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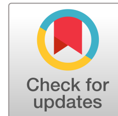

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/78 (2)

Diagnostics

PM 7/78 (2) *Verticillium nonalfalfae* and *V. dahliae*

Specific scope

This Standard describes a diagnostic protocol for *Verticillium nonalfalfae* and *V. dahliae* on hop (*Humulus lupulus*).

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

Specific approval and amendment

First approved in 2007–09. Revised in 2020–04.

1. Introduction

Verticillium wilt of hops (*Humulus lupulus*) is a vascular disease caused by the soil-borne fungal plant pathogens *Verticillium nonalfalfae* (formerly *V. albo-atrum sensu lato*) and *V. dahliae*. The disease is particularly devastating when infections are caused by highly virulent (lethal) pathotypes of *V. nonalfalfae* (Radišek *et al.*, 2006). *V. nonalfalfae* is most frequently isolated from hop and causes the majority of outbreaks, whereas *V. dahliae* infections are comparatively rare and less severe (Neve, 1991). Recent taxonomic revision of the genus *Verticillium* (Inderbitzin *et al.*, 2011; Inderbitzin & Subbarao, 2014) split *V. albo-atrum sensu lato* into three different species: *V. albo-atrum sensu stricto*, *V. nonalfalfae* and *V. alfalfae*. The new taxonomic concept recognizes *V. albo-atrum sensu lato* isolates pathogenic to hop as *V. nonalfalfae*. All three new species share an overall similar morphology, including resting mycelium, the main morphological character which distinguishes them from *V. dahliae*. *V. albo-atrum sensu stricto* can be morphologically differentiated from *V. alfalfae* and *V. nonalfalfae*, while *V. alfalfae* and *V. nonalfalfae* are morphologically indistinguishable. Nevertheless, they could be clearly differentiated based on host range, mating types and DNA characters (Inderbitzin *et al.*, 2011). *V. nonalfalfae* is reported from Canada, Cuba, Japan, Germany, the UK and Slovenia, and infects several hosts, including hop, potato, petunia and spinach (Inderbitzin *et al.*, 2011), while *V. alfalfae* causes disease only on alfalfa and is known from Iran, France, Sweden, Germany, Canada, Japan, New Zealand, the former USSR and the USA (Pegg & Brady, 2002). *V. albo-atrum sensu stricto* infects primarily potato and is reported from Canada, USA, the UK and Germany. *V. dahliae* is common and widespread on many herbaceous and woody plants, including hop, throughout much of the temperate and subtropical regions (Pegg & Brady, 2002; Inderbitzin & Subbarao, 2014).

Verticillium wilt on hops appears in fluctuating (mild) or progressive (lethal) disease forms. In general, mild wilt varies in intensity from year to year and rarely causes plant death, whereas lethal wilt is less influenced by seasonal climatic variations and causes very severe symptoms with rapid plant withering. Lethal wilt is caused by highly virulent pathotypes of *V. nonalfalfae* which are currently present only in England (Keyworth, 1942), Slovenia (Radišek *et al.*, 2003, 2006) and Germany (Seefelder *et al.*, 2009). There are also reports of mild verticillium hop infections from Germany, Poland, Belgium, France, Slovenia and, outside Europe, from New Zealand and the USA (Oregon), caused either by less virulent strains of *V. nonalfalfae* or *V. dahliae* (Neve, 1991).

Both species are primarily soil-borne. Once infection is established in plants, there are no effective chemical treatments to control the disease. Hops are non-grafted long-lived perennials which, due to the requirement for expensive fixed support systems, are often replanted on the same sites. Contamination of hop fields can lead to repeated infection and abandonment of sites, although the use of highly resistant varieties and four years' crop rotation by non-host plants is often effective in reducing losses.

A flow diagram describing the diagnostic procedure for *V. nonalfalfae* and *V. dahliae* on hop is presented in Fig. 1.

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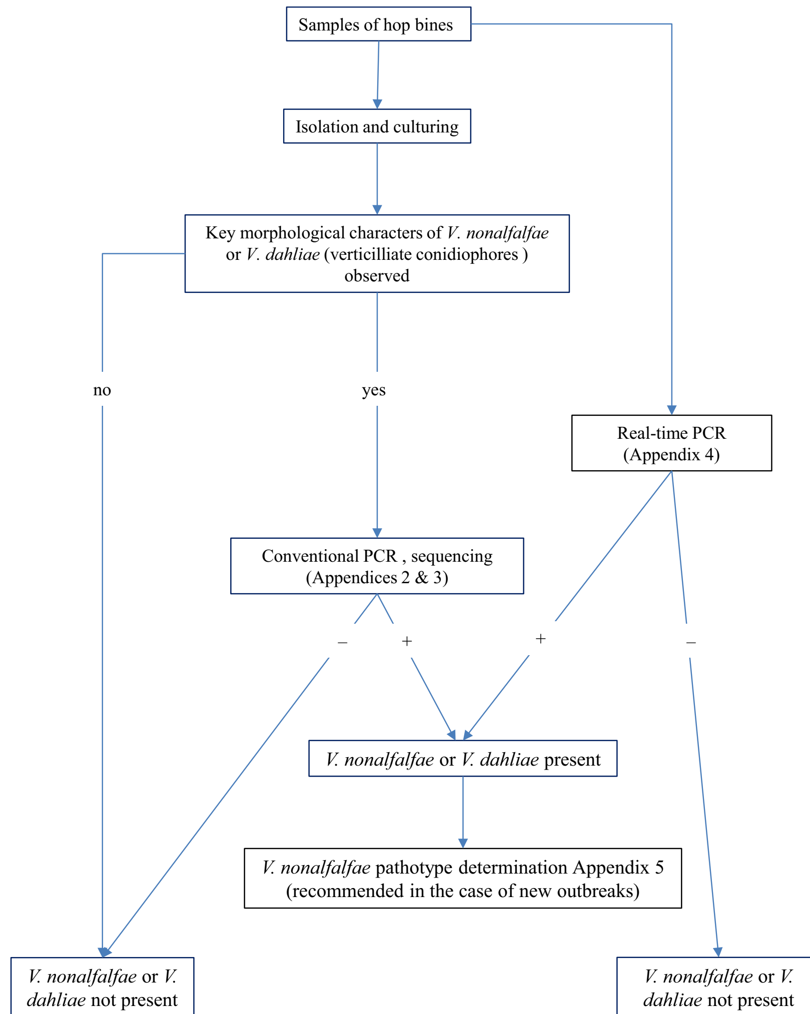

Fig. 1 Flow diagram describing the diagnostic procedure for *V. nonalfalfae* and *V. dahliae*. [Colour figure can be viewed at wileyonlinelibrary.com]

2. Identity

Name: *Verticillium nonalfalfae* Inderb., H. W. Platt, R. M. Bostock, R. M. Davis & K. V. Subbarao
Synonyms: None
Taxonomic position: Fungi, Ascomycota, Pezizomycotina, Sordariomycetes, Glomerellales, Plectosphaerellaceae.
EPPO Code: VERTNO (*V. nonalfalfae*)
Phytosanitary categorization: EPPO A2 list; EU Annex IV (RNQP)

Notes on taxonomy and nomenclature: Before the last taxonomic revision (Inderbitzin *et al*., 2011), *V. albo-atrum sensu lato* included two accepted and molecularly clearly distinguished groups (Grp): Grp1, which includes a subgroup of isolates pathogenic to *Medicago sativa* (lucerne) (lucerne pathotype) and a subgroup of isolates pathogenic to all other hosts including *Humulus lupulus* (hop) (non-lucerne pathotype), and Grp 2, which includes isolates that form resting mycelia and microsclerotia and primary infect potato.

The new species *V. alfalfae* and *V. nonalfalfae* refer to the lucerne and non-lucerne pathotypes of *V. albo-atrum sensu lato*, respectively (Barbara & Clewes, 2003). The species *V. albo-atrum sensu stricto* represents isolates previously recognized as Grp2 (Robb *et al*., 1993; Mahuku & Platt, 2002).

Name: *Verticillium dahliae* Klebahn
Synonyms: *Verticillium albo-atrum* f. *angustum* Wollenw., *Verticillium albo-atrum* var. *chlamydosporale* Wollenw., *Verticillium albo-atrum* var. *dahliae* (Kleb.) R. Nelson, *Verticillium albo-atrum* var. *medium Wollenw.*, *Verticillium dahliae* f. *angustum* Wollenw.) J.F.H. Beyma, *Verticillium dahliae* f. *cerebriforme* J.F.H. Beyma, *Verticillium dahliae* f. *chlamydosporale* (Wollenw.) J.F.H. Beyma, *Verticillium dahliae* f. *medium* (Wollenw.) J.F.H. Beyma, *Verticillium dahliae* f. *dahliae* Kleb., *Verticillium dahliae* f. *zonatum* J.F.H. Beyma, *Verticillium ovatum*

G.H. Berk. & A.B. Jacks., *Verticillium tracheiphilum* Curzi,
Taxonomic position: Fungi, Ascomycota, Pezizomycotina, Sordariomycetes, Glomerellales, Plectosphaerellaceae.
EPPO Code: VERTDH (*V. dahliae*)
Phytosanitary categorization: EPPO A2 list; EU Annex IV (RNQP)

3. Detection

3.1. Commodities concerned

Both fungi can be spread by planting material and possibly also in associated soil. The fungi are not associated with those parts of the crop normally traded for use in the brewing industry, i.e. the dried flowers (cones) and various types of extract made from them. They may occur in field harvest waste, but this is usually disposed of locally, at worst leading to only short-distance spread.

Fig. 2 Yellowing and wilting of leaves caused by Verticillium wilt on hop. Courtesy of S. Radišek.

3.2. Disease symptoms

The disease on hops appears in mild or lethal forms, depending on the pathogen virulence, the sensitivity of the cultivars and ecological factors. Disease symptoms that are known in both disease forms include:

(1) Yellowing and wilting of leaves, which is initiated at the base of the bine, and extends upwards. Leaf discoloration starts between the main veins and is followed by marginal and interveinal necrosis (Fig. 2). The margins of the leaves turn upwards and the affected leaves drop off very easily.
(2) Affected bines, up to 1.5 m from the base, become swollen and show rough epidermis and brown discolouration of the vascular tissue. In contrast to wilting due to Fusarium canker, affected bines do not develop a restricted "neck" at the base and plants remain firmly anchored.

3.2.1. Mild wilt

This disease form arises from infections of sensitive hop cultivars by less virulent isolates of *V. nonalfalfae* or *V. dahliae* or from infection of tolerant/resistant cultivars by highly virulent *V. nonalfalfae* isolates. Mild wilt varies in intensity from season to season and rarely causes plant death. Plants affected in one season may thus look healthy the next season. Affected plants are usually scattered in distribution through the hop garden and the disease incidence is associated with excessive soil moisture. The first symptoms on leaves appear in late July or early August, and only some of the bines are affected (Fig. 3). The lateral shoots arising from the axial of affected leaves frequently show no symptoms. A considerable thickening of the bines usually occurs (Fig. 3), and the browning of the vascular tissue is limited to the centre (Fig. 4).

Fig. 3 Mild form of Verticillium wilt on hop: affected swollen and normal bine intertwined. Courtesy of S. Radišek.

3.2.2. Lethal wilt

This disease form arises from infections of sensitive hop cultivars by highly virulent isolates of *V. nonalfalfae*. In contrast to the mild form, lethal outbreaks exhibit extensive and rapid dieback of leaves and lateral shoots (Fig. 5), eventually leading to the death of the entire rootstock. Bines rarely become swollen and brown discoloration of

Fig. 4 Transverse (left) and longitudinal (right) sections of hop bine infected with Verticillium wilt: brown vascular tissue is limited to the centre, which is typical of the mild form of the disease. Courtesy of S. Radišek.

Fig. 5 Lethal form of Verticillium wilt on hop: extensive and rapid dieback of leaves and lateral shoots. Courtesy of S. Radišek.

the whole vascular tissue (Fig. 6) is evident from an early stage. Affected plants that survive the following winter often put forth only a few weak bines the next season, and these soon (beginning of June) develop symptoms and die. Plants that are infected during the season usually show the first symptoms at the stage of forming cones. In 2–3 weeks all leaves are dead and usually fallen while the cones stay and wither with the bine. The disease incidence is less influenced by ecological factors and its spread in hop gardens is related to the cultivation pattern.

3.3. Detection in plants

As root-invading pathogens, *V. nonalfalfae* and *V. dahliae* may be located from the roots upwards in the vascular system. Cross-sectional cutting of the stem will often reveal browning of the tissue, even when external symptoms are not apparent. Hop is most frequently propagated by cuttings which are obtained from the rhizomes of the hop plant during the dormant season in autumn or early spring. No external symptoms are apparent on cuttings, so visual inspections and diagnostic procedures are best applied to the parental material prior to propagation.

Because of the high number of cuttings produced from an individual hop garden, only random sampling (in Slovenia up to 3% of the cuttings) is possible. Cross-sectioning of the cuttings may reveal brown vascular tissue, which is caused by both *Verticillium* species. Similar discolouration of vascular tissues can also be induced by *Fusarium* sp., *Phytophthora citricola* species complex or *Pseudoperonospora humuli.* Unlike *Fusarium* canker, the aerial parts of the plant affected by Verticillium wilt remain firmly anchored. Isolation of the fungus and identification by morphology and molecular means is therefore required for confirmation.

3.4. Detection in soil

A soil test based on sieving and wet or dry plating on semi-selective medium can be used for detecting *V. dahliae* but since this species is relatively unimportant in hops, it is not used in conjunction with this crop. Several PCR-based tests have been developed (Debode *et al.*, 2011; Bilodeau *et al.*, 2012, 2012; Tzelepis *et al.*, 2017 and others) and can be used to detect and quantify *V. dahliae* inoculum in soil. No effective soil test is available for *V. nonalfalfae.*

3.5. Isolation

Isolation of the fungus is performed by plating of infected material on prune lactose yeast agar (PLYA) or potato dextrose agar (PDA) Appendix 1. A section of hop bine, about 10 cm in length, is taken from just above ground level. The segment is surface sterilized and the epidermis is then

Fig. 6 Longitudinal section of hop bine infected with Verticillium wilt (left): brown discoloration of whole vascular tissue, which is typical of the lethal form of the disease. Longitudinal section of bine from healthy plant (right). Courtesy of S. Radišek.

peeled away from a short section using a sterile scalpel. A wedge-shaped segment is cut to the middle of the stem. This is excised and placed onto a Petri dish containing agar media (see Appendix 1). The procedure is repeated for the other half of the stem so that an entire cross-section is covered. As the fungus may be localized within the stem, it may be necessary to take a duplicate sample from elsewhere on the section of bine. Plates should be incubated at 20°C in the dark for up to 2 weeks.

3.5.1. Culture description

Verticillium forms white, fluffy mycelium, which appears after an incubation period of 3–5 days.

3.5.1.1. V. nonalfalfae (descriptions are from Inderbitzin et al., 2011). Colonies reach 35–55 mm in 2 weeks on PDA. Colonies are initially white, then darkening due to the formation of resting mycelium. Aerial mycelium is usually abundant (Fig. 7).

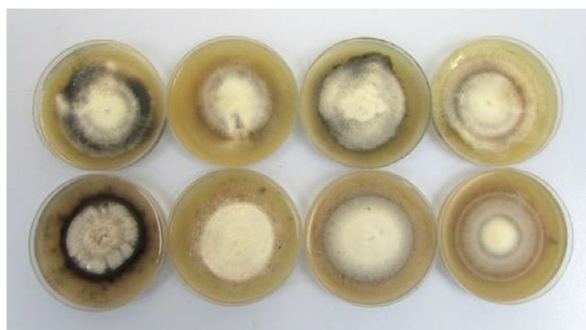

Fig. 7 Four-week-old colonies of *V. nonalfalfae* (upper line) and *V. dahliae* (bottom line) on PLYA. Courtesy of S. Radišek.

3.5.1.2. V. dahliae (descriptions are from Inderbitzin et al.,2011). Colonies reach 40–60 mm in 2 weeks on PDA. Colonies are initially white, then becoming dark due to the formation of microsclerotia. Aerial mycelium is abundant (Fig. 7).

3.5.2. Possible confusion with other species

Isolates growing from within infected hop tissue and producing either dark resting mycelium (*V. nonalfalfae*) or microsclerotia (*V. dahliae*) are unlikely to be confused with other *Verticillium* species except possibly for the weak pathogen *V. tricorpus*. The uncommon *V. tricorpus* produces both resting structures and chlamydospores as well as a copious yellow-orange pigment, clearly visible in young colonies and less intense after long culturing. The genus *Verticillium* includes seven other plant pathogenic species which are associated with other hosts and not relevant for Verticillium wilt in hop. An overview of main morphological characters of hop infecting *Verticillium* species is presented in Table 1 (based on Hawksworth and Talboys, 1970a,1970b; Hawksworth, 1970; Inderbitzin *et al.*, 2011).

4. Identification

Identification of *Verticillium* species based on morphological characters is possible provided that clearly defined, properly maintained and stable reference material is available for comparison. However, in atypical isolates the resting structures important for diagnostics may be absent or lost, leading to misidentification. Consequently, molecular identification is highly recommended for reliable species identification and confirmation of morphological identification. For a positive identification, the fungi should be identified unambiguously by any of the following methods:

Table 1. Morphological descriptions of hop infecting *Verticillium* species[a]

	V. nonalfalfae	*V. dahliae*	*V. tricorpus*[b]
Mycelium[c]			
Colour	Greyish black	Black	Golden black
Hyaline sectors	+	+	+
Resting mycelium (μm)	Up to 9	–	3.5–7
Microsclerotia (μm)	–	Solitary 25–100, aggregates up to 200	60–85
Chlamydospores (μm)	–	–	7.5–11, solitary or in chains
Conidiophores			
Colour	Hyaline	Hyaline	Hyaline
Number of phialides	(1–) 2–5 (–7)	(1–) 2–4 (–6)	3–4
Phialide size (μm)	Terminal 40–60, lateral 30–45	Terminal 40–60, lateral 25–50	12–25 × 2–3
Conidia			
Shape and colour	Hyaline, cylindrical to oval, single-celled	Hyaline, cylindrical to oval, single-celled	Hyaline, cylindrical to oval, single-celled
Size	(4.0–) 6.0 (–10.5) × (2.5–) 3.0 (–3.5)	(3.5–) 6.5 (–13.5) × (2.0–) 3.0 (–4.5)	3.5–10 × 1.5–3.5
Host (*Humulus lupulus*)	+	+	+
Pathogenicity	High	High	Low
Soil saprophyte	–	–	+

[a]Presence (+); absence (–).
[b]Colonies of *V. tricorpus* show a typical yellowing as soon as the colonies become visible (usually within 1 week) which is not the case with *V. nonalfalfae* and *V. dahliae* where either resting mycelium or microsclerotia form in 1–2 weeks.
[c]Description of cultures growing on (PDA) at 20°C for 2–3 weeks in the dark.

- isolation and morphological analysis (Appendix 1, Table 1) confirmed by conventional PCR (Appendix 2);
- conventional PCR (Appendix 2);
- sequencing (Appendix 3);
- real-time PCR (Appendix 4);
- conventional PCR for the identification of *V. nonalfalfae* pathotypes (Appendix 5).

4.1. Morphological identification

The most conspicuous character of *Verticillium* is verticilliate conidiophores (Fig. 8), which can be observed under a dissecting microscope. Further identification is based on formation of resting structures, macroscopically visible as a darkening of the cultures after 1–2 weeks of incubation (Fig. 7). Examination of darkened cultures under microscope (100× magnification), either *in situ* or squashed onto a slide, should reveal the nature of the resting structures: resting mycelia in the case of *V. nonalfalfae* (Fig. 9) and microsclerotia in the case of *V. dahliae* (Fig. 10).

4.1.1 Species description

V. nonalfalfae (from Inderbitzin *et al.,* 2011) Hyphae 1.5–3 µm, conidiophores erect, branched or unbranched, phialides arranged in 2–6 whorls along conidiophores, usually 2–5 phialides in each whorl. Resting mycelium is immersed in agar and consists of brown pigmented thick-walled hyphae up to 9 µm wide, straight or curved, arranged solitary or in aggregates (Fig. 9). Microsclerotia are absent. *V. nonalfalfae* is morphologically indistinguishable from *V. alfalfae* but differs in host range and DNA characters.

V. dahliae (from Inderbitzin *et al.,* 2011) Hyphae 2–4 µm wide, conidiophores erect, branched or unbranched, phialides arranged in 2–3 whorls along conidiophores, usually 2–4 phialides per whorl. Microsclerotia, composed of round, brown pigmented cells (a single cell can be up to 13 µm wide), are immersed in agar and distributed throughout the colonies, solitary or in aggregates (Fig. 10). Morphological characteristics of *V. dahliae* are similar to *V. longisporum*, apart from the smaller size of conidia.

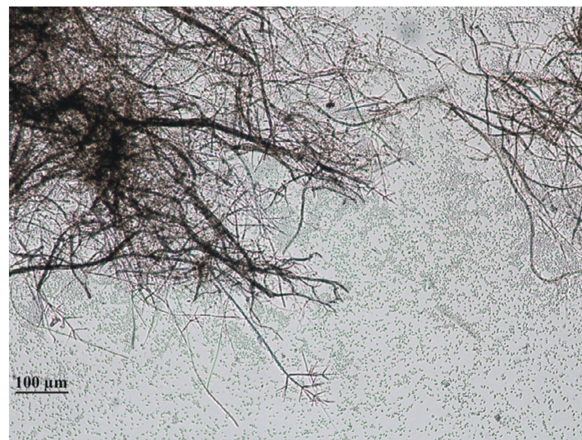

Fig. 9 Dark resting mycelium of *V. nonalfalfae*. Courtesy of S. Radišek.

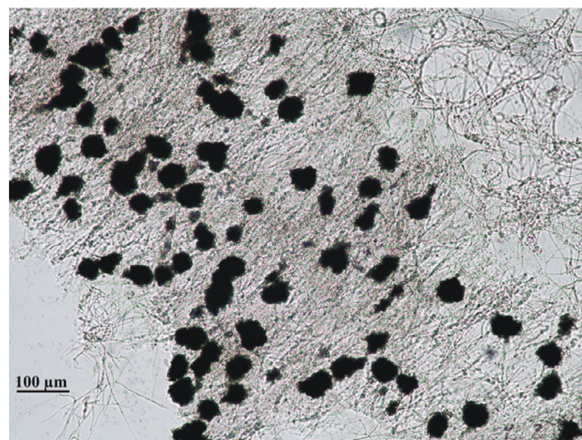

Fig. 10 Microsclerotia of *V. dahliae*. Courtesy of S. Radišek.

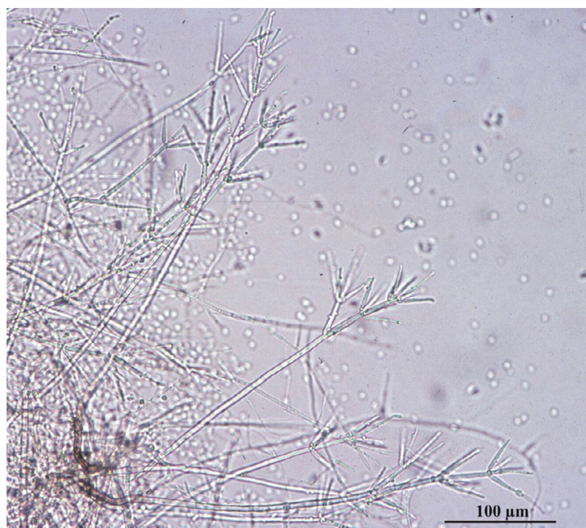

Fig. 8 Verticillate conidiophores of *V. nonalfalfae*. Courtesy of S. Radišek.

Both species are variable in culture and are often prone to sectoring (Fig. 7). Cultures may be maintained by repeated subculturing on PLYA or PDA but this may lead to loss of diagnostic resting structures (hyaline forms). They are best stored as spore suspensions in 20% glycerol (preferably dispersed on 3-mm ceramic craft beads for easy recovery) at ultra-low temperatures.

4.1.2 Possible confusion with other species

An overview of the main morphological characters of hop infecting *Verticillium* species is presented in Table 1 (based on Hawksworth and Talboys, 1970a,1970b; Hawksworth, 1970; Inderbitzin *et al.*, 2011).

4.2 Molecular methods

Due to their different host ranges and pathogenicity, plant pathogenic *Verticillium* species have been subjected to extensive molecular research which has resulted in numerous PCR-based tests. Reliable PCR primers for species identification were developed by Carder *et al.* (1994), Nazar *et al.* (1991), Robb *et al.* (1993) and recently Inderbitzin *et al.* (2013). This Standard recommends primers and PCR tests developed by Inderbitzin *et al.* (2013) and Maurer *et al.* (2013), Appendices 2 and 4. Conventional PCR developed by Inderbitzin *et al.* (2013) requires DNA isolation from fungal pure culture. The test has been validated with all recognized plant pathogenic *Verticillium* species. The real-time PCR test developed by Maurer *et al.* (2013) was recognized as highly reliable, specific and sensitive as well as less time-consuming since the DNA isolation step is performed directly from hop bines.

In addition to species identification, identification of *V. nonalfalfae* strains (pathotypes) is important. Jakše, Štajner and Radišek, (2018, (unpublished) developed the first diagnostic primers which can differentiate lethal from mild pathotypes regardless of the geographical origin (Appendix 5). Before that Radišek *et al.* (2004) developed primers which are specific only for the lethal *V. nonalfalfae* pathotype from Slovenia. The 'universal' primers ITS1 and ITS4 (which amplify the ribosomal RNA gene internal transcribed spacer regions; rRNA ITS) (White *et al.*, 1990) should be used to test the quality of DNA extracts. Additionally, if sequenced, the amplicons from this last primer pair can be used to identify *V. nonalfalfae* and *V. dahliae* (Appendix 3).

4.3 Pathogenicity and virulence tests

Virulence testing is performed by artificial inoculation of hop cultivars (cv.) of known resistance (tolerance). These tests are important in the case of new outbreaks to establish proper disease management strategies. As an inoculation method, root dipping in a conidial suspension (Radišek *et al.*, 2006; Flajšman *et al.*, 2017) or amending the soil with colonized straw or hop bines could be used (Sewell & Wilson, 1984; Clarkson & Heale, 1985). Plants can then be assessed for visible symptoms throughout the growing period. Infections have to be confirmed by re-isolation of the pathogen. To avoid differences in environmental conditions, virulence testing should be performed in a growth chamber or glasshouse. For standardization, the pathotype nomenclature of *V. nonalfalfae* isolates that was established in England (Sewell & Wilson, 1984; Clarkson & Heale, 1985) should be used:

- mild pathotype (M): isolates that cause mild wilt on susceptible cv. Fuggle;
- lethal pathotype (PV1): isolates that cause lethal wilt on susceptible cv. Fuggle;
- lethal pathotype (PV2): isolates that cause lethal wilt on moderately resistant cv. Wye Challenger;
- lethal pathotype (PV3): isolates that cause lethal wilt on resistant cultivar Wye Target.

In addition, rapid virulence characterization of *V. nonalfalfae* pathotypes has been developed for all lethal pathotypes (Jakše, Štajner and Radišek, 2018, unpublished), and for pathotype PV1 (genotype PG2) from Slovenia, by using a PCR test and specific primers (Radišek *et al.*, 2004).

5. Reference material

Centraalbureau voor Schimmelcultures (CBS), Uppsalalaan 8, 3584, CT Utrecht, Netherlands (*V. nonalfalfae* CBS 130339; *V. dahliae* CBS 130341)
NRRL, ARS Culture Collection, National Center for Agricultural Utilization Research 1815 N. University Street, Peoria, USA (*V. nonalfalfae* NRRL 54791, *V. dahliae* NRRL 54785).

6. Reporting and Documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 (1) *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 that this database is consulted 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 S. Radišek, Slovenian Institute for Hop Research and Brewing, Plant Pathology Department, Cesta Žalskega tabora 2, 3310 Žalec, Slovenia; sebastjan.radisek@ihps.si.

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 review process is in place to identify the need for revision of diagnostic protocols (every 18 months for pest specific protocols and every year for horizontal Standards). Standards 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.

11. Acknowledgements

This protocol was originally drafted by Dr G. Down, Disease Management, Horticulture Research International, East Malling, West Malling, Kent (GB); Dr D. Barbara, Sustainable Disease Resistance, Horticulture Research International, Wellesbourne, Warwick (GB); and Dr S. Radišek, Slovenian Institute for Hop Research and Brewing, Žalec (SI). This revision was prepared by Dr A. Munda, Agricultural Institute of Slovenia (KIS), Hacquetova ulica 17, Ljubljana and Dr S. Radišek. Dr E. Seigner, Bavarian State Research Center for Agriculture, Freising, (DE), provided information for the real-time PCR protocol. Dr J. Jakše and Dr N. Štajner, University of Ljubljana, Biotechnical Faculty (SI), developed primers for identification of *V. nonalfalfae* pathotypes.

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Appendix 1 – Media

Prune lactose yeast agar

Agar	20 g
Prune extract	100 mL
Lactose	5 g
Yeast extract	1 g
Distilled water	900 mL

Preparation of prune extract requires 50 g of prunes and 1000 mL of distilled water. Chop and stone the prunes, boil for 1 hour in a saucepan with the lid on, strain through muslin, filter and dispense in 100 or 200 mL lots. For storage, autoclave at 121°C for 20 min. More simply, ready-to-use pure prune juice for food consumption can be purchased from supermarkets, diluted 1:4 in water, dispensed and autoclaved for storage. To prepare prune lactose yeast agar (PLYA), add 100 mL of prune extract to distilled water in a beaker, add the remaining ingredients and stir. Heat in a microwave for 5 min, stir, then continue to heat until the agar has dissolved. Dispense and sterilize by autoclaving at 121°C for 20 min.

Potato dextrose agar (PDA): Commercially available.

Appendix 2 – Identification of *V. nonalfalfae* and *V. dahliae* by conventional PCR (Inderbitzin *et al.*, 2013)

1 General information

1.1. Conventional PCR is used for identification of *V. nonalfalfae* and *V. dahliae* from fungal culture.
1.2. The protocol was established in 2013 by Inderbitzin *et al.* (2013).
1.3. Nucleic acid source: mycelium and conidia from fungal pure culture.
1.4. The PCR primers for *V. dahliae* are selected in the ITS region of the rDNA gene. Sequences of the ITS region for *V. dahliae* and tryptophan synthase (TS) gene for *V. nonalfalfae* may be retrieved from Genbank, accession numbers HQ206718 and JN188035, respectively.
1.5. The PCR primers for *V. nonalfalfae* produce an amplicon of 1310 bp and those for *V. dahliae* are 490 bp.
1.6. Oligonucleotides:

V. nonalfalfae		
Forward primer	NoF	5′-CCT CGA AAA ATC CAC CAG CTC TA-3′
Reverse primer	NoNuR	5′-GTG GTT GAG ATC CTCA CGC TTC-3′
V. dahliae		
Forward primer	Df	5′-CCG GTC CAT CAG TCT CTC TG-3′
Reverse primer	Dr	5′-CTG TTG CCG CTT CAC TCG-3′

1.7. PCR reactions can be performed in any thermocycler with a heated lid.

2. Method

2.1. Nucleic acid extraction
2.1.1. DNA is extracted from mycelium scraped from a 1 cm^2 plug taken from a culture of the fungus or from mycelium growing on cellophane membranes placed on PDA or PLYA plates. The sample is ground in a mortar by a pestle with liquid nitrogen or another efficient grinding technique.
2.1.2 Nucleic acid extraction: DNA is extracted with the hexadecyltrimethylammonium bromide (CTAB) method (Kump & Javornik, 1996) or with the sodium dodecyl sulphate (SDS) method of Lee & Taylor (1990). Commercial DNA extraction kits with protocols for filamentous fungi are also suitable.
2.1.3. Extracted DNA is used immediately or stored at –20°C.
2.1.4. Before PCR amplification it is advisable to measure the concentration and purity of extracted DNA by using DNA fluorimeters or spectrophotometers.
2.2. Polymerase chain reaction
2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	NA	5.7	NA
5 × reaction buffer (Promega)	5×	4.0	1×
$MgCl_2$	25 mM	1.6	2.0 mM
Forward primer	10 µM	1.0	0.5 µM
Reverse primer	10 µM	1.0	0.5 µM
dNTPs	2.5 mM each	1.6	0.2 mM each
DNA polymerase (Promega)	5 U/µL	0.1	0.5 U
Subtotal		15	
DNA extract (dilution 1:10)		5	(optimal 20 ng)
Total		20	

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

2.2.2. PCR conditions
The PCR reaction includes initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation (94°C, 30 s), annealing (64°C, 30 s for *V. nonalfalfae* and 67°C, 30 s for *V. dahliae*) and extension (72°C, 60 s). A final extension is carried out at 72°C for 10 min.

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 isolation 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 of clean extraction buffer or molecular-grade water.
- **Positive isolation control (PIC)** to ensure that nucleic acid of sufficient quality and quantity is isolated: nucleic acid extraction and subsequent amplification of the target organisms.
- **Negative amplification control (NAC)** to rule out false positives due to contamination during the preparation of the reaction mix: amplification of PCR-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 organisms. This can include nucleic acid extracted from the target organisms, whole-genome amplified DNA or a synthetic control (cloned PCR product). The PAC should preferably be near the limit of detection of the method.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Before target-specific PCR, DNA samples should be subjected to PCR amplification with universal fungal ribosomal genes primers ITS1/ITS4 (White *et al.*, 1990) (Appendix 3). In the case of a negative PCR signal the sample is not valid for target-specific PCR.

3.2. Interpretation of results

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 IC if used) should produce amplicons of expected size (1310 bp for *V. nonalfalfae* and 490 bp for *V. dahliae*).

When these conditions are met

- A test will be considered positive if an amplicon of expected size (1310 bp for *V. nonalfalfae* and 490 bp for *V. dahliae*) is 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

Data from Inderbitzin *et al.* (2013).

4.1. Analytical sensitivity data

Analytical sensitivity (detection limit) is 1 pg of DNA per reaction.

4.2. Analytical specificity data

Verticillium nonalfalfae test

The inclusivity of the test was evaluated on 9 *V. nonalfalfae* isolates (Inderbitzin *et al.*, 2013) and additionally on 50 *V. nonalfalfae* isolates from the culture collection of the Slovenian Institute of Hop Research (Radišek *et al.*, unpublished).

The exclusivity of the test was evaluated on isolates of the following fungal species: *Gibellulopsis nigrescens*, *Musicillium theobromae*, *V. albo-atrum*, *V. alfalfae, V. isaacii*, *V. klebahnii*, *V. longisporum lineage A1/D1*, *V. longisporum lineage A1/D2*, *V. longisporum lineage A1/D3*, *V. nonalfalfae*, *V. nubilum*, *V. tricorpus* and *V. zaregamsianum* (Inderbitzin *et al.*, 2013)

Verticillium dahliae test

The inclusivity of the test was evaluated on 6 *V. dahliae* isolates (Inderbitzin *et al.*, 2013) and additionally on 12 *V. dahliae* isolates from the culture collection of the Slovenian Institute of Hop Research (Radišek *et al.*, unpublished).

The exclusivity of the test was evaluated on isolates of the following fungal species: *Gibellulopsis nigrescens*, *Musicillium theobromae*, *V. albo-atrum*, *V. alfalfae*, *V. isaacii*, *V. klebahnii*, *V. longisporum lineage A1/D1*, *V. longisporum lineage A1/D2*, *V. longisporum lineage A1/D3*, *V. nonalfalfae*, *V. nubilum*, *V. tricorpus* and *V. zaregamsianum* (Inderbitzin *et al.*, 2013)

Appendix 3 – Identification of *V. nonalfalfae* and *V. dahliae* by sequencing the internal transcribed spacer 1 and 2 of the nuclear ribosomal gene

1 General information

1.1. The target region is the internal transcribed spacer (ITS) region 1 and 2 of the fungal ribosomal RNA gene.
1.2. Nucleic acid source: mycelium and conidia.
1.3. Amplicon location: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2; and 28S ribosomal RNA gene, partial sequence.
1.4. Oligonucleotides (White *et al.*, 1990):

Forward primer	ITS1	5′-TCC GTA GGT GAA CCT GCG G-3′
Reverse primer	ITS4	5′-TCC TCC GCT TAT TGA TAT GC-3′

1.5. PCR reactions can be performed in any thermocycler with a heated lid.

2 Method

2.1. Nucleic acid extraction
2.1.1. DNA is extracted from mycelium scraped from a 1 cm^2 plug taken from a culture of the fungus or from mycelium growing on cellophane membranes placed on PDA or PLYA plates. The sample is ground in a mortar by a pestle with liquid nitrogen or another efficient grinding technique.
2.1.2. Nucleic acid extraction: DNA is extracted with the hexadecyltrimethylammonium bromide (CTAB) method (Kump & Javornik, 1996) or with the sodium dodecyl sulphate (SDS) method of Lee & Taylor (1990). Commercial DNA extraction kits with protocols for filamentous fungi are also suitable.
2.1.3. Extracted DNA is used immediately or stored at –20°C.
2.1.4. Before PCR it is advisable to measure the concentration and purity of extracted DNA by using DNA fluorimeters or spectrophotometers.
2.2. Polymerase chain reaction
2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	21.75	N.A.
5× reaction buffer (Promega)	5×	10.0	1×
$MgCl_2$	25 mM	4.0	2.0 mM
Primer ITS1	10 µM	2.5	0.5 µM
Primer ITS4	10 µM	2.5	0.5 µM
dNTPs	2.5 mM each	4.0	0.2 mM each
DNA polymerase (Promega)	5 U/µL	0.25	0.025 U/µL
Subtotal		45	
DNA extract (dilution 1:10)		5	(optimal 20 ng)
Total		50	

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

2.2.2. PCR conditions
The PCR reaction includes initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s) and extension (72°C, 60 s). A final extension is carried out at 72°C for 10 min.
2.2.3. Sequencing of amplicons
Run 5 µL of the amplified mixture on a 1.5% agarose gel to check for positive test reactions. Purify the remaining 45 µL from positive test reactions using a suitable PCR purification kit such as a QIAquick PCR purification kit (Qiagen, Cat. no. 28106) following the manufacturer's instructions. Sequencing with forward primer ITS1 and reverse primer ITS4.

3 Essential procedural information

Compare consensus sequences for the test sample with reference sequences (accession numbers JN187973, KT223526, NR126130.1 for *V. nonalfalfae* and NR_126124.1, HQ206718, HE972023 for *V. dahliae*) in NCBI database GenBank (http://www.ncbi.nlm.nih.gov). For the preparation and analysis of consensus sequence see Appendix 7 of PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests*.

4 Performance characteristics available

None available.

Appendix 4 – Identification of *V. nonalfalfae* and *V. dahliae* by real-time PCR (Maurer *et al.*, 2013)

1 General information

1.1. This multiplex real-time PCR method is used for simultaneous qualitative identification of *V. nonalfalfae* and *V. dahliae* directly from hop bine samples.
1.2. The protocol for the duplex TaqMan® based real-time PCR was published in 2013 by K.A. Maurer, S. Radišek, G. Berg and S. Seefelder.
1.3. The PCR primers and hydrolysis (TaqMan®) probes were designed using the software Beacon Designer™ 7 (Premier Biosoft, Palo Alto, CA, USA) based on primer sequences published by Carder *et al.* (1994) to differentiate *V. nonalfalfae* (formerly *V. albo-atrum*) and *V. dahliae*.
1.4. The PCR primers produce an amplicon of 150 bp for *V. nonalfalfae* and 123 bp for *V. dahliae.*
1.5. Oligonucleotides

V. nonalfalfae		
Forward primer	Vaa_f	5′-GGC TTT TGC TTT CTC TTG-3′
Reverse primer	Vaa_r	5′-GAC CAA ATG TAA TTG TCC AG-3′
Probe	Vaa_probe	5′-FAM-CGG CTA CGG CTC ATG CTA AC-BHQ1-3′
V. dahliae		
Forward primer	Vd_f	5′-GGC TCA AGT TAA CTA CGG-3′
Reverse primer	Vd_r	5′-CTG TCA TGT ATA TAA GAT ACT ACT G-3′
Probe	Vd_probe	5′-Cy5-AGG TAT AAG GTC CAT ATC CAA CAC GAG-BHQ2-3′

1.6. PCR reactions and analyses can be carried out in a real-time PCR detection system (e.g. CFX96™ Real-time detection system in combination with the manufacturer´s CFX Manager™ software, BioRad, Hercules, CA, USA).

2 Methods

2.1. Nucleic acid extraction and purification

2.1.1. Tissues source: plant material
From each plant to be tested at least three bines (symptomatic or asymptomatic hop plants), preferably near-base sections of a bine, should be tested individually. The bark of the hop bine is removed and then the xylem is excised and cut into small pieces. Then 100 mg of fresh tissue and 400 µL of lysis buffer (from the nucleic acid extraction kit) are added to a 2-mL tube containing 180 mg of granite matrix and two 6-mm ceramic beads. The plant tissue is ground in a homogenizer (e.g. using FastPrep 24® homogenizer, MP Biomedicals, Santa Ana, CA, USA) at 5.5 m s^{-1} for 40 s.
Different commercial kits are suitable and can be used for DNA isolation following the manufacturer's instructions (e.g. Invisorb® Spin Plant Mini Kit by Invitek, now Stratec Molecular, Berlin, Germany).
2.1.2. It is recommended that the concentration and quality of extracted DNA are determined with a spectrometer.
2.1.3. The samples are used immediately or stored at −20°C.
2.2. Real-time polymerase chain reaction
2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	NA	3.8	NA
Real-time PCR mix including buffer system, $MgCl_2$, PCR enhancer, dNTPs and DNA polymerase (e.g. SensiFAST™ Probe No-ROX Kit, Bioline)**	2×	10	1×
Forward primer 1 (Vaa_f)	10 µM	0.6	300 nM
Reverse primer 1 (Vaa_r)	10 µM	0.6	300 nM
Probe 1 (Vaa_probe)	10 µM	0.4	200 nM
Forward primer 2 (Vd_f)	10 µM	0.6	300 nM
Reverse primer 2 (Vd_r)	10 µM	0.6	300 nM
Probe 2 (Vd_probe)	10 µM	0.4	200 nM
Subtotal		17	
DNA extract (dilution 1:10)		3	(optimal 20 ng)
Total		20	

*Molecular-grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.
**The SensiFAST™ Probe No-ROX Kit from Bioline, Luckenwalde, Germany, is an alternative to SsoFast TM Probes Supermix from BioRad used by Maurer *et al.* (2013), which is no longer available.

2.2.2. PCR conditions
Real-time PCR amplifications include an initial activation step of the polymerase at 95°C for 2 min followed by 39 cycles of denaturation at 95°C for 5 s and annealing/extension at 61°C for 30 s. At the end of each cycle the fluorescence of the respective reporter dye (FAM and Cy5) is monitored; the threshold line for each run is set automatically.

3 Essential Procedural Information

3.1. Controls
Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue 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 nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near the detection limit of the method.

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

- To confirm the effectiveness of isolation of each sample and to prevent false negatives Maurer *et al.* (2013) performed a specific PCR with the primer pair 5′-ACC ATC ACT TTG GAG GTG GA-3′ (forward primer) and 5′-GAG ACG GAG GAC AAG GTG AA-3′ (reverse primer) for polyubiquitin (Oberhollenzer, 2012). The 186-bp amplicon would indicate the presence of plant DNA.
- Alternatively, as internal positive control specific co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample can be included (e.g. plant cytochrome oxidase [COX] gene or eukaryotic 18S rDNA). Validation of a triplex real-time assay with the simultaneous detection of *V. nonalfalfae* and *V. dahliae* and the plant COX gene as IPC (Weller *et al.*, 2000) is currently in progress.

3.2. Interpretation of results
Verification of the controls

- The PIC, PAC and IPC (as well as other applicable controls) 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.
- Testing of each sample should be conducted twice and if contradictory or unclear results are obtained, the test should be repeated.

4 Performance characteristics available

The validation was not carried out in accordance with PM 7/98.

4.1. Analytical sensitivity data:

The analytical sensitivity (limit of detection) of the TaqMan® assay as determined by Maurer *et al.* (2013) for detecting *V. nonalfalfae* DNA is 0.05 pg and for *V. dahliae* is 0.15 pg.

4.2. Analytical specificity data

Inclusivity was evaluated with a reference collection of 23 different *V. nonalfalfae* isolates and six *V. dahliae* isolates. Inclusivity was 100%.

Exclusivity was evaluated by Maurer *et al.* (2013) with 27 non-target microorganisms (17 fungi and 10 bacteria) isolated from healthy hop bines. No cross-reaction was noted.

Comments

In general, no amplification curve is seen for healthy hop plants and they are negative. However, for samples producing an amplification curve with high Ct values it is difficult to determine whether these samples are infected at a low level or if these are "false positives". Amplification with Ct values (quantification cycle = Ct for threshold cycle as used by Maurer *et al.*, 2013) up to 32 (under the conditions given in the Institute for Crop Science and Plant Breeding, Bavarian State Research Centre for Agriculture, Freising) can be verified reproducibly by conventional PCR (see Appendix 2) and thus the result is interpreted as positive. To clarify the result of samples with Ct values higher than 32, the product of conventional PCR should be sequenced (see Appendix 3). If the result is positive the presence of *Verticillium* is confirmed. If conventional PCR does not produce an amplicon, the presence of *Verticillium* cannot be confirmed as the analytical sensitivity of the conventional PCR is lower than the analytical sensitivity of the real-time PCR. For the confirmation of the absence of *Verticillium* sp. all three bines tested should be negative.

The Ct values mentioned in this comment section were obtained by using the specific material, equipment and chemicals described here and should be re-checked and adapted in each laboratory when implementing this test.

Appendix 5 – Identification of *V. nonalfalfae* hop pathotypes based on Jakše, Štajner and Radišek, (2018, unpublished)

1 General information

1.1. This PCR test is used for identification of highly virulent *V. nonalfalfae* pathotypes that cause the lethal form of Verticillium wilt with rapid plant withering and dieback.

1.2. Nucleic acid source: mycelium and conidia from fungal pure culture.

1.3. The PCR primers were designed based on a genome sequence (Gene model.chr4_116292YY.801) of a lethal strain of *V. nonalfalfae* published by Jakše *et al.* (2018). Primers 4CHR4-F/4CHR4-R amplify 585 bp DNA product (GenBank accession number MH588400).

1.4. Oligonucleotides:

Forward primer	4CHR4-F	5′-CGA GGG CCC TTA TAC ATC AA-3′
Reverse primer	4CHR4-R	5′-CTA ATG AAG GCG GTG GGT AA-3′

1.5. PCR reactions and analyses can be carried out in any thermocycler with a heated lid.

1.6. Molecular-grade water is used for primer dilutions and in all reactions.

2 Methods

2.1. Nucleic acid extraction and purification (see Appendix 2)

2.2. Polymerase chain reaction

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water*	NA	5.7	NA
5× reaction buffer (Promega)	5×	4.0	1×
$MgCl_2$	25 mM	1.6	2.0 mM
Forward primer	10 μM	1.0	0.5 μM
Reverse primer	10 μM	1.0	0.5 μM
dNTPs	2.5 mM each	1.6	0.2 mM each
DNA polymerase (Promega)	5 U/μL	0.1	0.5 U
Subtotal		15	
DNA extract (dilution 1:10)		5	(optimal 20 ng)
Total		20	

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

2.2.2. PCR conditions

The PCR reaction includes initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation (94°C, 30 s), annealing (64°C, 30 s) and extension (72°C, 60 s). A final extension is carried out at 72°C for 10 min.

3 Essential procedural information

3.1. Controls

- **Negative isolation control (NIC)** to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of clean extraction buffer or molecular-grade water.
- **Positive isolation control (PIC)** to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a matrix sample that contains the target organism.
- **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 nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near the detection limit of the method.

Other possible controls

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

3.2. Interpretation of results

Verification of the controls

- NIC and NAC should give no amplification.
- PIC and PAC (and IC if used) should produce amplicon of expected size.

When these conditions are met

- A test will be considered positive if amplicon of expected size (585 bp) is 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

4.1. Analytical sensitivity data:

Not relevant.

4.2. Analytical specificity data:

The inclusivity was evaluated with DNA from 50 *V. nonalfalfae* isolates from hop from which 33 isolates present lethal strains and 17 isolates mild strains from different geographic origin. All *V. nonalfalfae* lethal hop isolates tested positive.

The exclusivity was evaluated on other non-target *Verticillium* species (*V. dahliae*, *V. tricorpus*, *V. isaacii*, *V. alfalfa*, *V. albo-atrum sensu stricto*, *V. nubilum* and *V. longisporum*). Two *V. dahliae* isolates from green pepper tested positive (Jakše, Štajner and Radišek, (2018, unpublished)).

Comments

In the case of lower annealing temperatures than 64°C, unspecific PCR fragments of higher size (>600 bp) can be observed in *V. nonalfalfae* mild pathotype (M) isolates. Since the primers amplify PCR products in few *V. dahliae* isolates, conventional PCR for species identification is prerequisite before *V. nonalfalfae* pathotype analysis.