DOI: 10.1111/epp.12959

EPPO STANDARD ON DIAGNOSTICS

PM 7/93 (2) *Melampsora medusae*

Specific scope: This Standard describes a diagnostic protocol for *Melampsora medusae* on poplar.[1]

This Standard should be used in conjunction with PM 7/76 Use of EPPO diagnostic protocols.

Specific approval and amendment: First approved in 2009–09. Revision approved in 2023–09.

Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Melampsora medusae is a heteroecious rust fungus with *Populus* spp. as telial hosts (primary hosts on which uredinia and telia are produced) and various conifers as aecial hosts (on which spermagonia and aecia are produced). As is the case for other *Melampsora* species infecting poplars, *M. medusae* causes 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, for younger trees, even lead to death.

Shain (1988) showed evidence of the existence of two *formae speciales* within *M. medusae* and named them *M. medusae* f. sp. *deltoidae* (syn *deltoidis*) and *M. medusae* f. sp. *tremuloidae* (syn '*tremuloidis*'). Unlike *M. medusae* f. sp. *deltoidae* which is pathogenic on the poplars of the sections *Aigeiros* and *Tacamahaca*, i.e. *Populus nigra*, *P. deltoides*, *P. trichocarpa*, *P. balsamifera*, *P. maximowiczii* and their interspecific hybrids (*P.* × *canadensis*, *P.* × *generosa* and *P.* × *jackii*) (Frey et al., 2005; Vialle et al., 2011), the only telial host of *M. medusae* f. sp. *tremuloidae* with reported infections in nature in North America is *P. tremuloides*, belonging to the section *Populus* (EPPO, 2023a; Vialle et al., 2011).

Melampsora medusae originates from North America. 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 concluded that only *M. medusae* f. sp. *deltoidae* has been reported in Europe so far (EPPO, 2023a; Pinon, 1991; Pinon & Frey, 2005).

In addition to *M. medusae* f. sp. *deltoidae*, eight *Melampsora* species have been reported on poplars in Europe to date (Cellerino, 1999; Pinon, 1973; Vialle et al., 2011, 2013). Two of them, namely *M. laricis-populina* and *M. allii-populina* are pathogenic on the poplars of the sections *Aigeiros* and *Tacamahaca*. The six other species, namely *M. aecidioides*, *M. laricis-tremulae*, *M. magnusiana*, *M. pinitorqua*, *M. pulcherrima* and *M. rostrupii*, are only pathogenic on species of the section *Populus*, i.e. *P. alba*, *P. tremula* and their interspecific hybrids (Frey et al., 2005; Vialle et al., 2011). The eight other *Melampsora* species have been described on poplar in Europe are also present in other parts of the world (Vialle et al., 2011).

For more information on the biology of the pest see the EPPO Datasheet (EPPO, 2023b) and in the Scientific Opinion on the pest categorization of *Melampsora medusae* (EFSA, 2018).

A Flow diagram describing the diagnostic procedure for *Melampsora medusae* (including the specific diagnostic of *M. medusae* f. sp. *tremuloidae*) is presented in Figure 1.

2 | IDENTITY

Name: *Melampsora medusae* Thümen.

Other Scientific Names: *Melampsora albertensis* J.C. Arthur, *Uredo medusae* Thümen (uredinial stage). *Caeoma faulliana* Hunter (aecial stage) is considered as a synonym in Global Database.

Taxonomic position: Fungi: Basidiomycota: Uredinales.

Notes on taxonomy and nomenclature:
Note on the name used in this protocol: in Mycobank the name Melampsora medusae f. sp. tremuloidis *and* Melampsora medusae f. sp. deltoidis *are used, however, in this protocol the name* M. medusae f. sp. tremuloidae *and* M. medusae f. sp. deltoidae *are used as they are the original names given by Shain (1988) and are the names most often used in publications.*

EPPO Code: MELMME.

Phytosanitary categorization: EPPO A2 list no. 74, EU annex designation IIA for *Melampsora medusae* f. sp. *tremuloidis* only.

[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.

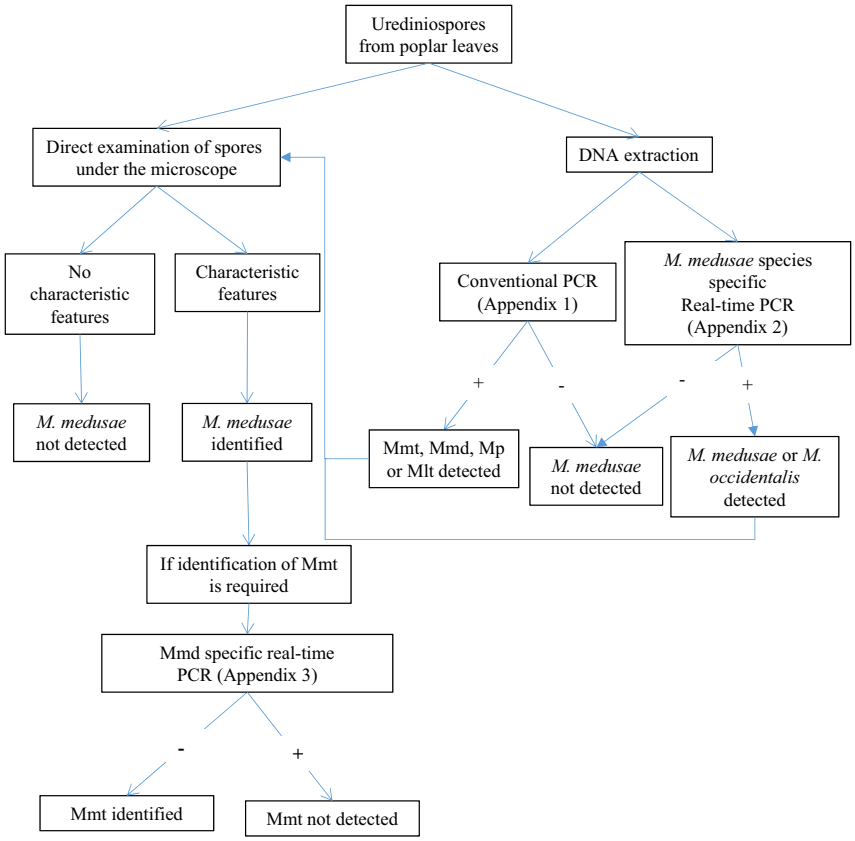

FIGURE 1 Flow diagram describing the diagnostic procedure for *Melampsora medusae* and of f. sp. *tremuloidae* when required. This flow diagram is intended to provide an overview of the diagnostic process and may not cover all possible scenarios (*Mlt*, *Melampsora laricis-tremulae*; *Mmd*, *Melampsora medusae* f. sp. *deltoidae*; *Mmt*, *Melampsora medusae* f. sp. *tremuloidae*; *Mo*, *Melampsora occidentalis*; *Mp*, *Melampsora pinitorqua*).

3 | DETECTION

3.1 | Disease symptoms

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

3.1.1 | 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 on the abaxial leaf surface (Figure 2a,b). The uredinia may be scattered on the leaves or, in severe attacks, be so crowded that the entire surface looks powdery and is yellowish orange (Figures 3 and 4). This can lead to partial or total defoliation. 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 late summer by mid-autumn, telia appear on either side of the leaves, resembling small brown to black raised spots and giving a crusty appearance (Figure 5). These telia contain large numbers of thick-walled resting spores (teliospores) that will overwinter on fallen leaves, and then germinate in the following spring to release basidiospores. The position of the telia is variable according to the *Melampsora* species: *M. medusae* and *M. allii-populina* form mainly hypophyllous telia, whereas *M. laricis-populina* forms mainly epiphyllous telia.

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

In EPPO countries, *M. medusae* is only reported on poplars and so far, has never been observed on its potential aecial hosts. 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 (Figure 6). These structures usually develop on the

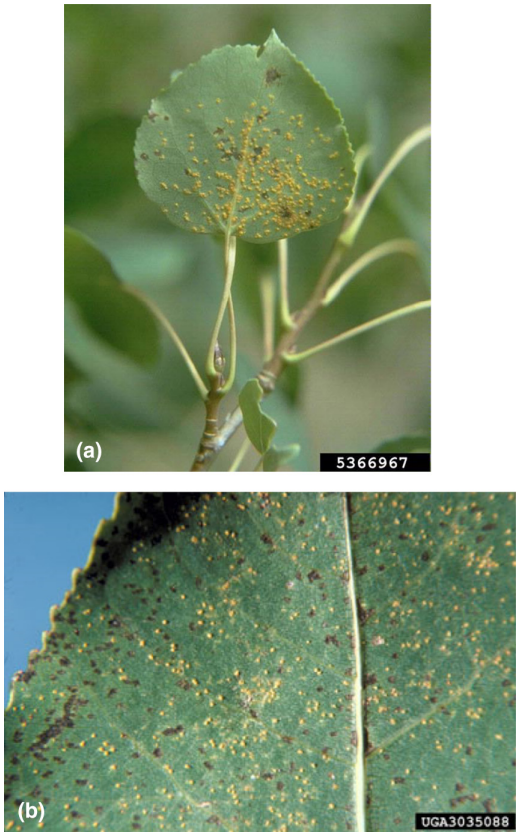

FIGURE 2 (a) *Populus tremuloides* leaf showing signs of *Melampsora* sp. rust. Courtesy Schomaker M, Colorado State Forest Service, Bugwood.org. (b) Uredinia of *Melampsora medusae* (yellow pustules) on the underside of a poplar leaf. Courtesy Robert L. Anderson, USDA Forest Service, Bugwood.org.

FIGURE 3 Typical *Melampsora* rust symptoms (uredinia) on a poplar leaf. Courtesy of Frey P, INRAE Nancy (FR).

current year's needles, but may be observed, although they are rare, on the conifer cones or on young shoots. Aecia produce large numbers of yellow aeciospores, arranged in chains. These wind-borne aeciospores can then infect a susceptible *Populus* host in the spring and the fungus will then rapidly produce urediniospores to complete its life cycle.

FIGURE 4 Young poplar heavily infected by *Melampsora* rust. Courtesy of Frey P, INRAE Nancy (FR).

FIGURE 5 *Melampsora medusae* telia (black) and uredinia (yellow) on the underside of a *Populus* spp. leaf. Courtesy: Whitney Cranshaw, Colorado State University, Bugwood.org.

FIGURE 6 Aecia of *Melampsora medusae* on *Larix* needles. Courtesy Steven Katovich, USDA Forest Service, Bugwood.org.

3.2 | Test sample requirements

The sample should consist of symptomatic poplar leaves bearing uredinia (dimensions, shape, and other morphological features of teliospores of *M. medusae* are not discriminating features in comparison with other indigenous species of poplar *Melampsora*). Samples should be sent to the laboratory within 2 days of collection. The best period for sampling is in late summer when symptoms are most obvious and uredinia are abundant.

It should be noted that since the aecial stage of *M. medusae* has never been reported on the conifer (aecial) hosts in the EPPO region, the sampling procedure is restricted to poplar trees, where the likelihood of detecting *M. medusae* will be the highest.

4 | IDENTIFICATION

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

- Morphological identification (Section 4.1): 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. In case of positive detection and when identification to *formae speciales* level is required, a molecular test (real-time PCR, Appendix 2) should be performed as it is not possible to discriminate *M. medusae* f. sp. *deltoidae* from *M. medusae* f. sp. *tremuloidae* on the basis of morphological criteria (see Figure 1).
- Molecular methods (Section 4.2 and Figure 1).

4.1 | Morphological identification

Morphological characters allow the distinction of *M. medusae* from other *Melampsora* species. However, the two *formae speciales* of *M. medusae* cannot be distinguished morphologically since they share similar grossly identical morphological features and can only be distinguished according to their respective host range (Shain, 1988).

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 compound microscope at magnifications between ×400 and ×1000. At least 10 urediniospores and paraphyses per uredinium are observed and measured in both dimensions. Their shape

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*	
	M. medusae f. sp. *deltoidae*	*M. laricis-populina*	*M. allii-populina*	*M. medusae f.* sp. *tremuloidae*	Other
Urediniospores					
Shape	Mainly ellipsoid to ovoid	Mainly pyriform	Mainly ellipsoid to ovoid	Mainly ellipsoid to ovoid	–
Apex echinulation	Echinulate	Smooth patch	Smooth patch	Echinulate	Echinulate
Equator echinulation	Smooth patch, or completely smooth	Echinulate	Echinulate	Smooth patch, or completely smooth	Echinulate
Wall	Thickened at the equator	Thickened at the equator	Uniformly thick	Thickened at the equator	–
Paraphyses					
Shape	Capitate to clavate	Clavate	Capitate	Capitate to clavate	–
Wall	Uniformly thick	Thickened at the apex	Uniformly thick	Uniformly thick	–

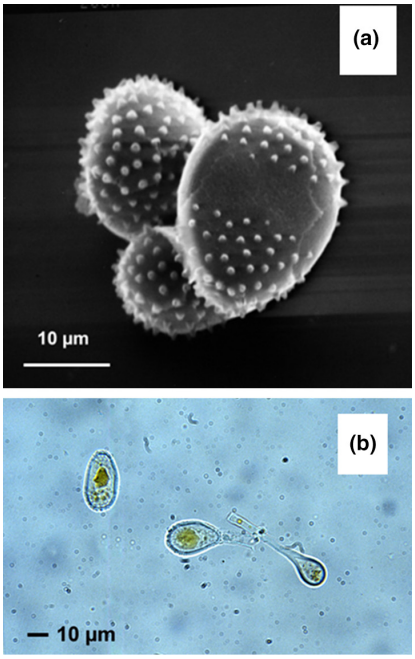

FIGURE 7 (a) *M. medusae* f. sp. *deltoidae* urediniospores observed by scanning electron microscopy (Courtesy of Le Thiec D, INRAE Nancy, FR) and (b) *M. medusae* f. sp. *deltoidae* paraphyse and urediniospores observed by light microscopy. Courtesy of Frey P, INRAE Nancy (FR).

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.

M. medusae urediniospores are obovate (Figure 7a,b) to oval or occasionally 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 thickened at equator 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 (Figure 8).

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, Figures 7–10). *M. medusae* has an equatorial smooth patch; *M. laricis-populina* and *M. allii-populina* both have smooth apical patches; all the other *Melampsora* species, only encountered on aspens, have uniformly echinulate urediniospores (*M. pulcherrima*, *M. pinitorqua*, *M. laricis-tremulae*, *M. rostrupii*, *M. magnusiana* and *M. aecidioides*). Therefore, based on morphological characters in Table 1, *M. medusae* can

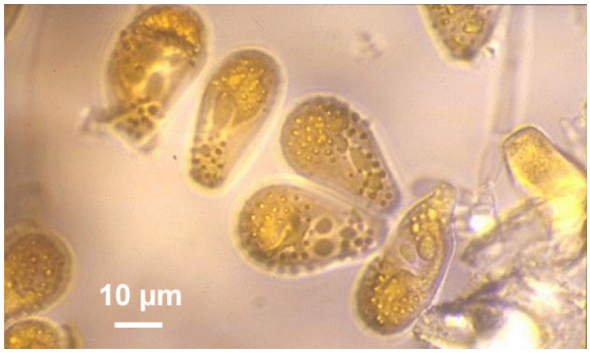

FIGURE 8 Urediniospores of *M. medusae* f. sp. *deltoidae* with the typical smooth equatorial patch. Courtesy of Hubert J, Anses LSV Malzéville (FR).

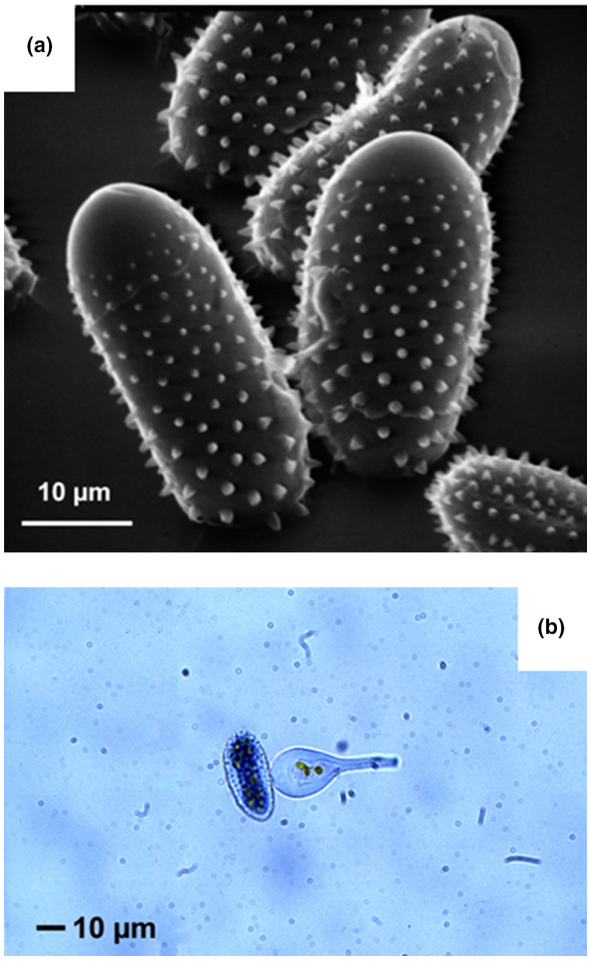

FIGURE 9 (a) *M. laricis-populina* urediniospores observed by scanning electron microscopy (Courtesy of Le Thiec D, INRAE Nancy, FR) and (b) *M. laricis-populina* paraphyse and urediniospore observed by light microscopy. Courtesy of Frey P, INRAE Nancy (FR).

be distinguished from the other *Melampsora* species 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

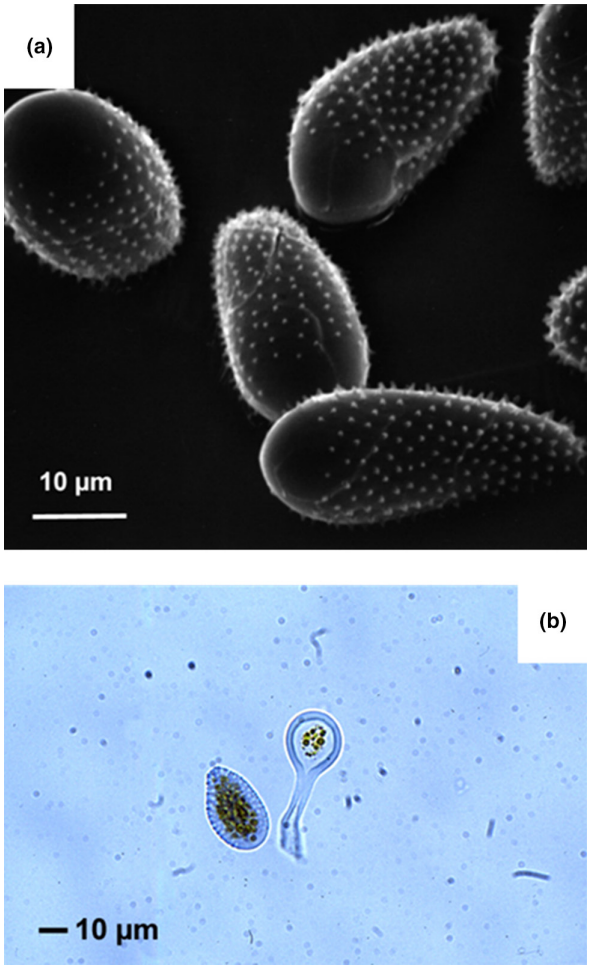

FIGURE 10 (a) *M. allii-populina* urediniospores observed by scanning electron microscopy (Courtesy of Le Thiec D, INRAE Nancy, FR) and (b) *M. allii-populina* paraphyse and urediniospore observed by light microscopy. Courtesy of Frey P, INRAE Nancy (FR).

enough urediniospores of *M. medusae* among a large number of other *Melampsora* spp. urediniospores, in which case the use of molecular methods may be considered.

4.2 | Molecular methods

4.2.1 | Husson et al. (2013) (Appendix 1) Mm species-specific conventional PCR

Husson et al. (2013) described a series of PCR primer pairs within the rDNA regions of *Melampsora* spp. that specifically target the ITS region of *M. allii-populina* (ITS-Map-F/R), *M. laricis-populina* (ITS-Mlp-F/R) and *M. medusae* f. sp. *deltoidae* (ITS-MMD-F/ITS-MMD-R). Analysis of *M. medusae* f. sp. *tremuloidae* ITS sequences shows that DNA from this *formae speciales* would also be successfully amplified by PCR with ITS-MMD-F/ITS-MMD-R primers since the target sequences are identical for both *formae speciales* (R. Ioos, ANSES, personal observation). However, the detection of *M. medusae* f. sp. *deltoidae* should not be based on this test alone since ITS-MMD-F/ITS-MMD-R primers cross-react with DNA from two other *Melampsora* species present in Europe, namely *M. pinitorqua* and *M. laricis-tremulae*, infecting poplars of the section *Populus* (Table 2). In case of a positive result, further tests should be performed (see Figure 1).

4.2.2 | Boutigny et al. (2013b) (Appendix 2) Mm species-specific real-time PCR

Boutigny et al. (2013b) selected primers (Mm-F and Mm-R) and dual-labelled fluorescent probe (Mm-P) in the ITS region of the rDNA for the detection of both *formae speciales* of *Melampsora medusae* by real-time PCR. This test does not cross-react with other rust pathogens found on poplars in Europe (Table 2). However, cross-reactions have been observed with *Melampsora occidentalis*, a species never reported in Europe.

4.2.3 | Boutigny et al. (2013a) (Appendix 3) Mmd specific real-time PCR

Boutigny et al. (2013a) selected primers (Mmd-F and Mmd-R) and dual-labelled fluorescent probe (Mmd-P) in the 28S region of the rDNA for the specific detection of *Melampsora medusae* f. sp. *deltoidae* by real-time PCR. This test does not cross-react with other rust pathogens found on poplars, including *M. medusae* f. sp. *tremuloidae* (Table 2).

4.2.4 | Advantages and limitations of the molecular tests

The detection of *M. medusae* by molecular test enables the simultaneous handling of many samples each containing thousands of urediniospores (1 mg = approximately 4×10^5 spores), which cannot be achieved with morphological identification.

The molecular tests developed by Husson et al. (2013) and Boutigny et al. (2013b, Appendix 3) can be used for screening for negative samples. However, when positive results are obtained, further tests should be carried out due to cross-reactions of the two molecular tests with *M. medusae* f. sp. *deltoidae* or other *Melampsora* species (see flow diagram).

The test developed by Boutigny et al. (2013a) (Appendix 2) is the only molecular test that can

TABLE 2 Specificity of the PCR primer combinations.

Primer name	Test	Mmd[a]	Mmt[b]	Mlp[a]	Map[a]	Mlt[b]	Mp[b]	Mr[b]	Mae[b]	Mo[c]	Appendix
ITS-MMD-F, ITS-MMD-R	Conventional PCR (Husson et al., 2013)	+	+[d]	–	–	+	+	–	–	na	1
ITS1, ITS4	Conventional PCR (White et al., 1990)	+	+	+	+	+	+	+	+	+	1–3
Mmd-F, Mmd-R, Mmd-P	Real-time PCR (Boutigny et al., 2013a)	+	–	–	–	–	–	–	–	–	2
Mm-F, Mm-R, Mm-P	Real-time PCR (Boutigny et al., 2013b)	+	+	–	–	–	–	–	–	+	3
Mel-F, Mel-R, Mel-P	Real-time PCR (Boutigny et al., 2013a)	+	+	+	+	+	+	+	+	+	2 and 3

Abbreviations: Mae, *M. aecidioides*; Map, *M. allii-populina*; Mlp, *M. larici-populina*; Mlt, *M. larici-tremulae*; Mmd, *M. medusae* f. sp. *deltoidae*; Mmt, *M. medusae* f. sp. *tremuloidae*; Mo, *M. occidentalis*; Mp, *M. pinitorqua*; Mr, *M. rostrupii*; na, not available.

[a]Species occurring only on poplars of the section *Aigeiros* and *Tacamahaca*, or their hybrids.

[b]Species occurring only on poplars of the section *Populus*.

[c]Species occurring mainly on *Populus trichocarpa*.

[d]Not validated in the publication of Husson et al. (2013) but same sequence for Mmt and Mmd in the region selected by the authors for the design of PCR primers.

discriminate between both *formae speciales* of *M. medusae*. When discrimination is required, this test should be used for positive samples after morphological identification or the two other molecular tests (see flow diagram).

Primers developed for the tests detecting Mmd (Boutigny et al., 2013a; Husson et al., 2013) cross-react with *M.* × *columbiana* and *M. medusae-populina*, which are interspecific hybrids containing the genome of *M. medusae*.

The previous version of this protocol included a PCR RFLP test (Bourassa et al., 2005). As new tests are available and better adapted to use in routine diagnostics, the Panel on Diagnostics in Mycology considered that it should not be maintained in this revision.

5 | REFERENCE MATERIAL

Subcloned positive controls for Husson et al. (2013), and Boutigny et al. (2013a) may be available upon request. Contact R. Ioos (ANSES, FR) by e-mail (see address below).

6 | REPORTING ANDDOCUMENTATION

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

7 | PERFORMANCE CRITERIA

When performance criteria are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

Dr R Ioos, ANSES, Laboratoire de la santé des végétaux, unité de mycologie. Domaine de Pixérécourt, Bâtiment E, CS40009, F54220 Malzéville (FR), e-mail: renaud.ioos@anses.fr.

Dr P Frey, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE) UMR Interactions Arbres - Microorganismes, 54280 Champenoux (FR). e-mail: pascal.frey@inrae.fr.

9 | FEEDBACK ON THIS DIAGNOSTIC PROTOCOL

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int.

10 | PROTOCOL REVISION

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website. When errata and corrigenda are in press, this will also be marked on the website.

ACKNOWLEDGEMENTS

This protocol was originally drafted by R. Ioos (ANSES, FR), P. Frey (INRAE, FR) and C. Husson (MASA/DSF, FR). The revision was prepared by A. Chandelier (CRAW, BE), R. Ioos (ANSES, FR) and P. Frey (INRAE, FR).

REFERENCES

Bourassa M, Bernier L & Hamelin RC (2005) Direct genotyping of the poplar leaf rust fungus, *Melampsora medusae* f. sp. *deltoidae*, using codominant PCR-SSCP markers. *Forest Pathology* 35, 245–261.

Boutigny AL, Guinet C, Vialle A, Hamelin RC, Andrieux A, Frey P, Husson C, Ioos R (2013a). Optimization of a real-time PCR assay for the detection of the quarantine pathogen *Melampsora medusae* f. sp. *deltoidae*. *Fungal Biology* 117, 389-398.

Boutigny AL, Guinet C, Vialle A, Hamelin R, Frey P, Ioos R (2013b). A sensitive real-time PCR assay for the detection of the two *Melampsora medusae formae speciales* on infected poplar leaves. *European Journal of Plant Pathology* 136, 433-441.

Cellerino GP (1999) *Review of fungal diseases in poplar*. International Poplar Commission, FAO. Available at website https://www.fao.org/3/ac492e/AC492E.pdf (accessed 2023/06/07)

CMI (1975) *Melampsora medusae. IMI descriptions of pathogenic fungi and bacteria N° 480*. Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, England.

EFSA Panel on Plant Health (PLH), Jeger M, Bragard C, Caffier D, Candresse T, Chatzivassiliou E, Dehnen-Schmutz K, Gilioli G, Grégoire J-C, Jaques Miret JA, MacLeod A, Navajas Navarro M, Niere B, Parnell S, Potting R, Rafoss T, Rossi V, Urek G, Van Bruggen A, Van der Werf W, West J, Winter S, Boberg J, Gonthier P & Pautasso M, (2018). Scientific Opinion on the pest categorisation of *Melampsora medusae*. *EFSA Journal*;16(7):5354, 28 pp. https://doi.org/10.2903/j.efsa.2018.5354

EPPO (2023a) *Melampsora medusae*. EPPO datasheets on pests recommended for regulation. https://gd.eppo.int (accessed 2023-02-26).

EPPO (2023b) EPPO Global Database (available online). https://gd.eppo.int (accessed 2023-09-01).

Frey P, Gérard P, Feau N, Husson C and Pinon J (2005) Variability and population biology of *Melampsora* rusts on poplars. In: Pei MH and Mc Cracken AR Eds. *Rust Diseases of willow and poplar*, CAB International, Wallingford, GB, pp. 63-72.

Henrion B, Chevalier G and Martin F (1994) Typing truffle species by PCR amplification of the ribosomal DNA spacers. *Mycological Research* 98, 37-43.

Husson C, Frey P, Boussouel N, Loevenbruck P, Pinon J (2003) Deux méthodes de diagnostic moléculaire des espèces de *Melampsora*, agent de la rouille foliaire des peupliers. *Journées du réseau de Mycologie de la Société Française de Microbiologie*, Nancy, 15-17 jan. 2003.

Husson C, Ioos R, Andrieux A, Frey P (2013) Development and use of new sensitive molecular tools for diagnosis and detection of *Melampsora* rusts on cultivated poplar. *Forest Pathology* 43, 1-11.

Pinon J (1973) Les rouilles du peuplier en France. Systématique et répartition du stade urédien. *European Journal of Forest Pathology* 3, 221-228.

Pinon J (1991) Eléments de répartition des rouilles des peupliers cultivés en France. *Comptes-Rendus de l'Académie d'Agriculture de France* 77, 109-115.

Pinon J & Frey P. (2005) Interactions between poplar clones and *Melampsora* populations and their implications for breeding for durable resistance. In: Pei MH, McCracken AR (Eds.), *Rust diseases of Willow and Poplar*. CAB International, Wallingford, UK, pp. 139-154.

Shain L (1988) Evidence for *formae speciales* in the poplar leaf rust fungus *Melampsora medusae*. *Mycologia* 80, 729-732.

Vialle A, Frey P, Hambleton S, Bernier L and Hamelin R (2011). Poplar rust systematics and refinement of Melampsora species delineation. *Fungal Diversity* 50, 227-248.

Vialle A, Feau N, Frey P, Bernier L, Hamelin RC (2013). Phylogenetic species recognition reveals host-specific lineages among poplar rust fungi. *Molecular Phylogenetics and Evolution* 66, 628-644.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ and White TJ eds. *PCR protocols: a guide to method and applications*. Academic Press, New York. 315-322.

How to cite this article: EPPO (2023) PM 7/93 (2) *Melampsora medusae*. *EPPO Bulletin*, 53, 580–593. Available from: https://doi.org/10.1111/epp.12959

APPENDIX 1 - CONVENTIONAL PCR IDENTIFICATION USING ITS-BASED PRIMERS (HUSSON ET AL., 2013)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1 This conventional PCR is used for the identification of *Melampsora medusae* f. sp. *deltoidae* and *M. medusae* f. sp. *tremuloidae*. The use of this test should be limited to urediniospores collected from poplar leaves (Table 2, flow diagram).

1.2 The protocol was established in 2003 (Husson et al., 2003) and later modified in 2013 (Husson et al., 2013).

1.3 The PCR primers are selected in the ITS region of the rDNA gene (sequences of the ITS region for *M. medusae* f. sp. *deltoidae* may be retrieved from Genbank accesssions AY375273 to AY375275). The sequence of primers is conserved between both *formae speciales*.

1.4 Oligonucleotides:

Primers	Sequence	Amplicon size
Forward primer (ITS-MMD-F)	5′-GAG TTG CTT AAA TGC GAT TC-3′	575 bp
Reverse primer (ITS-MMD-R)	5′-CTA AAG GTA AAT TCA ATG GG-3′	

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 The uredinia are scraped from leaves using a sterile scalpel blade or a spatula (ideally, approx. 2 mg) and transferred into a 2 mL microcentrifuge tube.

2.1.2 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 guanidium isothianate/chaotropic salts and silica-coated beads or a spin column with a silica membrane. For both techniques, urediniospores are first disrupted mechanically with 100 µL of lysis buffer, using either ten 2 mm diameter sterile glass beads (5 min with an orbital shaker, 5000 rpm) or two 3 mm diameter sterile steel or tungsten carbide beads (1 min with an oscillating bead-beater, 30 Hz). 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 50 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.

2.1.3 Extracted DNA should be stored at 2–8°C for immediate use or at approximately −20°C if testing is not to be performed on the same day.

2.2 Polymerase chain reaction (PCR)

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	12.7	N.A.
PCR buffer (Sigma Aldrich, France)	10×	2	1×
$MgCl_2$ (Sigma Aldrich, France)	25 mM	1.2	1.5 mM
dNTPs	10 mM	0.4	200 µM
Forward primer (ITS-MMD-F)	5 µM	0.8	0.2 µM
Reverse primer (ITS-MMD-R)	5 µM	0.8	0.2 µM
Taq DNA polymerase (Sigma Aldrich, France)	5 U/µL	0.1	0.5 U
Subtotal		18	
DNA from urediniospores		2	
Total		20	

2.2.2 PCR conditions

Amplifications are carried out in PCR tubes in a thermocycler with heated lid programmed as follows: denaturation step at 94°C for 3 min followed by 30 cycles of denaturation, annealing and elongation for respectively 30 s at 94°C, 30 s at 55°C and 90 s at 72°C, and a final extension step at 72°C for 10 min.

3. Essential procedural information

3.1 Controls

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction (molecular grade water following all the steps of the DNA extraction/purification).
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: uredinia collected maintained at approximately −20°C (if available), if such material is not available (as *Melampsora medusae* cannot be cultured).

- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, or a synthetic control (e.g. cloned PCR product).

Other possible controls:
Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same spore suspension spiked with nucleic acid from the target organism.

The quality of the DNA extract should be tested by a relevant means e.g. by spectrophotometry, or by testing the extract in PCR with the 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/ITS-MMD-R primers with ITS1/ITS4 primers 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.

3.2 Interpretation of results

Verification of the controls

- NIC and NAC should produce no amplicon.
- PIC, PAC and IC (if relevant) should produce amplicons of 575 bp.

When these conditions are met:

- A test will be considered as positive if an amplicon of 575 bp is produced.
- A test will be considered negative if it produces no band, or if it produces a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

The validation data presented below are for *Melampsora medusae* f. sp. *deltoidae but also valid for Melampsora medusae* f. sp. *tremuloidae since both species share identical target sequences.*

4.1 Analytical sensitivity data
The limit of detection was estimated to be 0.1 pg μL^{-1} of DNA of the pathogen. In terms of spores, the test was capable to detect approx. seven urediniospores of *M. medusae* f. sp. *deltoidae* in approx. 800 000 urediniospores, of *Melampsora laricis-populina*.

4.2 Analytical specificity data
The inclusivity was evaluated from 30 isolates of *M. medusae* f. sp. *deltoidae*. All *Melampsora medusae* f. sp. *deltoidae* were amplified.

The exclusivity was evaluated from 79 isolates belonging to 8 *Melampsora* species: *M. allii-populina* (28), *M. laricis-populina* (30), *M. laricis-tremulae* (4), *M. pinitorqua* (4), *M. aecidioides* (3), *M. magnusiana* (3), *M. pulcherrima* (3) and *M. rostrupii* (*4*). Cross-reactions were observed with *M. laricis-tremulae* and *M. pinitorqua* isolates (Table 2). No amplicon was obtained from poplar or larch DNA extracts.

4.3 Data on repeatability
No data available.

4.4 Data on reproducibility
No data available.

APPENDIX 2 - REAL-TIME PCR (BOUTIGNY ET AL., 2013B)

The test below differs from the one described in the original publication.

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1 This real-time PCR method is used for the identification of both *formae speciales* of *Melampsora medusae* (*M. medusae* f. sp. *deltoidae* & *M. medusae* f. sp. *tremuloidae*) in urediniospores from poplar leaves.
1.2 The protocol was published in 2013.
1.3 The PCR primers are selected in the ITS region of the rDNA gene (sequences of the ITS region for *M. medusae* f. sp. *deltoidae* may be retrieved from Genbank, accessions AY375273 to AY375275, sequences of the ITS region for *M. medusae* f. sp. *tremuloidae* may be retrieved from Genbank, accession numbers JN881746.1 and GQ479884.1).
1.4 Oligonucleotides:

Primers	Sequence	Amplicon size
Forward primer (Mm-F)	5′ GCT TAA ATG CGA TTC TTT GTA TAC TAT 3′	77 bp
Reverse primer (Mm-R)	5′ CTG CTA ACC TAA TTA AAG GCC A 3′	
Taqman probe (Mm-P)	5′ FAM-ACC CCC ACC AAC CCA GAG GT-BHQ1 3′	

1.5 Thermocycler RotorGene 6500 (Corbett Research), threshold line set manually at 0.02 for all experiments.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 The uredinia are scraped from leaves using a sterile scalpel blade or a spatula (ideally, approx. 2 mg) and transferred into a lysing matrix C tube (MP Biomedicals) or into a 2 mL microcentrifuge tube.

2.1.2 The extraction and the purification of the nucleic acids from urediniospores may be carried out using commercially available plant DNA extraction kits (for instance DNeasy Plant Mini Kit (Qiagen)).

2.1.3 Before DNA extraction urediniospores are disrupted. Four hundred µL lysis buffer and 4 µL RNase A (supplied with the Qiagen kit) are added to the harvested urediniospores. If using the lysing matrix C tube the suspension is shaken for 2 min at a frequency of 6.5 units with the FastPrep® system (MP Biomedicals); If 2 mL tubes are used, either ten 3 mm diameter sterile glass beads are added to the urediniospores suspension and the suspension is shaken for 5 min with an orbital shaker, at 5000 rpm, or 1 mm diameter sterile glass beads (approx. 8 mg) are added to the urediniospores suspension and the suspension is shaken for 2 min with an oscillating bead-beater at 30 Hz. DNA is then extracted following the manufacturer's instructions.

2.1.3 Extracted DNA should then be stored at 2–8°C for immediate use or at approximately −20°C if testing is not to be performed on the same day.

2.2 Polymerase chain reaction (PCR)

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	11.7	N.A.
PCR buffer (qPCR core kit no ROX, Eurogentec)	10×	2	1×
$MgCl_2$ (qPCR core kit no ROX, Eurogentec)	50 mM	2	5 mM
dNTPs (qPCR core kit no ROX, Eurogentec)	10 mM	0.4	0.2 mM
Forward primer (Mm-F)	5 µM	0.8	0.2 µM
Reverse primer (Mm-R)	5 µM	0.8	0.2 µM
Taqman probe (Mm-P)	5 µM	0.2	0.05 µM
Taq DNA polymerase	5 U/µL	0.1	0.5 U
Subtotal		18	
DNA from urediniospores		2	
Total		20	

2.2.2 PCR conditions

Real-time PCR amplifications include an initial denaturation step at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 64°C for 45 s. The fluorescence is measured during the annealing/elongation step.

3. Essential procedural information

3.1 Controls

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction (molecular grade water following all the steps of the DNA extraction/purification).
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, or a synthetic control (e.g. cloned PCR product).

Other possible controls:

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same spore suspension spiked with nucleic acid from the target organism.

There are three other possible ways to check the quality of DNA extracts:

- Real-time PCR on a sample that contains the target organism.
- Real-time PCR targeting a region of the 28S rDNA that is highly conserved in the *Melampsora* genus (Boutigny et al., 2013a). The primers and probe Mel-F, Mel-R and Mel-P should be used as indicated in the table below and the annealing temperature should be set at 62°C (instead of 64°C).

  Mel-F: 5′ TGA TAC GGT TTC TAA GAG TCG AG 3′

  Mel-R: 5′ CAT CTT TCC CTC ACG GTA CTT G 3′

  Mel-P: 5′ JOE-TTG GGA ATG CAG CTC AAA GTG GG-BHQ1 3′

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	10.7	N.A.
PCR buffer (qPCR core kit no ROX, Eurogentec)	10×	2	1×
$MgCl_2$ (qPCR core kit no ROX, Eurogentec)	50 mM	2	5 mM
dNTPs (qPCR core kit no ROX Eurogentec)	10 mM	0.4	0.2 mM
Forward primer (Mel-F)	5 µM	1.2	0.3 µM
Reverse primer (Mel-R)	5 µM	1.2	0.3 µM
TaqMan probe (Mel-P)	5 µM	0.4	0.1 µM
Taq DNA polymerase	5 U/µL	0.1	0.5 U
Subtotal		18	
DNA from urediniospores		2	
Total		20	

- Conventional PCR with primers ITS1 and ITS4 (White et al., 1990). The PCR conditions are those described in the conventional PCR method (Appendix 1), simply replacing the ITS-MMD-F/ITS-MMD-R primers with ITS1 and ITS4 primers 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 (Table 2).

3.2 Interpretation of results

The C_t cut-off value given below is as established in ANSES. As a C_t cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.

Verification of controls:

- The PIC and PAC amplification curves should be exponential (when the quality of the DNA extract is evaluated by conventional PCR with primers ITS1/ITS4, amplicons of 660–680 bp should be produced).
- NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve and a C_t value ≤30.
- A test will be considered negative if does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained, in particular when a C_t value above 30 is obtained.

4. Performance characteristics available

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

4.1 Analytical sensitivity data

The test can detect approx. 2 urediniospores of *M. medusae* f. sp. *deltoidae* in approx. 800 000 urediniospores of *Melampsora laricis-populina*.

4.2 Analytical specificity data

The inclusivity was evaluated on 39 isolates of *M. medusae* f. sp. *deltoidae*, and 2 isolates of *M. medusae* f. sp. *tremuloidae*. All isolates were detected.

The exclusivity was evaluated from 82 isolates belonging to 11 *Melampsora* species. (*M. abietis-canadensis*, *M. aecidioides*, *M. allii-populina*, *M. laricis-populina*, *M. laricis-tremulae*, *M. magnusiana*, *M. medusae-populina*, *M. occidentalis*, *M. pinitorqua* and *M. rostrupii*). The PCR test yielded negative results with DNA from all species, except with *Melampsora medusae*, the interspecific hybrids (*M. medusae-populina*) and the species *M. occidentalis*.

4.3 Data on repeatability

No data available.

4.4 Data on reproducibility

No data available.

APPENDIX 3 - MMD SPECIFIC REAL-TIME PCR (BOUTIGNY ET AL., 2013A)

The test below differs from the one described in the original publication.

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1 This real-time PCR method is used for the identification of *M. medusae* f. sp. *deltoidae* in urediniospores from poplar leaves.

Note concerning the interpretation of the test result: *Melampsora medusae* f. sp. *tremuloidae* is considered *present* in the case of a *negative result* with this test after positive results obtained by morphological identification, and PCR using the tests of Husson et al. (2013) (Appendix 1) or Boutigny et al. (2013b) (Appendix 2), see Figure 1. The protocol was published in 2013.

1.2 The PCR primers are selected in the 28S region of the rDNA gene (sequences for *M. medusae* f. sp. *deltoidae* may be retrieved from Genbank, accession JN934959).

1.3 Oligonucleotides:

Primers	Sequence	Amplicon size
Forward primer (Mmd-F)	5′ GTT GGA AAA AGG GCT CGA G 3′	94 bp
Reverse primer (Mmd-R)	5′ AGC TTA CTA CGC GTT CCT CA 3′	
Taqman probe (Mmd-P)	5′ FAM-TTG GGA CCT CGA ATA CAA CGC TC-BHQ1 3′	

1.4 Thermocycler RotorGene 6500 (Corbett Research), threshold line set manually at 0.02 for all experiments.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 The uredinia (ideally, approx. 2 mg in total) are scraped from leaves using a sterile scalpel blade or a spatula and transferred into a lysing matrix C tube (MP Biomedicals) or into a 2 mL microcentrifuge tube.

2.1.2 The extraction and the purification of the nucleic acids from urediniospores may be carried out using commercially available plant DNA extraction kits (for instance DNeasy Plant Mini Kit (Qiagen)).

2.1.3 Before DNA extraction urediniospores are disrupted. Four hundred µL lysis buffer and 4 µL RNase A (supplied with the Qiagen kit) are added to the harvested urediniospores. If using the lysing matrix C tube the suspension is shaken for 2 min at a frequency of 6.5 units with the FastPrep® system (MP Biomedicals); If 2 mL tubes are used, either ten 3 mm diameter sterile glass beads are added to the urediniospores suspension and the suspension is shaken for 5 min with an orbital shaker, at 5000 rpm, or 1 mm diameter sterile glass beads (approx. 8 mg) are added to the urediniospores suspension and the suspension is shaken for 2 min with an oscillating bead-beater at 30 Hz. DNA is then extracted following the manufacturer's instructions.

2.1.4 Extracted DNA should then be stored at 2–8°C for immediate use or at approximately −20° if testing is not to be performed on the same day.

2.2 Real-time PCR

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	12.1	N.A.
PCR buffer (qPCR core kit no ROX, Eurogentec)	10×	2	1×
$MgCl_2$ (qPCR core kit no ROX, Eurogentec)	50 mM	2	5 mM
dNTPs (qPCR core kit no ROX, Eurogentec)	10 mM	0.4	0.2 mM
Forward primer (Mmd-F)	5 µM	0.4	0.1 µM
Reverse primer (Mmd-R)	5 µM	0.8	0.2 µM
Taqman probe (Mmd-P)	5 µM	0.2	0.05 µM
Taq DNA polymerase	5 U/µL	0.1	0.5 U
Subtotal		18	
DNA from urediniospores		2	
Total		20	

2.2.2 PCR conditions

Real-time PCR amplifications include an initial denaturation step at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 62°C for 45 s. The fluorescence is measured during the annealing/elongation step.

3. Essential procedural information

3.1 Controls

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction (molecular grade water following all the steps of the DNA extraction/purification)
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, or a synthetic control (e.g. cloned PCR product).

Other possible controls:

Inhibition control (*IC*) to monitor inhibitory effects introduced by the nucleic acid extract. Same spore suspension spiked with nucleic acid from the target organism.

There are three other possible ways to check the quality of DNA extracts:

- Real-time PCR on a sample that contains the target organism.
- Real-time PCR targeting a region of the 28S rDNA that is highly conserved in the *Melampsora* genus performed on the same sample (Boutigny et al., 2013a). The primers and probe Mel-F, Mel-R and Mel-P should be used as indicated in the table below. The PCR conditions are identical to those of the Mmd test.:
  Mel-F: 5′-TGA TAC GGT TTC TAA GAG TCG AG-3′
  Mel-R: 5′-CAT CTT TCC CTC ACG GTA CTT G-3′
  Mel-P: 5′ JOE-TTG GGA ATG CAG CTC AAA GTG GG-BHQ1 3′

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	10.7	N.A.
PCR buffer (PCR core kit no ROX, Eurogentec)	10×	2	1×
$MgCl_2$ (PCR core kit no ROX, Eurogentec)	50 mM	2	5 mM
dNTPs (PCR core kit no ROX, Eurogentec)	10 mM	0.4	0.2 mM
Forward primer (Mel-F)	5 µM	1.2	0.3 µM
Reverse primer (Mel-R)	5 µM	1.2	0.3 µM
Taqman probe (Mel-P)	5 µM	0.4	0.1 µM
Taq DNA polymerase	5 U/µL	0.1	0.5 U
Subtotal		18	
DNA from urediniospores		2	
Total		20	

- Conventional PCR with primers ITS1 and ITS4 (White et al., 1990) performed on the same sample. The PCR conditions are those described in the conventional PCR method (Appendix 1), replacing the ITS-MMD-F/ITS-MMD-R primers with ITS1 and ITS4 primers and decreasing the annealing temperature to 50°C. A positive signal (approximately 660–680 bp) following this test indicates that the DNA extract was amplifiable (Table 2).

3.2 Interpretation of results

The C_t cut-off value given below is as established in ANSES. As a C_t cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.

Verification of controls:

- The PIC and PAC amplification curves should be exponential (when the quality of the DNA extract is evaluated by conventional PCR with primers ITS1/ITS4, amplicons of 660–680 bp should be produced)
- NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve and a C_t value ≤30.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained, in particular when a C_t value above 30 is obtained.

4. Performance characteristics available

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or other modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

Data from Anses.

4.1 Analytical sensitivity data

The test can detect 240 copies of the amplification product (in a plasmid vector). This corresponds to approx. 1 urediniospore of *M. medusae* f. sp. *deltoidae* in 2 mg (~800 000) urediniospores of *Melampsora laricis-populina*.

4.2 Analytical specificity data

The inclusivity was evaluated on 39 isolates of *M. medusae* f. sp. *deltoidae*.

The exclusivity was evaluated from 93 isolates belonging to 11 *Melampsora* species. (*M. abietis-canadensis*, *M. aecidioides*, *M. allii-populina*, *M. laricis-populina*, *M. laricis-tremulae*, *M. magnusiana*, *M. medusae* f. sp. *tremuloidae*, *M. medusae-populina*, *M. occidentalis*, *M. pinitorqua* and *M. rostrupii*). The PCR test yielded negative results with DNA from all species, except with *Melampsora medusae* f. sp. *deltoidae* and the interspecific hybrid *M. medusae-populina*.

4.3 Data on repeatability

The intra-test coefficient of variation (CV) was between 0.61% and 1.26% (ten replicates of the same DNA sample tested in the same run).

4.4 Data on reproducibility

The inter-test CV was between 0.61% and 1.95% (one replicate of the same DNA sample tested in 10 different runs over a 4-week period with two different operators and using two different Rotor-Gene thermal cyclers).

4.5 Data on robustness

The results remained unaffected by a deliberate ±10% variation of the reaction volume.