Flow chart of methods to confirm the identity of *Austropuccinia psidii*.
* Confirm with DNA sequencing for a first detection in a country.
ITS, internal transcribed spacer; PCR, polymerase chain reaction.

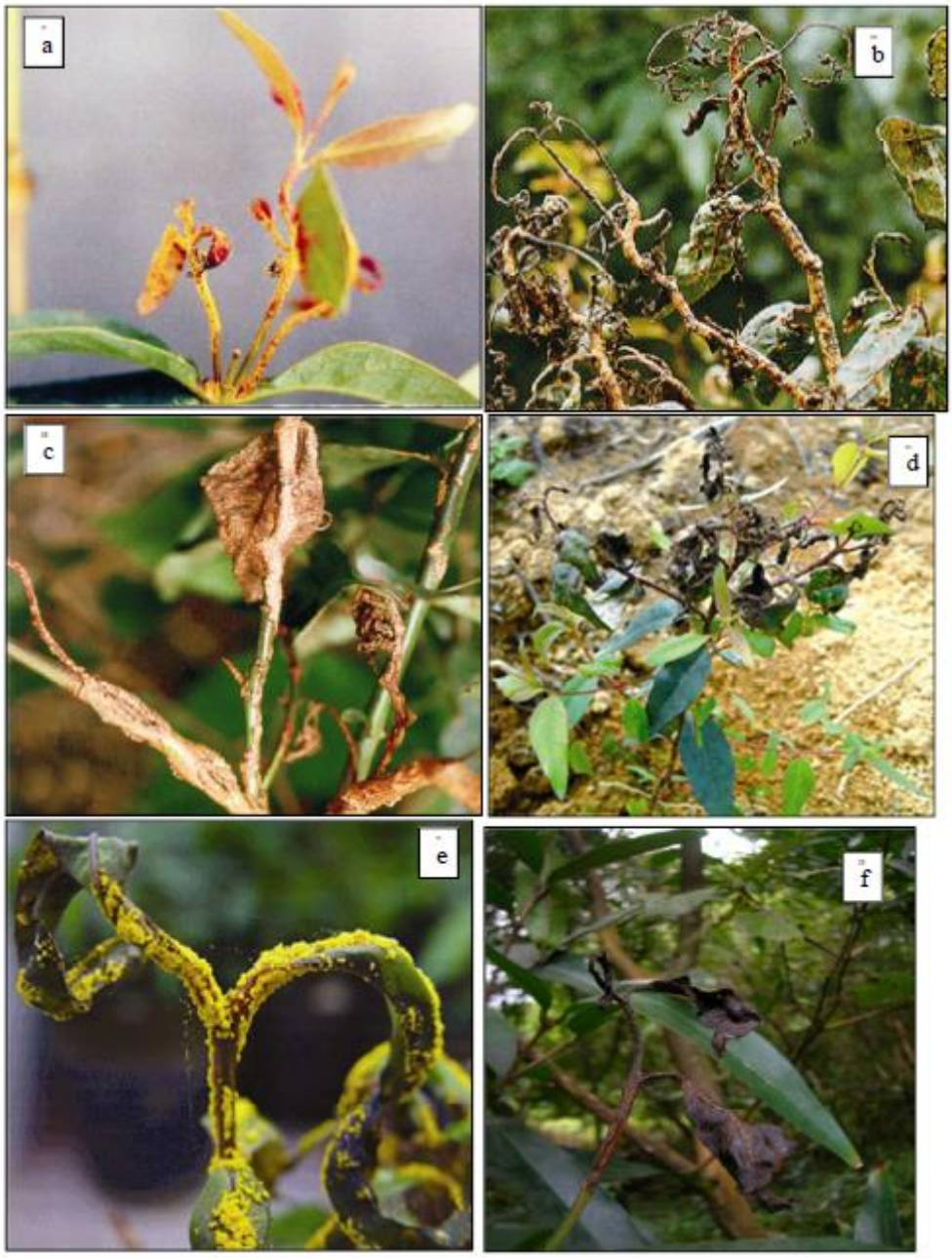

Figure 2. *Austropuccinia psidii* on *Eucalyptus grandis* causing (a) infection on leaves and stems, (b) defoliation and shoot death, (c) stem galls on a previous infection and (d) defoliation and shoot death on young leaves and stems. *A. psidii* on *Syzygium jambos* showing (e) uredinia on young leaves and stems and (f) leaf and shoot death.
Source: Photos courtesy of Forest Pathology and Genetics of Plant Pathogen Interactions Laboratory, Federal University of Viçosa, Brazil.

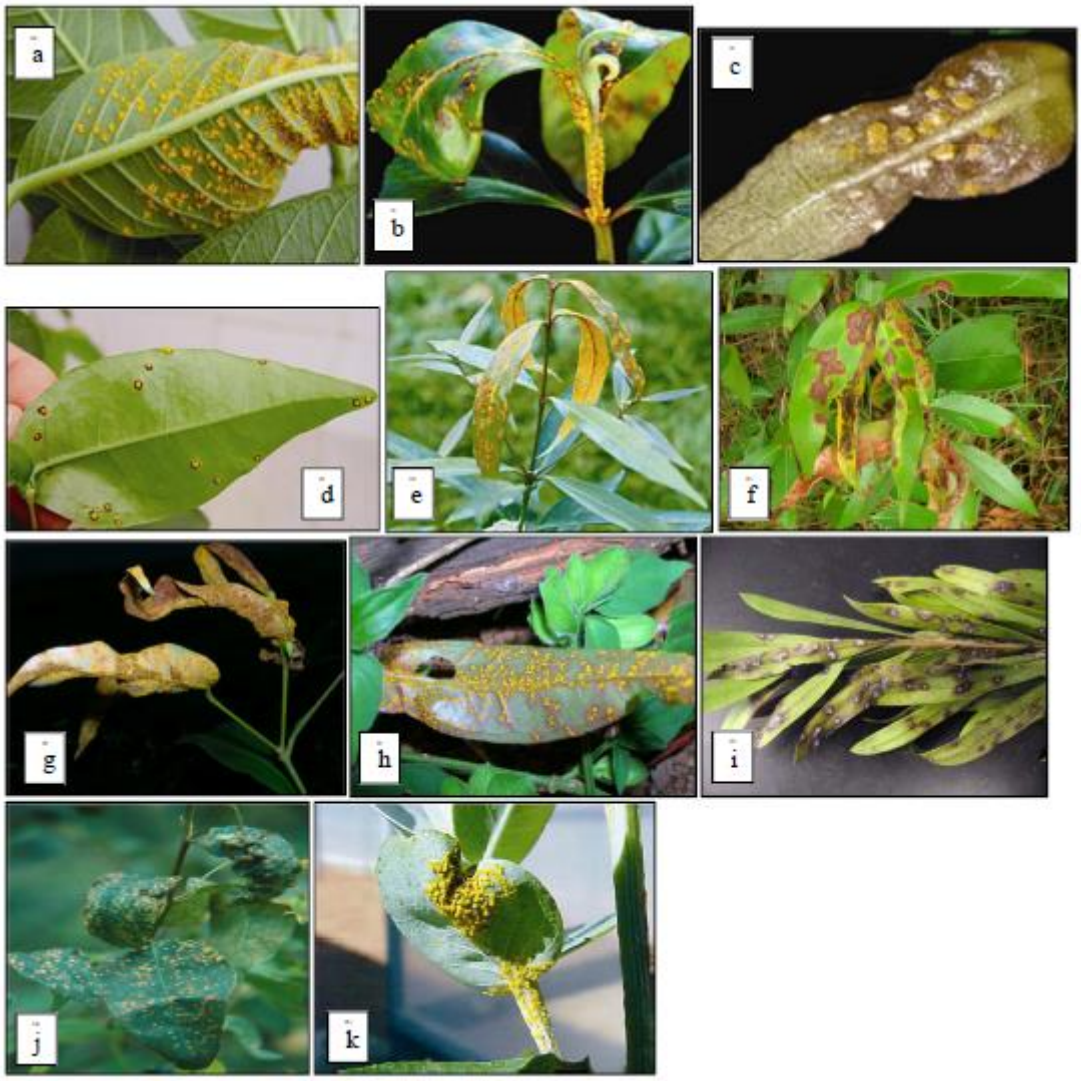

Figure 3. *Austropuccinia psidii* causing infection on leaves of (a) *Psidium guajava*, (b)–(c) *Heteropyxis natalensis*, (d) *Eugenia uniflora*, (e)–(h) *Syzygium jambos*, (i) *Callistemon citrinus*, (j) *Eucalyptus grandis* hybrid and (k) *Eucalyptus globulus*.
Source: Photos courtesy of Forest Pathology and Genetics of Plant Pathogen Interactions Laboratory, Federal University of Viçosa, Brazil.

Figure 4. *Austropuccinia psidii* infection on fruit of (a) *Psidium guajava*, (b) *Eugenia uniflora* and (c) *Myrciaria cauliflora*, and on flower buds of (d) *Syzygium jambos*.
Source: Photos courtesy of Forest Pathology and Genetics of Plant Pathogen Interactions Laboratory, Federal University of Viçosa, Brazil.

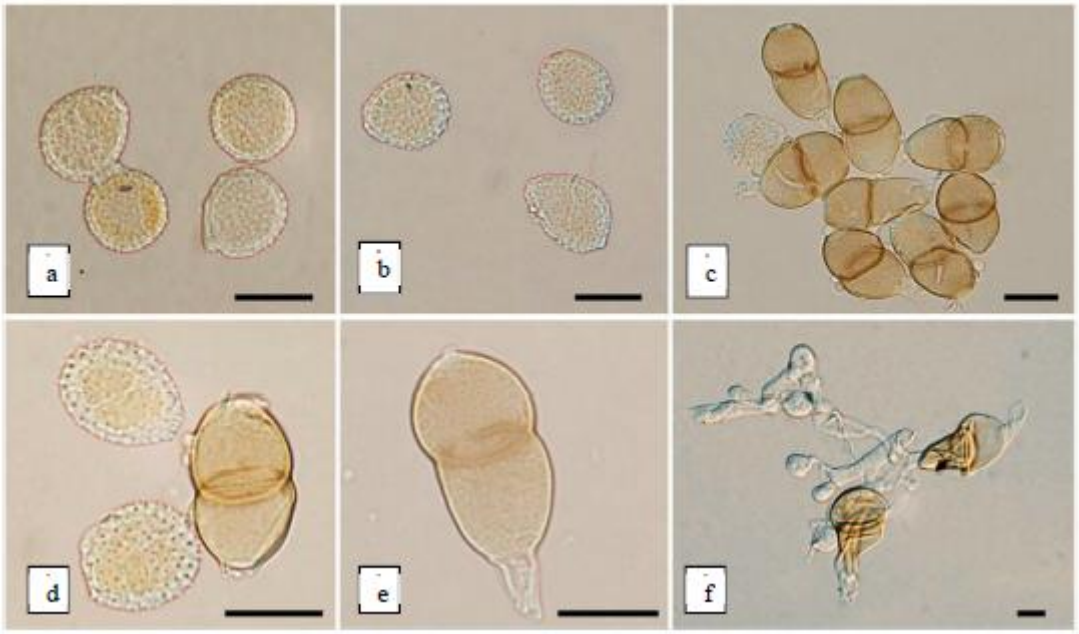

Figure 5. *Austropuccinia psidii*: (a)-(b) urediniospores; (c)–(d), teliospores and urediniospores; (e) teliospore; and (f), germinated teliospores and basidiospores. Scale bars: 20 µm.
Source: da S. Machado et al. *(2015).*

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This is not an official part of the standard

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2016-05 Expert consultation.

2016-06 Diagnostic Protocol (DP) drafting group and Discipline Lead revised the draft.

2016-09 Revised by Technical Panel on Diagnostic Protocols (TPDP).

2017-02 TPDP e-decision for submission to SC for approval to first consultation (2017_eTPDP_Feb_02).

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2017-11 Revised by the Lead based on consultation comments.

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