**Diagnostics**
**Diagnostic**

# *Melampsora medusae*

## Specific scope

This standard describes a diagnostic protocol for *Melampsora medusae*[1]

## Specific approval and amendment

Approved in 2009–09.

## Introduction

*Melampsora medusae* Thümen is one of the causal agents of poplar rust. The main species involved in this disease in Europe on cultivated poplars are *Melampsora larici-populina* and *Melampsora allii-populina*. All three species cause abundant uredinia production on leaves, which can lead to premature defoliation and growth reduction. After several years of severe infection leading to repeated defoliation, the disease may predispose the trees to dieback or even death for the younger trees. *Melampsora* rust is a very common disease of poplar trees, which can cause severe economic losses in commercial poplar cultivation because of the emergence and spread of new pathotypes of *M. larici-populina*.

*Melampsora medusae* originates from North America and has spread to other continents. In addition to the USA, Canada and Mexico, it has spread during the twentieth century to Europe (see below), South America (Bolivia, Brazil, Chile), Southern Africa (South Africa, Zimbabwe), Asia (Japan), and Oceania (Australia, New Zealand). In the EPPO region, the occurrence of *M. medusae* was reported in restricted areas in Belgium, France and Portugal (EPPO, 1997).

The telial hosts are hybrids of *Populus* spp. and the aecial hosts are conifers such as *Larix* spp., *Pseudotsuga* spp. and *Pinus* spp. However, *M. medusae* has never been found on any aecial host in the EPPO region (Pinon, 1986; EPPO/CABI, 1997).

There are a total of eight *Melampsora* spp. reported on poplars in Europe up to now (Pinon, 1973; Cellerino, 1999). However, only three of them, namely *M. larici-populina*, *M. allii-populina* and *M. medusae,* are pathogenic on the poplars of the sections *Aigeiros* and *Tacamahaca*, i.e. *Populus nigra*, *Populus deltoides*, *Populus trichocarpa,* and their interspecific hybrids (Frey *et al.*, 2005). In Europe, most of the cultivated poplars are *P.* × *euramericana* (*P. deltoides* × *P. nigra*) and *P.* × *interamericana* (*P. trichocarpa* × *P. deltoides*) hybrids. In addition, Shain (1988) showed evidence for the existence of two *formae speciales* within *M. medusae* and named them *M. medusae* f. sp. *deltoidae* and *M. medusae* f. sp. *tremuloidae* according to their primary host, *Populus deltoides* and *Populus tremuloides*, respectively. Neither the EU directive, nor the EPPO make the distinction between these two *formae speciales* but only refer to *M. medusae*. Notwithstanding, since *M. medusae* was only reported in Europe on poplars from the sections *Aigeiros* and *Tacamahaca*, and in respect with the host specialization, it can be stated that only *M. medusae* f. sp. *deltoidae* was reported in Europe so far (Pinon, 1991; Pinon & Frey, 2005).

The five other species, namely *Melampsora larici-tremulae*, *Melampsora magnusiana*, *Melampsora pinitorqua*, *Melampsora rostrupii* and *Melampsora pulcherrima,* are only pathogenic on species of the section *Populus* (formerly *Leuce*) also called 'aspens': *P. alba*, *P. tremula*, and their hybrids (Frey *et al.*, 2005). In the scientific literature, the first four taxa are sometimes merged in the *M. populnea* (Pers.) P. Karst complex but recent multigene phylogeny suggests that this complex can be clearly divided in different groups (Feau *et al.*, 2009). Since these aspen species are not widely planted for commercial cultivation and exist mainly as wild and ornamental trees in Europe, the incidence of these five *Melampsora* rust species on *P. alba*, *P. tremula* and their hybrids has a much lower economic impact (Frey *et al.*, 2005), than *M. medusae* f. sp. *tremuloidae* would have, if ever introduced into Europe.

In addition, it is worth noting that *M. medusae* f. sp. *deltoidae* was reported to form interspecific hybrids with other *Melampsora* sp. First, *M. medusae-populina*, an interspecific hybrid of *M. medusae* and *M. larici-populina*, was discovered in 1991 in New Zealand (Spiers & Hopcroft, 1994) and subsequently found

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.


 

in South Africa (Frey *et al.*, 2005). Second, *M.* × *columbiana*, an interspecific hybrid of *M. medusae* and *M. occidentalis*, was discovered in 1995 in the Pacific Northwest of the USA in 1995 (Newcombe *et al.*, 2000). Although none of these hybrid *Melampsora* have been reported from the EPPO region so far, special attention should be paid in the regions where both *M. medusae* and *M. larici-populina* occur.

## Identity

**Name**: *Melampsora medusae* Thümen

Macrocyclic development stage names: *Caeoma faulliana* Hunter (aecial stage), *Uredo medusae* Thümen (uredinial stage).

**Synonyms**: *Melampsora albertensis* J.C. Arthur

**Taxonomic position**: *Fungi: Basidiomycota: Uredinales*

**Common names**: Poplar rust

Notes on taxonomy and nomenclature: see the introduction section

**EPPO code**: MELMME

**Phytosanitary categorization**: EPPO A2 list no. 74, EU annex designation I/AII.

## Detection

### Symptoms

The symptoms caused on their telial host by the poplar rust fungi native to Europe (*M. allii-populina* and *M. larici-populina*) are hardly distinguishable from those caused by *M. medusae* and therefore are not sufficient for diagnosis.

*Symptoms on the telial host (Poplar)*

Early symptoms of infection by *Melampsora* rust fungi, regardless of the species involved, are small yellowish pustules (uredinia, containing masses of urediniospores, Fig. 1) on the abaxial leaf surface (Fig. 2). The uredinia may be scattered on the leaves or be so crowded that the entire surface finally looks like powdery and yellowish orange (Fig. 3). Numerous asexual generations of uredinia and urediniospores occur during the whole growing season when favourable climatic conditions are met. After primary infection, urediniospores are easily windborne to the whole tree crown and to nearby susceptible trees where infection occurs in moist conditions. In severe attacks, the pustules may cover the whole leaf surface giving a golden appearance, and then cause partial or total defoliation. In late summer by mid-autumn, telia appear on either side of the leaves, looking like small brown to black raised spots giving a crustlike appearance. These telia contain large numbers of thick-walled resting spores (teliospores) that will overwinter on fallen leaves, and then will germinate in the following spring to release basidiospores. The position of the telia is variable

**Fig. 1** Uredinia of *Melampsora* sp. on the abaxial face of a poplar leaf (courtesy of Frey P, INRA Nancy, FR).

**Fig. 2** Typical *Melampsora* rust symptoms on a poplar leaf (uredinia) (courtesy of Frey P, INRA Nancy, FR).

**Fig. 3** Young poplar heavily infected by *Melampsora* rust (courtesy of Frey P, INRA Nancy, FR).


**Fig. 4** Sampling of uredinia using a spatula (courtesy of P Loevenbruck, LNPV Malzéville, FR).

according to the *Melampsora* spp.: *M. medusae* and *M. allii-populina* form mainly hypophyllous telia, whereas *M. larici-populina* forms mainly epiphyllous telia.

Since it is heteroecious, like *M. larici-populina* and *M. allii-populina*, *M. medusae* needs an aecial host to overwinter sexually. There is no published evidence that the uredinial stage of *M. medusae* is able to overwinter asexually under a temperate climate. However, *M. medusae* is thought to overwinter asexually in Australia, through a 'green bridge' provided by semi-evergreen poplars (Wilkinson & Spiers, 1976). In EPPO countries, *M. medusae* is only reported on poplars and was so far never observed on its potential aecial hosts.

*Symptoms on the aecial hosts* (*Larix* spp., *Pseudotsuga* spp., *Pinus* spp.)

The basidiospores infecting the aecial hosts in spring infect the needles and produce pale yellow spots (spermogonia) on the upper leaf surface. Within a few days, following plasmogamy, pale yellow pustules (aecia) will appear on the lower leaf surface. These structures develop preferably on the current year's needles, but may be observed, although scarcely, on the conifer cones or on young shoots. Aecia produce large numbers of yellow aeciospores, arranged in chains. These wind-borne aeciospores will infect a susceptible *Populus* host in the spring and the fungus will soon produce urediniospores to complete the life cycle.

## Sampling procedure

Since the aecial stage of *M. medusae* was never reported on the conifer (aecial) hosts in the EPPO region, the sampling procedure will be restricted to the poplar trees, where the likelihood of detecting *M. medusae* will be the highest. As the dimensions, shape, and other morphological features of teliospores of *M. medusae* are not discriminant in comparison with other indigenous species of poplar *Melampsora*, only symptomatic poplar leaves bearing uredinia should be collected. Inspection is best carried out in late summer when symptoms are most obvious and uredinia levels are at their highest level. Infected leaves may be sent directly to the laboratory for analysis. For molecular analysis, it is preferable to scrape uredinia from the leaves with a scalpel blade or a spatula and transfer into a sterile microcentrifuge tube (Fig. 4) to be sent to the laboratory.

**Table 1** Morphological description of the urediniospores and paraphyses for poplar *Melampsora* spp.

| | Species infecting poplars of the sections Aigeiros and Tacamahaca | | | *Melampsora* spp. infecting poplars of the section Populus*** |
|---|---|---|---|---|
| | *Melampsora medusae* | *Melampsora larici-populina* | *Melampsora allii-populina* | |
| **Urediniospores** | | | | |
| Shape | Mainly ellipsoid to ovoid | Mainly pyriform | Mainly ellipsoid to ovoid | – |
| Size (µm) | 26–35 × 15–19*<br>22–36 × 13–21** | 30–44 × 14–19*<br>30–42 × 13–20** | 24–38 × 12–19** | – |
| Apex echinulation | Echinulate | Smooth patch | Smooth patch | Echinulate |
| Equator echinulation | Smooth patch, or completely smooth | Echinulate | Echinulate | Echinulate |
| Wall | Thickened at the equator | Thickened at the equator | Uniformly thick | – |
| **Paraphyses** | | | | |
| Shape | Capitate to clavate | Clavate | Capitate | – |
| Size (µm) | ≤70*<br>32–58** | 65–75*<br>40–70** | 50–60** | – |
| Capitate apex size (µm) | 14–17*<br>9–15** | 14–21*<br>14–18** | 14–22** | – |
| Wall | Uniformly thick | Thickened at the apex | Uniformly thick | – |

*After CMI description of pathogenic fungi and bacteria for *M. medusae*, *M. larici-populina* and *Melampsora allii-populina*, respectively. **After Pinon, 1973. ***For *Melampsora* spp. infecting aspens (i.e. *P. tremula*, *P. alba*) in Europe (*M. larici-tremulae*, *M. magnusiana*, *M. pinitorqua*, *M. pulcherrima*, and *M. rostrupii*), the observation of dimensions and shape features is not necessary since these species are uniformly echinulate.

## Identification

For a positive identification the fungus should have been identified unambiguously by any one of the following:

- Morphological identification: observation of echinulate urediniospores with a typical equatorial smooth patch, equatorial wall thickening, and capitate to clavate paraphyses with a uniform wall thickness. Dimensions of urediniospores and paraphyses should match those listed in Table 1,
- Conventional PCR, satisfying the criteria listed in Appendices 1 or 2.

### Morphological characterization

Individual uredinia are recovered from the infected leaves using a sterile needle, mounted in lactic acid, lactophenol or lactoglycerol (using water is not recommended since it may affect the urediniospore's shape and size) and observed under a light microscope at magnifications between ×400 and ×1000. At least 10 urediniospores and paraphyses per uredinia are observed and measured in both dimensions. Their shape and ornamentation are carefully observed. As several species may be simultaneously present on an individual leaf, it is very important to examine separately each of the uredinia collected. This method is not able to distinguish the two *formae speciales* of *M. medusae* since they share grossly identical morphological features and can only be distinguished according to their respective host range (Shain, 1988).

*Melampsora medusae* urediniospores are obovate to oval or in a few occasions pyriform, apex rounded, display a truncated base and a golden yellow content. Urediniospore dimensions are (23) 26–35 (37) × 15–19 (21) µm. The wall is colourless, 1–1.5 µm thick above to 2 µm at base and is often thicker [3–5 (5.5) µm] at the equatorial level. The wall surface is echinulate, except for a distinctive smooth equatorial patch, commonly extending half to three quarters of the way around the spore (Fig. 5).

The paraphyses are capitate, up to 70 µm long, with a 4–6 µm wide stalk. The capitate apex is roughly spherical, 14–17 (19) µm in diameter, or oval to less commonly clavate 18–22 × 12–16 µm. The wall is uniformly thick (1.5–3 µm) or occasionally slightly thicker at the apex (4 µm). (CMI, 1975)

Combination of shape, position of the echinulations, and wall thickening is typical for *M. medusae* (Table 1, Figs 6–8). *Melampsora medusae* has an equatorial smooth patch; *M. larici-populina* and *M. allii-populina* both have smooth apical patches; all the other *Melampsora* spp., only encountered on aspens, have uniformly echinulate urediniospores (*M. pulcherrima*, *M. pinitorqua*, *M. larici-tremulae*, *M. rostrupii*, *M. magnusiana*). Therefore, based on morphological characters in Table 1 *Melampsora medusae* can be distinguished from the other *Melampsora* sp. present in Europe.

This method is straightforward, requires only minimal taxonomic skill and a light microscope. Though it is a highly specific method it may be difficult to detect a few urediniospores of *M. medusae* in large number of other *Melampsora* urediniospores, in which case the use of molecular methods may be considered.

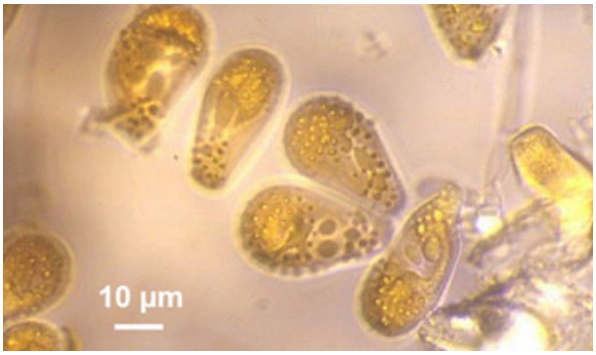


**Fig. 5** Urediniospores of *Melampsora medusae* f. sp. *deltoidae* with the typical smooth equatorial patch (courtesy of Hubert J, LNPV Malzéville, FR).

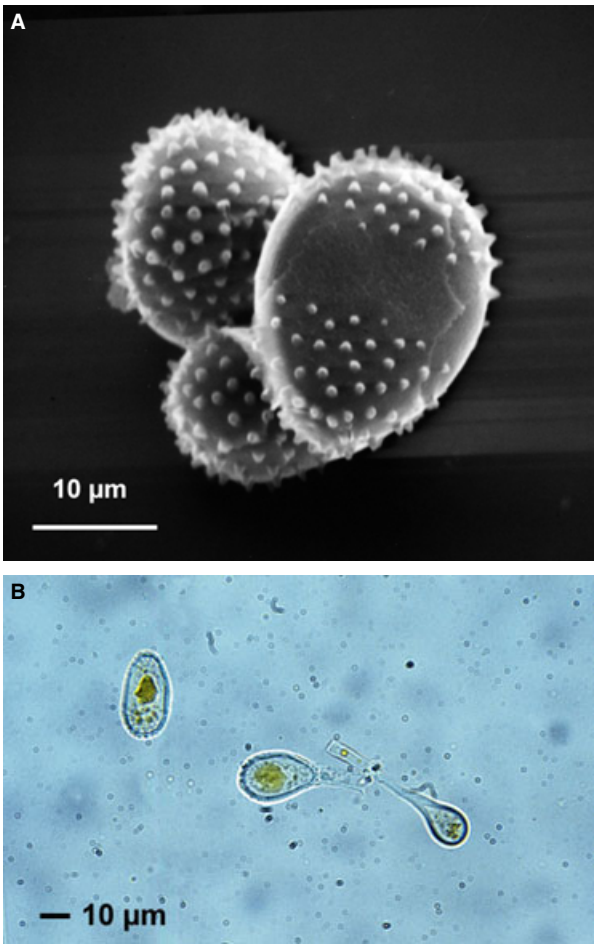


**Fig. 6** (A) *Melampsora medusae* f. sp. *deltoidae* urediniospores observed by scanning electron microscopy (courtesy of Le Thiec D, INRA Nancy, FR) and (B) *M. medusae* f. sp. *deltoidae* paraphyse and urediniospores observed by light microscopy (courtesy of Frey P, INRA Nancy, FR).

### Molecular methods

*Method A (Husson et al.*, 2003) (Appendix 1)
Husson *et al.* (2003, 2009) described a series of PCR primer pairs within the rDNA regions of *Melampsora* spp. that specificially target the ITS region of *M. allii-populina* (ITS-Map-F/R), *M. larici-populina* (ITS-Mlp-F/R) and *M. medusae* f. sp. *deltoidae* (ITS-Mmd-F/R). Besides, analysis of *M. medusae* f. sp. *tremuloidae* ITS sequences shows that DNA from this *forma specialis* would also be successfully amplified by PCR with ITS-Mmd-F/R primers since the target sequences are identical for both *formae speciales*. (R. Ioos, LNPV, pers. obs.).

*Method B (Bourassa et al.*, 2005) (Appendix 2)
Bourassa *et al.* (2005) developed two PCR-SSCP (Single Strand Conformation Polymorphism) markers derived from SCARs isolated from *M. medusae* f. sp. *deltoidae*. Initially developed for genotyping isolates of *M. medusae* f. sp. *deltoidae*, the two PCR primer pairs developed (targeting locus 'A', 502 bp, and locus 'B', 594 bp, respectively) may also be useful for the detection of this taxon. Both primer pairs also proved to yield positive signals with *M. medusae* f. sp. *tremuloidae*, except for a few strains whose DNA could not be amplified, or with a low PCR efficiency (DL Joly and N Feau, Canadian Forest Service, pers. comm.).

*Advantages and limitations of the molecular methods A and B*
The detection of both *formae speciales* of *M. medusae* by molecular analyses enables the simultaneous handling of many samples each containing thousands of urediniospores (1 mg = approximately $4 \times 10^5$ spores), which cannot be achieved with morphological identification. The methods are very sensitive and selective due to the ability to handle large number of urediniospores in comparison to the morphological

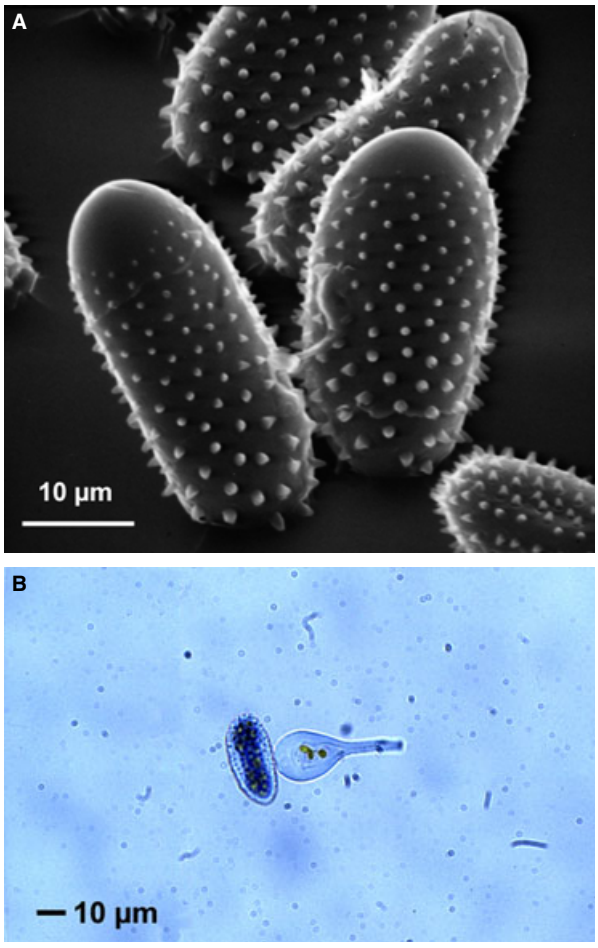


**Fig. 7** (A) *Melampsora larici-populina* urediniospores observed by scanning electron microscopy (courtesy of Le Thiec D, INRA Nancy, FR) and (B) *Melampsora larici-populina* paraphyse and urediniospore observed by light microscopy (courtesy of Frey P, INRA Nancy, FR).

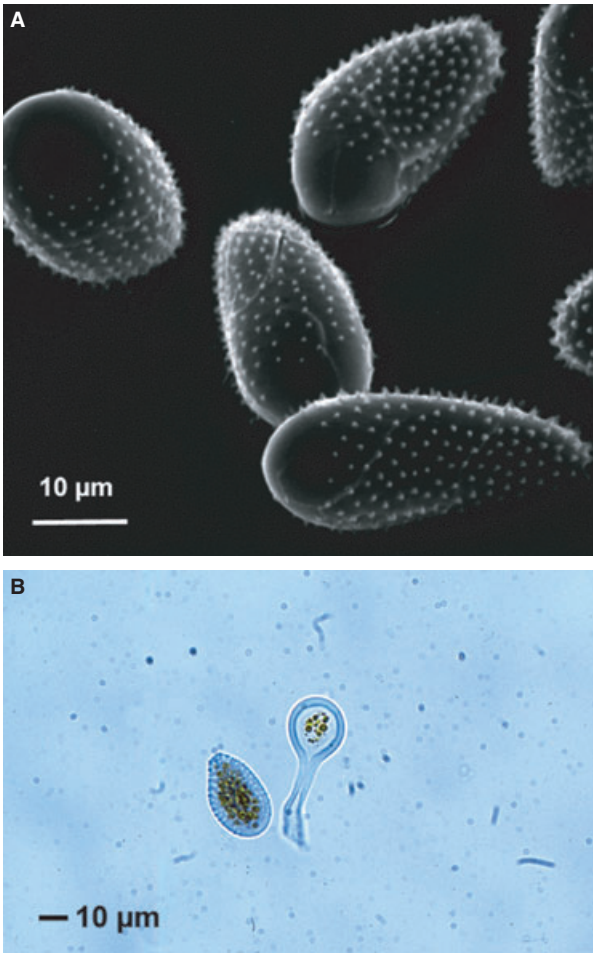


**Fig. 8** (A) *Melampsora allii-populina* urediniospores observed by scanning electron microscopy (courtesy of Le Thiec D, INRA Nancy, FR) and (B) *Melampsora allii-populina* paraphyse and urediniospore observed by light microscopy (courtesy of Frey P, INRA Nancy, FR).

**Table 2** Sequence and specificity of the PCR primer combinations

| Primer | Sequence (5′–3′) | Amplicon size (bp) | Target | Specificity | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Mmd*[a]** | *Mmt*[b]** | *Mlp*[c]* | *Map*[d]* | *Mlt*[e]** | *Mp*[f]** | *Mr*[g]** |
| ITS-Mmd-F | GAG TTG CTT AAA TGC GAT TC | 575 | ITS region | + | + | − | − | + | + | − |
| ITS-Mmd-R | CTA AAG GTA AAT TCA ATG GG | | | | | | | | | |
| c1c3a2f | GGG GGT CTT TAG GAC AAA | 502 | 'locus A'[$] | +[§] | +[§] | − | − | − | − | − |
| c1c3a2r | TTC GAG CCA GCA TGA AAC AC | | | | | | | | | |
| c1c3a3f | TTC GAG CCA GAA GTT GTT TC | 594 | 'locus B'[$] | + | +[§] | − | − | − | − | − |
| 5 | TTC GAG CCA GGA TCA CTT | | | | | | | | | |
| ITS1 | TCC GTA GGT GAA CCT GCG G | 660–680[&] | ITS region | + | + | + | + | + | + | + |
| ITS4 | TCC TCC GCT TAT TGA TAT GC | | | | | | | | | |

[a]*Melampsora medusae* f. sp. *deltoidae*
[b]*Melampsora medusae* f. sp. *tremuloidae*
[c]*Melampsora larici-populina*
[d]*Melampsora allii-populina*
[e]*Melampsora larici-tremulae*
[f]*Melampsora pinitorqua*
[g]*Melampsora rostrupii.*
*Species occurring only on poplars of the section *Aigeiros* and *Tacamahaca*, or their hybrids
**Species occurring only on poplars of the section *Populus*
[&]Depending on the *Melampsora* sp.
[$]Unknown location, single copy locus (Bourassa *et al.*, 2005)
[§]Except a few isolates (null alleles or low PCR efficiency).

method. Nevertheless, false-positive results may be obtained using method A if uredinia are collected on aspen leaves (*P. tremula* or *P. alba*) infected by *M. larici-tremulae* or *M. pinitorqua*. Similarly, false-negative results may be obtained using method B for some isolates of both *M. medusae* f. sp. *deltoidae* and *M. medusae* f. sp. *tremuloidae*. Table 2 illustrates the analytical specificity of the different primers.

## Reference cultures

Reference cultures are not available.

Subcloned positive controls for molecular methods A and B may be available upon request. Contact R. Ioos (LNPV, FR) by e-mail (see address below).

## Reporting and documentation

Guidelines on reporting and documentation are given in EPPO standard PM 7/77 *Documentation and reporting of a diagnosis.*

## Further information

Further information on this organism can be obtained from:

Dr R Ioos, Laboratoire National de la Protection des Végétaux (LNPV), Station de Mycologie, Domaine de Pixérécourt, BP 90059, F54220 Malzéville (FR). e-mail: renaud.ioos@agriculture.gouv.fr

Dr P Frey, Institut National de la Recherche Agronomique (INRA), UMR Interactions Arbres - Microorganismes, 54280 Champenoux (FR). e-mail: frey@nancy.inra.fr

## Acknowledgements

This protocol was originally drafted by:

Ioos R, Laboratoire National de la Protection des Végétaux, Malzéville (FR), Frey P and Husson C, Institut National de la Recherche Agronomique, Nancy (FR).

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## Appendix 1 – Method A: Conventional PCR identification using ITS-based primers (after Husson *et al.*, 2003)

*1. General information*

Husson *et al.* (2003) described a technique based on a conventional PCR designed from the rDNA Internal Transcribed Spacers (ITS) to identify *M. medusae* f. sp. *deltoidae* from urediospores scraped from poplar leaves, even in mixture with other species.

The PCR primers target two regions within the ITS and produce a 575 bp amplicon with *M. medusae* f. sp. *deltoidae* DNA (sequences of the ITS region for *M. medusae* f. sp. *deltoidae* may be retrieved from Genbank, accesssions AY375273 to AY375275).

A specific region of the ITS is amplified from *M. medusae* f. sp. *deltoidae* DNA using the primer pair ITS-Mmd-F (forward) and ITS-Mmd-R (reverse) (Table 2). The PCR successfully yielded a 575-bp band with DNA from isolates of *M. medusae* f. sp. *deltoidae* collected from different continents and on different poplar hybrid cultivars (*P.* × *interamericana*, *P.* × *euramericana*) (Husson *et al.*, 2003).

The detection threshold was estimated to be less than 50 *M. medusae* f. sp. *deltoidae* urediniospores within $8 \times 10^5$ *M. larici-populina* uredionospores (Husson *et al.*, 2003).

This method is suitable for the detection of *M. medusae* f. sp. *deltoidae* on poplars from the sections *Aigeiros* and *Tacamahaca*. Conversely, the PCR detection of *M. medusae* f. sp. *tremuloidae* should not use the ITS-Mmd-F/-R primer pair since it cross-reacts with DNA from two other indigenous *Melampsora* species infecting aspens (i.e. *P. tremula* and *P. alba*), namely *M. pinitorqua* and *M. larici-tremulae* (see Table 2). However, the latter species both infect aspens but are not pathogenic on poplars in the sections *Aigeiros* and *Tacamahaca*. In this respect, uredinia of *M. pinitorqua* and *M. larici-tremulae* cannot be found on these cultivated poplars and should not yield false positive results in the conditions herein described.

*2. Methods*

Nucleic acids extraction and purification

The extraction and the purification of the nucleic acids from urediniospores may be carried out using in-house prepared lysis buffers and organic solvent solutions, such as the CTAB/Phenol-chloroform method described by Henrion *et al.* (1994) or commercially available plant DNA extraction kits using for instance guanidium isothianate/chaotropic salts and silica-coated beads or a spin column with a silica membrane.

For both techniques, the urediniospores are collected (1–2 mg) and transferred into a microcentrifuge tube are first disrupted mecanically with 100 µL of lysis buffer, using either ten 3-mm diameter sterile glass beads (5 min with an orbital shaker, 2000 rpm) or two 3-mm diameter sterile steel or tungsten carbide beads (1 min with an oscillating bead-beater, 30 Hz). Then 300 additional microlitres of lysis buffer are added and the DNA is extracted following Henrion *et al.* (1994) or following the manufacturer's instructions in case commercial DNA extraction kits are used. Total DNA is eluted in 100 µL elution buffer or Tris EDTA (10 mM Tris HCl, pH 8, 1 mM ETDA, pH 8) and directly used as a template for PCR.

PCR reaction

A *M. medusae* f. sp. *deltoidae*-specific region is ampified by PCR as follows.

The PCR reaction mixture includes:

- 1 × PCR buffer supplied with the DNA polymerase,
- 1.5 mM $MgCl_2$,
- 0.2 µM of each ITS-Mmd-F and ITS-Mmd-R primers,
- 250 µM each dNTP,
- 0.0375 U/µL of DNA Polymerase,
- 200–300 ng of template DNA,
- Molecular grade water (MGW) is added to reach the final reactional volume (20 µL).

MGW should be purified (deionized or distilled), sterilized by autoclaving or 0.2-µm filtration, and DNase-free.

The PCR reactions should be carried out in a thermocycler equipped with a heated lid and includes an initial denaturation step at 95°C for 3 min (increased to 10 min if using a 'hotstart' DNA polymerase) followed by 30 cycles of denaturation, annealing and elongation for respectively 30 sec at 95°C, 30 sec at 50°C and 1 min at 72°C, and a final extension step at 72°C for 7 min.

The PCR products are resolved by electrophoresis on a 1% agarose gel followed by ethidium bromide staining. A DNA template containing amplifiable *M. medusae* f. sp. *deltoidae*

DNA will yield a 575-bp fragment following a ITS-Mmd-F/ITS-Mmd-R PCR.

*3. Essential procedural information*

A **DNA extraction negative control** (blank tube) should be included for each DNA extraction series in order to ensure the absence of contamination during this step.

A **PCR negative control** containing no target DNA should be included in every test in order to ensure the absence of contamination during PCR.

A **PCR positive control** should be used (subcloned *M. medusae* f. sp. *deltoidae* ITS-Mmd-F/R PCR product). The positive control should correspond to the limit of detection of the test (LOD, Limit of Detection). This **LOD positive control**[2] should be included in order to assess the performance of the PCR run and to ensure that the negative results are caused by an absence or a too low level of the PCR target in the DNA sample, rather than by an insufficient PCR efficiency.

The quality of the DNA extract should be assessed by a relevant means e.g. by spectrophotometry, or by testing the extract in PCR with the ribosomal genes primers ITS1 and ITS4 (White *et al.*, 1990). In the latter case, the PCR conditions are those described above, simply replacing the ITS-Mmd-F/-R primers with ITS1 and ITS4 primers (Table 2), and decreasing the annealing temperature to 50°C. A positive signal (approximately 660–680 bp) following this test would mean that the DNA extract was amplifiable: DNA was successfully extracted and the level of co-extracted inhibiting compounds was sufficiently low. Additional evidence of the absence of significant PCR inhibition may be provided by the use of an *ad hoc* **internal amplification control**.

Interpretation of results:

- A sample will be considered positive if it produces amplicons of 575 bp and provided that the contamination controls are negative.
- A sample will be considered negative, if it produces no band of 575 bp and provided that (i) the sample DNA extract proved to be amplifiable, (ii) that no significant inhibition occurred and if used (iii) that the LOD positive control tested in the PCR run yielded a 575 bp amplicon.
- Tests should be repeated if any contradictory or unclear results are obtained.

## Appendix 2 – Method B: conventional PCR identification using SCAR-based primers (after Bourassa *et al.*, 2005)

*1. General information*

Bourassa *et al.* (2005) described two techniques based on conventional PCR designed from two loci of unknown locations (derived from SCARs) which may be both used to identify *M. medusae* f. sp. *deltoidae* from urediospores scrapped from poplar leaves.

The PCR primers's targets are located in regions named 'Locus A' and 'Locus B', respectively. The two PCR tests produce a 512-bp and a 594-bp amplicon, respectively, with *M. medusae* f. sp. *deltoidae* DNA. Sequences of the loci targeted for *M. medusae* f. sp. *deltoidae* may be retrieved from Genbank, accessions AY489127 to AY489134 (locus A) and AY490805 to AY490813 (locus B), respectively.

Locus A is amplified from *M. medusae* f. sp. *deltoidae* DNA using the primer pair c1c3a2f (forward) and c1c3a2r (reverse), whereas Locus B region is amplified from *M. medusae* f. sp. *deltoidae* DNA using the primer pair c1c3a3f (forward) and c1c3a3r (reverse) (Table 2).

This method is suitable for the detection of *M. medusae* f. sp. *deltoidae* on poplars from the sections *Aigeiros* and *Tacamahaca* but with a risk of false-negative results due to null allele with some isolates. Indeed, when used in PCR against 80 isolates from other rust species it did not yield any signal or a fragment of a distinctive different size. In particular, no signal was obtained with DNA from *M. larici-populina*, *M. allii-populina*, *M. larici-tremulae*, *M. rostrupii*, and *M. pinitorqua* (Bourassa *et al.*, 2005). However, these primer pairs were not originally optimized for detection purposes and their respective sensivity has not been assessed. In addition, screening a world collection of 40 *M. medusae* f. sp. *deltoidae* isolates with these primer pairs showed that null alleles occur with locus A with a single isolate, and therefore lead to false negative results (P Frey, INRA Nancy, unpublished data).

The method is also suitable for the detection of *M. medusae* f. sp. *tremuloidae* on aspens but with a risk of false-negative results due to null alleles or low PCR efficiency with some isolates. (See Table 2).

*2. Methods*

Nucleic acid extraction and purification.

The urediniospores collected and transferred into a microcentrifuge tube are first lyophilized. A volume of diatomaceous earth (Sigma Chemical company, St Louis, USA) equivalent of that of spores is added to the tube. Spores are crushed in 400 µL of lysis buffer (100 mM Tris HCl, pH 9.5, 2% CTAB, 1.4 mM NaCl, 1% polyethyleneglycol 8000, 20 mM EDTA, pH 8, 1% ß-mercaptoethanol).

The suspension is mixed, incubated at 65°C for 1 h, extracted once with 400 µL of phenol:chloroform:iso-amyl alcohol (25:24:1) and centrifuged at 10 000 *g* for 5 min.

The aqueous phase is transferred into a new microtube, precipitated with one volume of cold isopropanol and centrifuged at 10 000 *g* for 5 min.

Resulting pellets are washed with 70% ethanol, air dried overnight and resuspended in 20 µL of Tris EDTA buffer (10 mM Tris HCl, pH 8, 1 mM ETDA, pH 8) to be used for PCR as a DNA template.

An alternative to this procedure described in Bourassa *et al.* (2005) for DNA extraction and purification would be to follow the procedures described in Appendix for method A.

PCR reaction

[2]LOD positive control is made of diluted subcloned *M. medusae* f. sp. *deltoidae* ITS-Mmd-F/R PCR product. It can be defined as the lowest target amount giving positive result in at least 95% of the times, thus ensuring a ≤5% false negative rate.

Two disctinct *M. medusae* f. sp. *deltoidae*-specific regions named 'locus A' and 'locus B' are amplified by separate PCR reactions, each carried out as follows.

The PCR reaction mixture includes:

- 10 mM Tris HCl (pH 8.3), 50 mM KCl, or the buffer supplied with the DNA polymerase
- 1.5 mM $MgCl_2$,
- 1 µM of each primer (c1c3a2f and c1c3a2r for Locus A or c1c3a3f and c1c3a3r for Locus B),
- 200 µM dNTPs,
- 0.05 U/µL of DNA Polymerase,
- 1 µL of template DNA (concentration not indicated),
- Molecular biology grade molecular grade water (MGW) is added to reach the final reaction volume (20 µL).

MGW should be purified (deionized or distilled), sterilized by autoclaving or 0.2-µm filtration, and DNase-free.

The PCR reaction for both Locus A and Locus B should be carried out in a thermocycler equipped with a heated lid and includes an initial denaturation step at 95°C for 3 min (increased to 10 min if using a 'hotstart' DNA polymerase) followed by 30 cycles of denaturation, annealing and elongation for respectively 30 sec at 92°C, 30 sec at 55°C and 1 min at 72°C, and a final extension step at 72°C for 10 min.

The PCR products are resolved by electrophoresis on a 1% agarose gel followed by ethidium bromide staining. A DNA template containing amplifiable *M. medusae* f. sp. *deltoidae* DNA will yield a 502-bp fragment (locus A) following a c1c3a2f/r PCR and a 594-bp fragment (locus B) following a c1c3a3f/r PCR.

*3. Essential procedural information*

A **DNA extraction negative control** (blank tube) should be included for each DNA extraction series in order to ensure the absence of contamination during this step.

A **PCR negative control** containing no target DNA should be included in every test in order to ensure the absence of contamination during PCR.

A **PCR positive control** should be used (subcloned *M. medusae* f. sp. *deltoidae* c1c3a2f/r or c1c3a3f/r PCR product). The positive control should correspond to the limit of detection of the test (LOD). **This LOD positive control** [3] should be included in order to assess the performance of the PCR run and to ensure that the negative results are caused by an absence or a too low level of the PCR target in the DNA sample, rather than by an insufficient PCR efficiency.

The quality of the DNA extract should be assessed by a relevant mean e.g. by spectrophotometry, or by testing the extract in PCR with the ribosomal genes primers ITS1 and ITS4 (White *et al.*, 1990). In the latter case, the PCR conditions are those described above, simply replacing the c1c3a2f/r or c1c3a3f/r PCR product primers with ITS1 and ITS4 primers (Table 2), and decreasing the annealing temperature to 50°C. A positive signal (approximately 660–680 bp) following this test would mean that the DNA extract was amplifiable: DNA was successfully extracted and the level of co-extracted inhibiting compounds was sufficiently low. Additional evidence of the absence of significant PCR inhibition may be provided by the use of an *ad hoc* **internal amplification control**.

Interpretation of results:

- A sample will be considered positive if it produces amplicons of 502-bp (locus A) or a 594-bp (locus B) and provided that the contamination controls are negative.
- A sample will be considered negative, if (i) it produces no band of 502-bp (locus A) or a 594-bp (locus B), (ii) provided that the sample DNA extract proved to be amplifiable and that no significant inhibition occurred, and (iii) if used, that the corresponding LOD positive control tested in the PCR run yielded a 502-bp (locus A) or a 594-bp (locus B) amplicon.
- Tests should be repeated if any contradictory or unclear results are obtained.

[3]LOD positive control is made of diluted subcloned *M. medusae* f. sp. *deltoidae* c1c3a2f/r or c1c3a3f/r PCR product. It can be defined as the lowest target amount giving positive result in at least 95% of the times, thus ensuring a ≤5% false negative rate.