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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/027 (2)

Diagnostics
Diagnostic

PM 7/027 (2) *Puccinia horiana*

Specific scope

This Standard describes a diagnostic protocol for *Puccinia horiana*.[1]

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

Specific approval and amendment

First approved in 2003-09.
Revision approved in 2020-03.

1. Introduction

Puccinia horiana (EPPO/CABI, 1997) is the causal organism of chrysanthemum white rust, an economically important disease of florist's chrysanthemum (*Chrysanthemum* × *morifolium*). It is an autoecious microcyclic rust fungus: it completes its life cycle on a single host and only produces teliospores and basidiospores. Teliospores germinate *in situ* and form basidiospores (sometimes called sporidia) without a period of dormancy. The basidiospores are dispersed by air and, under appropriate conditions, cause new infections. High humidity and a thin film of moisture on the leaf surface are essential for the germination of both the telio- and basidiospores. Typical symptoms of the disease are yellow spots on the upper leaf surface and raised pustules (telia) on the lower leaf surface. These pustules are white, hence the name white rust. This fungus infects fresh tissues of growing chrysanthemum plants although older leaves can also be infected.

Chrysanthemum white rust originates in Japan but has now been reported in most countries where florist's chrysanthemums are grown (EPPO, 2019). Although *Chrysanthemum* × *morifolium* (formerly *Dendranthema* × *morifolium*) is the main host of *P. horiana*, natural infections have also been observed on *Chrysanthemum japonense*, *Chrysanthemum japonicum* var. *wakasaense*, *Ajania shiwogiku*, *C. lavandulifolium* (formerly *Dendranthema boreale*), *C. japonicum* (formerly *Dendranthema japonicum*; Punithalingam, 1968). By inoculation, *C. arcticum* (formerly *Arctanthemum arcticum*), *Ajania pacifica* (formerly *Dendranthema pacificum*), *C. yoshinaganthum* and *Nipponanthemum nipponicum* were found to be susceptible (Hiratsuka, 1957). Species that did not develop symptoms when inoculated include annual chrysanthemums (*Chrysanthemum carinatum*, *Chrysanthemum coronarium*, *Chrysanthemum segetum*), *Tanacetum cinerariifolium*, *Tanacetum coccineum*, *Argyranthemum frutescens*, *Leucanthemum vulgare* and *Leucanthemum* × *superbum*. Other species reported as hosts are *Chrysanthemum indicum* L, *Chrysanthemum zawadskii* Herbich subsp. Yezoense (Maek.) Y. N. Lee, *Leucanthemella serotina* (L.) Tzvelev and *Nipponanthemum nipponicum* (Franch. ex Maxim.) Kitam (O'Keefe, 2014).

A flow diagram describing the diagnostic procedure for *Puccinia horiana* is presented in Fig. 1.

2. Identity

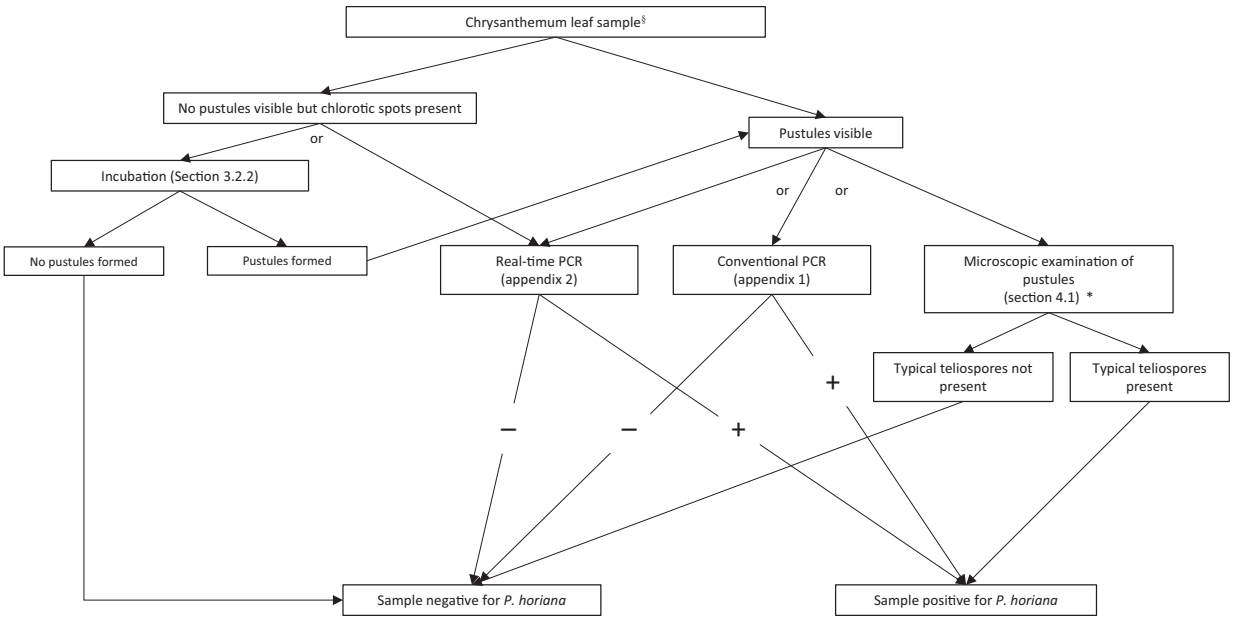
Name: *Puccinia horiana* P. Hennings
Synonyms: None
Taxonomic position: Fungi: Basidiomycota: Pucciniales
EPPO Code: PUCCHN
Phytosanitary categorization: EPPO A2 list: no. 80, EU Annex designation IV (RNQP)

3. Detection

3.1. Disease symptoms

The fungus primarily attacks the younger leaves of chrysanthemum, causing spotting and in severe cases rolling,

[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.



*For less experienced laboratory staff: confirmation of microscopic observation via molecular test is recommended.
§ In cases where a latent infection is suspected real-time PCR can be used (see section 3.2.3).

Fig. 1 Flow diagram for the diagnostic procedure for *P. horiana*.

Fig. 2 Symptoms of chlorosis on the upper (adaxial) leaf side of chrysanthemum due to *P. horiana*.

Fig. 3 Fresh pustules of *P. horiana* on the lower (abaxial) leaf side of chrysanthemum.

twisting and finally death (Firman & Martin, 1968; Smith, 1988). Infected dead leaves remain attached to the stems. During early stages of infection, light green to yellow spots can be observed on the upper leaf surface (Fig. 2).

These spots gradually enlarge up to 5 mm and become light brown. As the infection progresses, the spots become sunken. Raised white, often faintly pinkish white, and waxy pustules (telia) are formed on the corresponding lower leaf surface (Fig. 3), rarely on the upper surface. They are 2–4 mm in diameter, solitary or aggregated.

In some cases, small telia group together or a secondary ring of telia is formed around the original pustule. Maturing lesions as seen on the upper leaf surface can increase in size up to 2 cm in diameter, turn brown and become necrotic. Old pustules eventually become colonized by saprophytic organisms and turn dirty brown (Fig. 4). In severe cases infections of petioles, stems and flowers can occur.

The pathogen can move systemically and survive in the roots of overwintering plants, while the upper plant parts are killed by frost (O'Keefe & Davis, 2012; Bonde *et al.*, 2015). Newly developed shoots can develop symptoms in spring, as the pathogen transfers back from the roots to the upper parts. In such plants, the pustules will first be found on the lower (older) leaves of the new stems instead of on the upper (younger) leaves, in contrast with regular basidiospores-mediated infection via the air.

Fig. 4 Mainly older pustules of *P. horiana* on the lower (abaxial) leaf side of a commercial chrysanthemum plant.

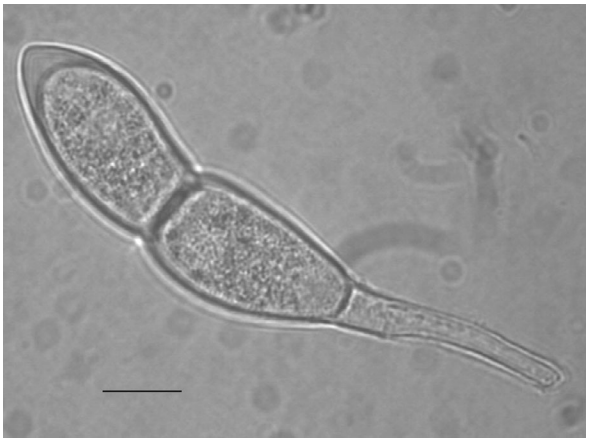


Fig. 5 A teliospore of *P. horiana*. Bar = 10 µm.

3.2. Test sample requirements

3.2.1. Leaves with pustules

Leaves with (preferentially fresh) pustules should be collected.

Pieces of the pustules (telia) are excised from the leaves with a scalpel and either used for microscopic observation of the teliospores or for DNA extraction and molecular analysis. For microscopic observation, leaves containing fresh pustules should be moistened and incubated in a closed container for 2–4 h at 17–21°C to allow germination of the teliospores and the development of the promycelium and basidiospores.

3.2.2. Leaves without pustules but with chlorotic spots

Leaves from plants without pustules but with suspect chlorotic leaf spots can also be sampled. If such plants have not been treated with fungicides, they are incubated for up to 10 days at 17–21°C to promote pustule development. Alternatively, leaves with chlorotic spots can be tested with real-time PCR (see Appendix 2).

3.2.3. Asymptomatic plants

Plants without pustules and without chlorotic spots are usually not sampled. However, if a latent infection is suspected, samples comprising up to ten leaves are collected for molecular analysis using real-time PCR (see Appendix 2). The leaves can be taken from different plants or different stems on the same plant. The leaves should be chosen randomly among the four most recent leaves per stem. When sampling larger plants more samples can be collected (e.g. with five leaves per plant).[2]

[2]ISPM 31 (IPPC, 2008) provides useful information on the number of plants to be sampled.

4. Identification

Being an obligate pathogen, isolation onto nutrient media is not an option. However, pustules are usually present in the case of infection, making direct morphological or molecular identification possible.

4.1. Morphological identification

For microscopical analysis, parts of the suspected telia should be mounted (e.g. in water with a drop of Tween) and examined at 400× magnification under normal microscopic light for the morphological characteristics. The teliospores (Fig. 5) are hyaline, cylindrical, fusiform and oblong to oblong clavate (rarely elliptical) with a smooth pale-yellow cell wall (1–2 µm thick, 3–10 µm thick at the apex). Predominantly the teliospores are slightly constricted at a single septum, rarely biseptate, 32–45 × 12–18 µm (excluding the pedicel). The pedicel is hyaline, persistent and up to 45 µm long.

From one or both cells, the teliospores germinate *in situ* to produce a promycelium, with one to four (often two) kidney-shaped basidiospores attached (Figs 6 and 7; Kapooria & Zadoks, 1973).

Other *Puccinia* species known to infect *Chrysanthemum* spp. and host species previously classified as *Chrysanthemum* are listed in Table 1 (Punithalingam, 1968; Alaei *et al.*, 2009b).[3] *P. horiana* is easily distinguished from other species by its smooth, hyaline teliospores that always germinate *in situ* on the living leaf. All the other species except *P. leucanthemi* have brown and/or verruculose teliospores that do not germinate *in situ*. *P. leucanthemi* is distinguished by its pale-yellow

[3]The nomenclature of *Chrysanthemum* species has changed several times, which should be taken into account when consulting older literature. For example, *Chrysanthemum* × *morifolium* was changed to *Dendranthema* × *morifolium* and later back to *Chrysanthemum* × *morifolium*.

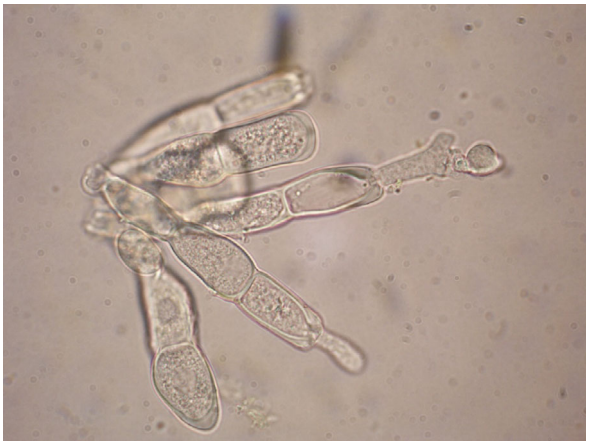


Fig. 6 Teliospores of *P. horiana*, some germinating with a promycelium. The central spore has a basidiospore attached to the promycelium.

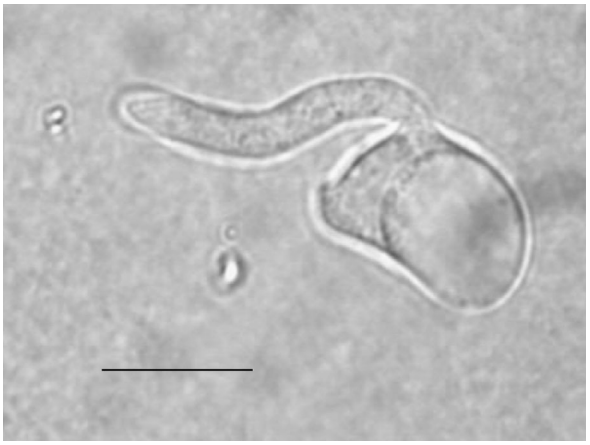


Fig. 7 Germinating basidiospore of *P. horiana*. Bar = 10 µm.

Table 1. *Puccinia* spp. other than *P. horiana* found on *Chrysanthemum* and related species

Puccinia spp.	Host range
P. chrysanthemi	*Argyranthemum frutescens*, *Chrysanthemum lavandulifolium (= C. boreale)*, *Chrysanthemum* × *morifolium*
P. chrysanthemicola	*Glebionis coronaria* (L.) Tzvelev
P. gaeumannii	*Tanacetum cinerariifolium*
P. heeringiana	*Argyranthemum frutescens*, *Tanacetum parthenium*
P. leucanthemi	*Leucanthemum vulgare*
P. pyrethri	*Tanacetum corymbosum*
P. tanaceti	*Argyranthemum frutescens*, *Chrysanthemum lavandulifolium (= C. boreale)*, *Tanacetum vulgare* and many other *Artemisia* and *Tanacetum* spp.

teliospores and a non-overlapping host range; it infects *Leucanthemum vulgare*. *P. chrysanthemi*, the other rust species that commonly infects the main host

Fig. 8 Uredinia of the non-target organism *P. chrysanthemi*.

Chrysanthemum × *morifolium*, also distinguishes itself from *P. horiana* in that it produces urediniospores (Fig. 8).

4.2. Molecular identification

Conventional PCR tests for *P. horiana* have been described by Alaei *et al.* (2009a) and Pedley (2009), in both cases based on the rDNA ITS regions. Analytical specificity (exclusivity) of conventional PCR tests has been evaluated most extensively in Alaei *et al.* (2009a), using 25 non-target rust fungi versus one non-target rust fungus in Pedley (2009). Alaei *et al.* (2009a) tests are also reported as more sensitive (10 pg versus 1 ng genomic DNA as analytical sensitivity), although direct comparison is difficult as not only fungal DNA but also plant DNA was included in the target genomic DNA sample used by Pedley (2009). Alaei *et al.* (2009a) also describe a very sensitive nested version of their conventional PCR test (analytical sensitivity of 10 fg genomic DNA); however, when DNA is extracted directly from pustules, such a level of analytical sensitivity is not needed. The main conventional PCR test from Alaei *et al.* (2009a) is described in Appendix 1.

Real-time PCR tests for *P. horiana* have been described by Alaei *et al.* (2009a) (with a slightly modified version in O'Keefe (2014)), Pedley (2009) and Demers *et al.* (2015). The main test described by Alaei *et al.* (2009a) is very sensitive (analytical sensitivity of 5 fg genomic DNA in the version with Sybr green) and specific (exclusivity tested with 25 non-target rusts). The primers provide full specificity so the test can be conducted with Sybr green. However, if desired it can be conducted in a version with a Taqman® MGB probe. Analytical specificity (exclusivity) tests of the Pedley (2009) and Demers *et al.* (2015) real-time PCR tests were limited to *P. chrysanthemi*, a species which is common on Chrysanthemum, but which is phylogenetically not closely related to *P. horiana*. Both Pedley (2009) and Demers *et al.* (2015) tests have a reported analytical sensitivity of 1 pg genomic DNA, but direct comparison with the Alaei *et al.* (2009a) test is difficult as the

gDNA used by Pedley (2009) and Demers *et al.* (2015) also included plant DNA. According to O'Keefe (2014), who simultaneously analysed the same samples with the Pedley (2009) and the Alaei *et al.* (2009a) tests, the C_t values were 4.1 to 5.3 cycles higher with the Pedley (2009) tests than with the main Alaei *et al.* (2009a) test. This difference was relevant only when analysing latently infected material. Based on the sensitivity and specificity data, the main test from Alaei *et al.* (2009a) is described in this protocol (Appendix 2). The adjustments to the Alaei *et al.* (2009a) test by O'Keefe (2014) relate to the cycling conditions only. The adjustments lowered the C_t values by 0.6 to 1.3 cycles compared to the original test but on a different platform, no differences were observed between the cycling conditions. The LNA-probe-based tests described by Demers *et al.* (2015) not described in this protocol do have the advantage that they can be conducted in duplex format, for simultaneous detection of *P. horiana* and *P. chrysanthemi,* for applications where this is relevant.

5. Reference material

Reference material in the form of the plasmid-cloned target region can be obtained from ILVO. See point 8 for contact information.

6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *Documentation and reporting on 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 Kurt Heungens, Institute for Agricultural, Fisheries and Food Research (ILVO), Burgemeester Van Gansberghelaan 96, 9820 Merelbeke, Belgium (kurt.heungens@ilvo.vlaanderen.be) or from Ann Barnes at Fera Science Ltd, Sand Hutton, York, YO41 1LZ, UK (ann.barnes@fera.co.uk).

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

A regular 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

The protocol was originally written by G. Szkuta and J. Butrymowicz (Main Inspectorate of Plant Protection, Torun, PL), R. Cook (Fera Science Ltd, York, GB) and M. Maes (ILVO, Merelbeke, BE). It has been revised by K. Heungens (ILVO, Merelbeke, BE) and reviewed by the EPPO Panel on Diagnostics in Mycology.

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Appendix 1 - Identification of *Puccinia horiana* by conventional PCR (Alaei *et al.*, 2009a)

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 test is used for the identification of *Puccinia horiana* in symptomatic leaves of *Chrysanthemum* × *morifolium* or other host species.

1.2. It was published in 2009 (Alaei *et al.*, 2009a).

1.3. The PCR primers were selected in the ITS regions of the rRNA gene (sequences of this region for *P. horiana* may be retrieved from GenBank, e.g. accession number EU013967). There is some intragenome sequence variability among the different copies of this gene, mostly related to small differences in the length of repeat regions (see details in Alaei *et al.*, 2009b). However, this does not affect the performance of this PCR test.

1.4. The amplicon covers a region spanning from bases 105–131 (in rDNA ITS1) to 652–679 (in rDNA ITS2) in the sequence reported in EU013967.

1.5. The amplicon size is approximately 575 bp. It can vary by a few bp due to the intragenome sequence variability reported in 1.3.

Forward primer Ph260F	5′-CCCTTTTAAATATATCACCCAAACTAT-3′
Reverse primer Ph256R	5′-GATTAATTTTGGGTTTTTAGAAGTCTT-3′

Alternative primer pairs are described in Alaei *et al.* (2009a) but Ph260F and Ph256R were recommended by the authors.

1.6. The test was developed on a GeneAmp PCR System 9700 (Applied Biosystems, later ThermoFisher Scientific). As far as known, there are no specific platform requirements for this test. The test was developed using AmpliTaq Gold polymerase and associated GeneAmp PCR buffer II but should perform well with other polymerase and buffer combinations.

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. Approximately 100 mg excised pustule material is placed in a microcentrifuge tube and ground with liquid nitrogen before DNA extraction. Alternatively, the material can be ground using commercial bead-beating equipment.

2.1.2. Ten DNA extraction methods were compared in Alaei *et al.* (2009a) in terms of efficiency in extraction of the target and in terms of co-extraction of PCR inhibitors. The best methods were the GenElute plant genomic DNA kit (Sigma), the Invisorb spin plant mini kit (Invitek, now Stratec), and a non-commercial CTAB method. When using up to 10 ng template DNA in the PCR reaction, no PCR inhibition was observed with these methods. Other (commercial) DNA extraction methods are expected to produce adequate results. In absence of previous experience with a given DNA extraction method for this pathogen and matrix combination, it is recommended to also include an extra sample with 1/10 diluted DNA extract. This should bring potential co-extracted PCR inhibitors below problematic levels while still allowing sufficient sensitivity.

2.1.3. DNA should preferably be stored at approximately −20°C or kept at 2–8°C for immediate use.

2.2. Polymerase chain reaction

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	19.75	NA
GeneAmp PCR buffer II (*ThermoFisher*)	10×	5	1×
$MgCl_2$ (*ThermoFisher*)	25 mM	5	2.5 mM
dNTPs (*Roche*)	2 mM	5	0.2 mM
Forward primer (Ph260F)	2 µM	5	0.2 µM
Reverse primer (Ph256R)	2 µM	5	0.2 µM
DNA polymerase (AmpliTaq Gold; *ThermoFisher*)	5 U μL^{-1}	0.25	0.025 U μL^{-1}
Subtotal		45	
Genomic DNA extract (maximum 10 ng total DNA amount)		5	
Total		50	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

NA, not applicable.

2.2.2. PCR conditions

After an initial denaturation of 5 min at 94°C, 35 cycles are conducted consisting of 30 s denaturation at 94°C, 30 s annealing at 60°C and 45 s extension at 72°C. This is followed by a final extension of 10 min at 72°C.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- 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: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue (mainly pustule material) or a synthetic control (cloned PCR product). The PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPCs) can be used to monitor each individual sample separately. IPCs can be either genes present in the matrix DNA or added to the DNA solutions.

Alternative IPCs can include:s

- Specific amplification or co-amplification of endogenous nucleic acid using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample, for example plant cytochrome oxidase gene (e.g. Weller *et al.*, 2000 or as described in EPPO, 2017 Appendix 3) or eukaryotic 18S rDNA.
- amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample

Laboratories should take additional care to prevent risks of cross-contamination when using high-concentration positive controls (e.g. cloned products)

3.2. **Interpretation of results**: In order to assign results from the PCR-based test the following criteria should be followed

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC and PAC (and if relevant IPCs containing the target sequence) should produce amplicons of approximately 575 bp.

When these conditions are met

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

4. Performance characteristics available

The validation data listed below were published in Alaei *et al.* (2009a). This validation preceded the publication of the relevant EPPO instructions in PM 7/98.

4.1. Analytical sensitivity data

The analytical sensitivity of the test is 10 pg genomic DNA (isolated from basidiospores, so excluding host DNA). When testing leaves at different times post inoculation, the pathogen was already detected 4 days after inoculation (during the latent phase), when the amount of target DNA was 4.6 ng per 100 mg infected plant tissue. This amount was 117 times smaller than 14 days after inoculation (538.5 ng target DNA per 100 mg randomly sampled plant tissue), when pustules were present. Amounts of target DNA were determined via simultaneous analysis of the samples with real-time PCR. When using infected plant material in a dilution series with healthy plant material, the pathogen could be detected when at least 0.1% of the plant material was fully symptomatic.

4.2. Analytical specificity data

The inclusivity of the test was confirmed by Alaei *et al.* (2009a) using 18 isolates of *P. horiana*. Inclusivity is further supported by Alaei *et al.* (2009b), who showed that no sequence variation was present in the primer binding areas of an international collection of isolates from eight countries and three continents.

Exclusivity of the test was demonstrated by Alaei *et al.* (2009a) using 1 ng template DNA and/or 5×10^6 cloned target copies from 25 non-target rust species, including phylogenetically closely related species.

Appendix 2 - Identification of *Puccinia horiana* by real-time PCR (Alaei *et al.*, 2009a)

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 test is used for the detection and identification of *Puccinia horiana* in symptomatic or non-symptomatic leaves of *Chrysanthemum* × *morifolium* or other host species.

1.2. The test was published in 2009 (Alaei *et al.*, 2009a). A version with slightly adjusted cycling parameters was published by O'Keefe (2014).

1.3. The PCR primers were selected in the ITS1 region of the rRNA gene (sequences of this region for *P. horiana* may be retrieved from GenBank, e.g. accession number EU013967). There is some intragenomic sequence variability among the different copies of this gene, mostly related to small differences in the length of repeat regions (see details in Alaei *et al.*, 2009b). However, this does not affect the success of this real-time PCR test.

1.4. The amplicon covers a region spanning from bases 103–131 to 238–266 (all in rDNA ITS1) in the sequence reported in EU013967.

1.5. The amplicon size is approximately 164 bp. It can vary by a few bp due to the intragenomic sequence variability reported in 1.3.

Forward primer Ph263F	5′-ACCCCTTTTAAATATATCACCCAAACTAT-3′
Reverse primer Ph264R	5′-CTTGTGTTATATAATAAAAAAGGGGGTAA-3′
Optional dual-labelled Taqman probe Ph706	6-FAM-ACTTGGTTGCATGAATT-MGB (minor groove binding version)

Alternative primer pairs have been described in Alaei *et al.* (2009a) but Ph263F and Ph264R were recommended by the authors.

1.6. The test was developed on an ABI Prism 7900 HT (Applied Biosystems, later ThermoFisher Scientific). It has been conducted successfully on other platforms, such as the Biorad CFX96 and the Cepheid Smart Cycler II. The test was developed using the SYBR Green I PCR Master Mix (Applied Biosystems) for the probe-free version and the Taqman Universal PCR Mastermix (Applied Biosystems) for the probe-based version.

These original mastermixes are no longer commercialized but the probe-free version has since been conducted successfully using the Sensimix Sybr PCR mastermix (Bioline) and the PerfeCTa Sybr Green SuperMix (Quanta Bio). Poor results have been obtained with this test and the Maxima Sybr Green qPCR Master Mix (ThermoFisher Scientific). The probe-based version has also been conducted successfully using Premix Ex Taq (probe qPCR) (Takara), PerfeCTa qPCR ToughMix (Quanta Bio), Maxima Probe qPCR Master Mix (ThermoFisher Scientific), and GoTaq qPCR Master Mix (Promega). Poor results have been reported in Demers *et al.* (2015) using the LightCycler 480 mastermix (Roche), although it is not clear whether this was due to the mastermix or the use of a different type of probe modification (ZEN internal quencher versus MGB).

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. When working with symptomatic plants, see the equivalent section in Appendix 1 for tissue source and homogenization method. When working with non-symptomatic plants, up to 10 potentially infected leaves are ground in liquid nitrogen, after which a 100 mg subsample can be processed as for samples from symptomatic plants.

2.1.2. See the equivalent section in Appendix 1 for DNA extraction method. Given the higher sensitivity of the real-time PCR test, optimal extraction of target DNA is not crucial in case of symptomatic sample material.

2.1.3. DNA should preferably be stored at approximately −20°C or kept at 2–8°C for immediate use.

2.2. Real-time polymerase chain reaction

2.2.1. Master Mix for probe-free test version

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	6	NA
Sensimix Sybr PCR Mastermix (*Bioline*)	2×	12.5	1×
Forward Primer (Ph263F)	10 µM	0.75	0.3 µM
Reverse Primer (Ph264R)	10 µM	0.75	0.3 µM
Subtotal		20	
DNA template (maximum 10 ng)		5	
Total		25	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

NA, not applicable.

2.2.2 Master Mix for probe-based test version

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	5.5	NA
Premix Ex Taq (Probe qPCR) (*Takara*)	2×	12.5	1×
Forward Primer (Ph263F)	10 µM	0.75	0.3 µM
Reverse Primer (Ph264R)	10 µM	0.75	0.3 µM
Probe (Ph706)	10 µM	0.5	0.2 µM
Subtotal		20	
DNA template (maximum 10 ng)		5	
Total		25	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

NA, not applicable.

2.2.3 PCR conditions

After an initial 10 min at 95°C, 40 cycles are conducted consisting of 15 s denaturation at 95°C and 60 s annealing and extension at 60°C.

3. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- 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: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue (mainly pustule material) or a synthetic control (cloned PCR product). The PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPCs) can be used to monitor each individual sample separately. IPCs can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative IPCs can include:

- Specific amplification or co-amplification of endogenous nucleic acid using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample, for example plant cytochrome oxidase gene (e.g. Weller *et al.*, 2000 or as described in EPPO, 2017 Appendix 3) or eukaryotic 18S rDNA.
- amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Laboratories should take additional care to prevent risks of cross-contamination when using high-concentration positive controls (e.g. cloned products).

3.2. **Interpretation of results**: In order to assign results from the PCR-based test the following criteria should be followed

Verification of the controls

- The PIC and PAC (as well as IPC as applicable) amplification curves should be exponential.
- 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.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- For the SYBR® Green based real-time PCR version of the test the melting temperature (TM) value should be approximately 75°C (exact temperature depends on Mastermix used and will be observed in e.g. the PAC sample).
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation data listed below were published in Alaei *et al.* (2009a) and O'Keefe (2014). The validation by Alaei *et al.* (2009a) preceded the publication of the relevant EPPO instructions in PM7/98.

4.1. Analytical sensitivity data

Based on Alaei *et al.* (2009a), the analytical sensitivity of the test is 5 fg genomic DNA (isolated from basidiospores, so excluding host DNA). When testing leaves at different time points after inoculation, the pathogen was already detected 9 h after inoculation, when the amount of target DNA was 1.3 ng per 100 mg infected plant tissue. This amount is 414 times smaller than 14 days after inoculation (538.5 ng target DNA per 100 mg randomly sampled plant tissue), when pustules were present. When using

infected plant material in a dilution series with healthy plant material, the pathogen could be detected when 0.001% of the plant material (= the highest dilution analysed) was fully symptomatic. Direct analysis of a dilution series of basidiospores allowed detection of as low as 8 basidiospores at a C_t value of 33.5.

O'Keefe (2014) reported a C_t value of 25.0 with symptomatic leaf material and 34.1 with asymptomatic leaf material when using the original Alaei *et al.* (2009a) test.

4.2. Analytical specificity data

The inclusivity of the test was confirmed by Alaei *et al.* (2009a), using 18 isolates of *P. horiana*. Inclusivity is further supported by Alaei *et al.* (2009b), who showed that no sequence variation was present in the primer binding areas of an international collection of isolates from eight countries and three continents.

Exclusivity of the test was demonstrated by Alaei *et al.* (2009a), using 1 ng template DNA and/or 5×10^5 cloned target copies from 25 non-target rust species, including phylogenetically closely related species.

4.3. Data on reproducibility

The coefficient of variation (CV) between the C_t values from three separate real-time PCR runs on three different dilution series was smaller than 5% at all template levels tested (Alaei *et al.*, 2009a).