DOI: 10.1111/epp.13001

EPPO STANDARD ON DIAGNOSTICS

PM 7/90 (2) *Anisogramma anomala*

Specific scope: This Standard describes a diagnostic protocol for *Anisogramma anomala*.[1] This Standard should be used in conjunction with PM 7/76 Use of EPPO diagnostic protocols.
Specific approval and amendment: Approved in 2009–09. Revision approved in 2024–03. Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Anisogramma anomala (Peck) E. Müller in E. Müller & Arx (Diaporthales) was first described by Peck as a pathogen of the native American hazelnut (also called American filbert), *Corylus americana* Walt. (Gottwald & Cameron, 1979). It is considered as an endemic pest of *C. americana*, but on the European (cultivated) hazelnut, *C. avellana* L., it causes eastern filbert blight, a devastating disease producing perennial cankers on limbs. Already reported as early as 1892 in the North-East USA (Halsted, 1892), the pathogen later caused serious problems on cultivated hazelnut in the North-West USA. Since its first detection in South-West Washington State in 1970 (Davison & Davidson, 1973), *A. anomala* has spread southwards into the Willamette Valley of Oregon and can now be found throughout the entire valley (OSU, 2023). This pathogen is distributed in Canada and the USA and information on its population structure (based on SSR markers) can be found in Cai et al. (2013). For an updated geographical distribution consult EPPO Global Database (EPPO, 2022). *A. anomala* has no known hosts other than *Corylus* spp.

Anisogramma anomala systematically colonizes the phloem, cambium and the outer xylem of branches and produces cankers after an incubation period of at least 14 months. The average growth of cankers in European hazelnut is 31 cm per year (Gottwald & Cameron, 1980), girdling branches, causing canopy dieback and death of trees (Johnson et al., 1996). The wild American hazelnut, *Corylus americana* Marshall, is generally resistant or highly tolerant of infection.

The pathogen infects actively growing shoots and buds from budbreak to early shoot elongation. It has the typical characteristics of an obligate, biotrophic parasite.

A flow diagram describing the diagnostic procedure for *A. anomala* is presented in Figure 1.

2 | IDENTITY

Name: *Anisogramma anomala* (Peck) E. Müller.
Synonyms: *Apioporthe anomala* (Peck) Höhn.
Cryptosporella anomala (Peck) Sacc.
Diatrype anomala Peck.
Cryptospora anomala (Peck) Ellis & Everh.
Taxonomic position: Fungi: *Ascomycota: Diaporthales.*
EPPO Code: CRSPAN.
Phytosanitary categorization: EPPO A1 list no. 201, EU A1 Quarantine Pest (Annex IIA).

3 | DETECTION

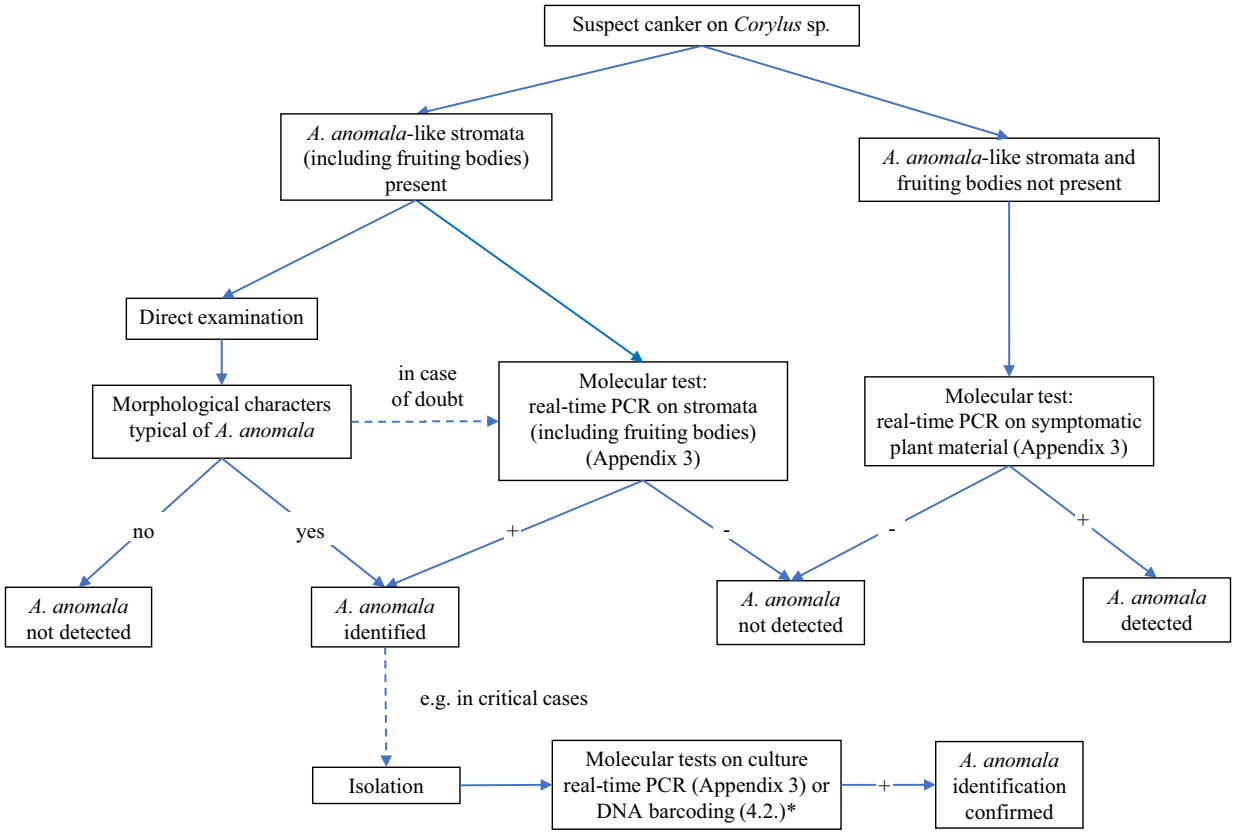
3.1 | Disease symptoms

Symptoms of *A. anomala* are very specific. The periods for observation of different symptoms given in this section are based on observations of the disease in Oregon state (USA).

Anisogramma anomala infects immature shoots in the spring following budbreak. Initial host invasion occurs by direct penetration of young epidermal cells by germinating ascospores and early establishment of intra-cellular hyphae (Pinkerton et al., 1995). Over the summer the pathogen colonizes cambium, phloem, and secondary xylem without producing visible symptoms. The latent infection period generally lasts 14–15 months but it can be longer (>24 months, https://web.engr.oregonstate.edu/~mendelse/EFB/lifecycle/lifecycle2.htm).

The first visible symptoms consist of bumps on branches, generally in late spring to early summer of the year following infection (Figure 2). As the cankers continue to develop, stromata emerge from the stems in the cankered area which is sunken due to cambium death (Gottwald & Cameron, 1979). First evident as whitish mycelial mats (Figure 3), they turn black in late summer-autumn (end of August–September) (Figure 4). This fungus produces multiple perithecia within a compact black stroma. The stroma is the main diagnostic character of the fungus. The lifecycle is completed in spring of the 2 years following the initial infection. Perennial

[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.



* If the real-time PCR was already used on plant material, barcoding should be performed.

FIGURE 1 Flow diagram describing the diagnostic procedure for *Anisogramma anomala*. This flow diagram is intended to provide an overview of the diagnostic process and may not cover all possible scenarios.

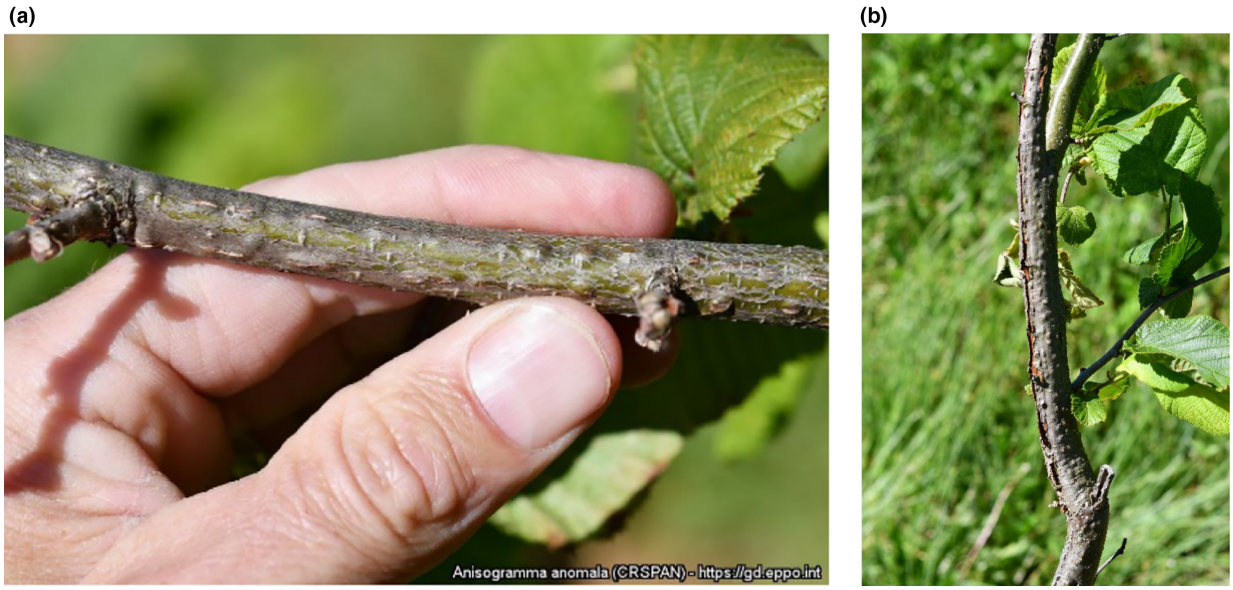


FIGURE 2 Very first symptoms in late spring or early summer the year following infection as rows of bumps along a branch. Courtesy T Molnar, Rutgers (US).

FIGURE 3 Immature stromata that have erupted through the bark on *Corylus avellana* 'Contorta' (courtesy Adams SM, The Morton Arboretum, Bugwood.org).

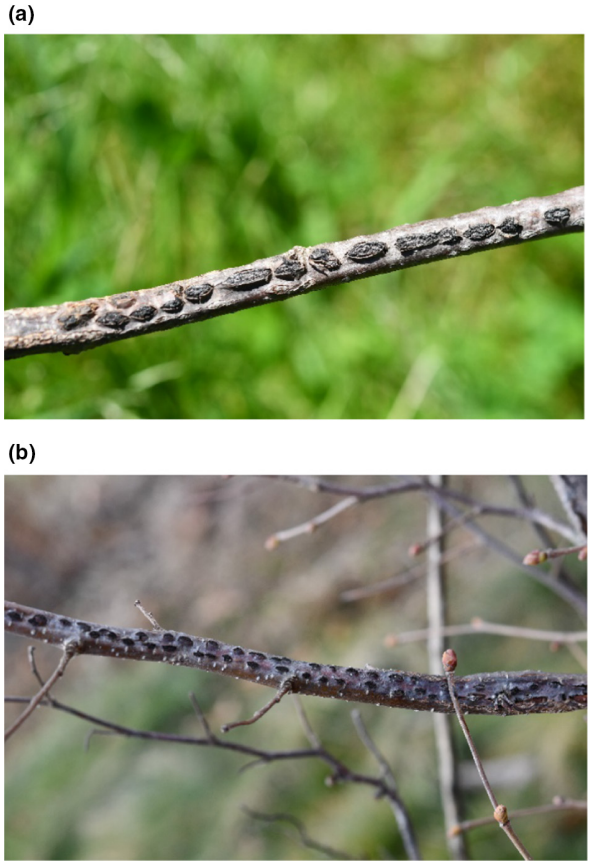


FIGURE 4 Mature, black stromata arranged in row(s) along a branch. Courtesy T Molnar, Rutgers (US).

cankers are formed (Figure 5) and expand laterally each year until branches are girdled over a period of 1–5 years. In susceptible cultivars of European hazelnut, *C. avellana*, such as Barcelona, Casina, Daviana, Ennis, Negret, Tonda Gentile Romana, and Tonda Gentile Trilobata (formerly named Tonda Gentile delle Langhe, TGL), canopy dieback occurs within 4–5 years in the absence of treatments (Iriti & Faoro, 2004; McCluskey et al., 2005; Pinkerton et al., 1993, 1998).

FIGURE 5 Severe Eastern filbert blight canker on susceptible *Corylus avellana*. Courtesy T Molnar, Rutgers (US).

On *C. avellana*, cankers contain up to 20 stromata (in single or double rows) in the first year (Figure 4) and hundreds of stromata (in three to five rows) from the second year on. Fungal spread and infection is mediated by ascospores (a conidial stage has not been observed). Spore maturation begins in late summer. Both the number of mature ascospores per perithecium and the proportion of ascospores that can germinate increase throughout autumn. By January, over 90% of the spores are morphologically mature. After January, the number of spores per perithecium decline until May, when few viable spores remain. The ascospores remaining after budbreak are the most important for infection, as this period coincides with the highest susceptibility to infection. Timing and amount of precipitation are the main variables affecting ascospore release (Pinkerton et al., 1998). Ascospores of *A. anomala* are released when stromata on the surface of hazelnut branches are wet due to rainfall, dew does not cause this release to occur. Release of ascospores ceases after branch surfaces dry. In eastern States of the USA, which have a different climate to Oregon, the ascospore release continues later into the growing season and allows for some later infection (T. Molnar, personal communication).

FIGURE 6 Eastern filbert blight on *Corylus americana*. Courtesy T. Molnar, Rutgers (US).

FIGURE 7 Mature erumpent black stromata of *A. anomala* ejecting ascospores (white globs) on top of the stromata. *Source*: JW Pscheidt, Oregon State University (US).

Corylus americana and *C. cornuta* var. *californica* (Beaked Hazelnut) are generally resistant or very tolerant to *A. anomala*. When exposed to the pathogen *C. americana* usually show no outward signs or symptoms of eastern filbert blight (Capik & Molnar, 2012; Revord et al., 2020). On occasion the fungus may cause an insignificant canker, measuring 1-10 cm in length (Figure 6).

3.2 | Direct examination

If symptoms of *A. anomala*, e.g., cankers with stromata and fruiting bodies (perithecia) (Figure 7), are observed on diseased parts of *C. avellana*, a preliminary diagnosis is possible by direct examination. Spores from fruiting bodies can be removed with the tip of a sterile needle and placed directly in a drop of distilled water on a microscope slide for examination under a compound microscope (×400, ×1000). Perithecial primordia generally appear in May and develop from the end of spring to the end of August–September.

3.3 | Molecular tests

A real-time PCR test targeting a ribosomal DNA internal transcribed spacer (Molnar et al., 2013; Appendix 3) can be used for the detection of *A. anomala* in symptomatic plant material. Thin slices of stem tissue approximately 1 cm long, 0.5 cm wide and 2 mm deep should be collected making sure to cut below bark into vascular tissue.

3.4 | Isolation

This fungus does not grow on standard mycological media. Methods for isolation, media for culturing and colony characteristics are presented in Appendices 1 and 2. Because the fungus grows very slowly and can only be isolated from fruiting bodies, isolation is not recommended as a method for detection, but it can be useful for confirmation in critical cases.

4 | IDENTIFICATION

4.1 | Morphological identification

Identification is based on morphological characters in vivo. The pest cannot be cultured from infested tissues without sporulating perithecia. The morphological characters of this fungus are very specific and confusion with other fungi is very unlikely.

4.1.1 | Morphological characters

Gottwald and Cameron (1979) described the fungus on *C. avellana*, European hazelnut. Mature stromata, which develop within a cankered area, are black and measure 1.5–3 ×2–10 mm and 1–2 mm in height (Figure 8). Perithecia are 40–60 per stroma, ovate to pyriform (Figure 9), 250–830 ×1040–2160 µm. Perithecium wall is 40–45 µm wide and the neck is 160–240 µm in diameter, which is often bulbous at the surface, near the ostiole, measuring up to 350 µm in diameter. The size of the neck

FIGURE 8 Magnification of a stroma of *A. anomala*. Courtesy: Jose R. Liberato, DPI&F (Liberato et al., 2016).

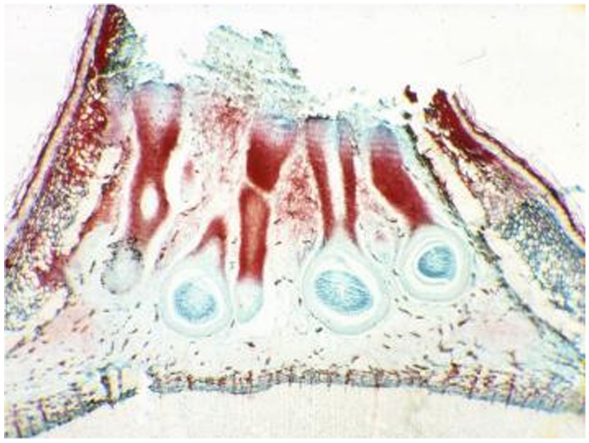


FIGURE 9 Cross section through a single stained stroma, showing pyriform perithecia containing blue masses of spores (copyright, for use contact tgottwald@ushrl.ars.usda.gov). *Source*: T. R. Gottwald (Pscheidt, 2006) USDA ARS/U.S. Horticultural Research Laboratory.

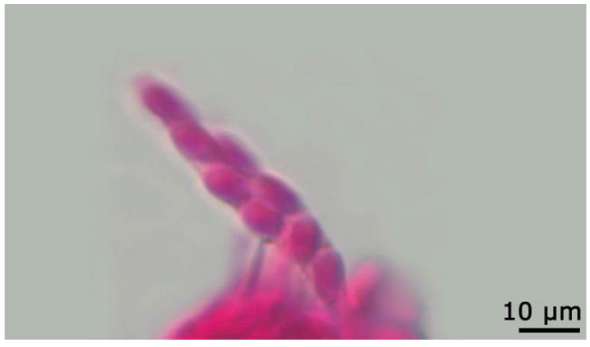


FIGURE 10 Stained ascus with 8 ascospores of *A. anomala*. Courtesy: Jose R. Liberato, DPI&F (Liberato et al., 2016).

depends upon the perithecium position in the stroma. Paraphyses and the ostiolar canal are evident in the perithecia at maturity. Asci are deliquescent, broadly clavate, 45–65 × 10–15 µm, with a long, threadlike stipe, 8-spored (Figure 10). There are about 8400 asci in a mature perithecium. Ascospores are hyaline, two-celled. The smaller cell

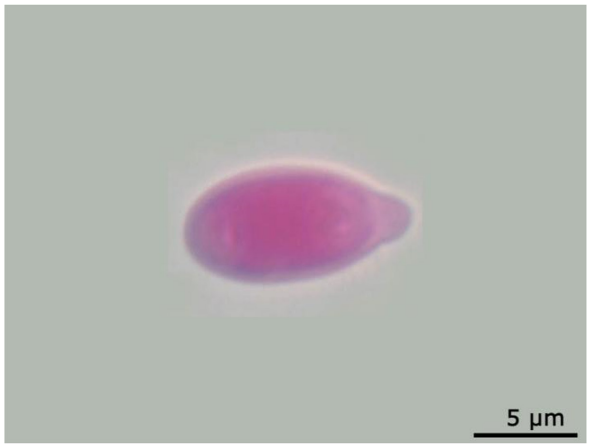


FIGURE 11 Stained ascospore of *A. anomala*. Courtesy: Jose R. Liberato, DPI&F (Liberato et al., 2016).

is completely degenerate and remains as a hemispherical cap cell on one end of the larger cell (Figure 11), measuring 1.1–1.4 × 1.1 µm. The larger cell measures 8–12 × 4–5 µm at maturity (Gottwald & Cameron, 1979).

4.2 | Molecular methods

A real-time PCR test targeting a ribosomal DNA internal transcribed spacer (Molnar et al., 2013; Appendix 3) may be performed on *A. anomala*-like stromata to identify the pathogen.

The following molecular tests may be performed on DNA from isolates to confirm the identification of *A. anomala* (e.g. in critical cases):

- A real-time PCR test targeting a ribosomal DNA internal transcribed spacer (Molnar et al., 2013; Appendix 3);
- DNA barcoding based on *ITS* sequencing using for example the test described in Appendix 3 of the EPPO Standard PM 7/129(2) *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, 2021). Sequence analysis should follow the guidelines described in Appendices 7 and 8 of PM 7/129 (EPPO, 2021).

5 | REFERENCE MATERIAL

No reference material available.

GenBank accession EU683064 (partial LSU gene) can be used as a reference.

6 | REPORTING AND DOCUMENTATION

Guidance on reporting and documentation is given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

7 | PERFORMANCE CHARACTERISTICS

When performance characteristics 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:

Molnar TJ, Rutgers, The State University of New Jersey 59 Dudley Road, New Brunswick, NJ 08901-8520 (US) (thomas.molnar@rutgers.edu).

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 Belisario A (C.R.A.-Plant Pathology Research Center, IT). Revision of the protocol was prepared by Heungens K (ILVO, BE) and was reviewed by the Panel on Diagnostics in Mycology.

REFERENCES

Anagnostakis SL (1982) An improved defined medium for growth of *Endothia parasitica*. *Mycologia* 74:826–830.

Cai G, Leadbetter CW, Muehlbauer MF, Molnar TJ & Hillman BI (2013) Genome-wide microsatellite identification in the fungus *Anisogramma anomala* using Illumina sequencing and genome assembly. *PLoS One* 8:e82408

Capik JM & Molnar TJ (2012) Assessment of host (*Corylus* sp.) resistance to eastern filbert blight in New Jersey. *Journal of the American Society for Horticultural Science.* 137:157–172.

Crouch J, Clarke BB, and Hillman BI. (2005) Phylogenetic relationships and fungicide sensitivities of *Colletotrichum graminicola* isolates from turfgrass in North America Int. *Turfgrass Soc. Res.* 10:186-195.

Davison AD & Davidson RM (1973) *Apioporthe* and *Monochaetia* cankers reported in western Washington. *Plant Disease Reporter* 57, 522-523.

EPPO (2021) PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests. *EPPO Bull*, 51: 100-143.

EPPO (2022) EPPO Global Database (available online). https://gd.eppo.int [last accessed: 2022-07-27]

Gottwald TR & Cameron HR. (1979) Studies in the morphology and life history of *Anisogramma anomala*. *Mycologia* 71, 1107- 1126.

Gottwald TR & Cameron HR. (1980) Disease Increase and the Dynamics of Spread of Canker Caused by *Anisogramma anomala* in European Filbert in the Pacific Northwest. *Phytopathology* 70:1087-1092.

Halsted BD. (1892). A serious filbert disease. New Jersey Agr. *Expt. Sta. Annu. Rpt*. 13:287–288.

Ioos R, Fourrier C, Iancu G, and Gordon TR (2009) Sensitive Detection of Fusarium circinatum in Pine Seed by Combining an Enrichment Procedure with a Real-Time Polymerase Chain Reaction Using Dual-Labeled Probe Chemistry. *Phytopathology* 99:582-590.

Iriti M, Faoro F (2004) L'avvizzimento del nocciolo in Italia: rischio reale o remota possibilità? *Petria* 14, 109-119.

Johnson KB, Mehlenbacher SA, Stone JK, Pscheidt JW & Pinkerton JN (1996) Eastern filbert blight of European hazelnut. It's becoming a manageable disease. *Plant Disease* 80, 1308-1316.

Liberato JR, Pscheidt JW, Johnson KB & Shivas RG (2016) *Anisogramma anomala*. Updated on 15/07/2016, Available online: PaDIL - http://www.padil.gov.au (last accessed 2 December 2022).

McCluskey RL, Azarenko AN, Mehlenbacher SA, Smith, DC, 2005. Advanced selection and cultivar performance of hazelnut trials planted in 1994 and 1998 at Oregon State University. *Acta Horticulturae*, 686, 71-78.

Molnar TJ, Walsh E, Capik JM, Sathuvalli V, Mehlenbacher SA, Rossman AY, Zhang N. (2013) A Real-Time PCR Assay for Early Detection of Eastern Filbert Blight. *Plant Dis.* 97:813-818.

OSU (2023) Location of Eastern Filbert Blight. https://web.engr.oregonstate.edu/~mendelse/EFB/location.htm (last accessed 14 June 2023).

Pinkerton JN, Johnson KB, Stone JK & Ivors KL (1998). Maturation and seasonal discharge pattern of ascospores of *Anisogramma anomala*. *Phytopathology* 88, 1165-1173.

Pinkerton JN, Stone JK, Nelson SJ & Johnson KB (1995). Infection of European hazelnut by *Anisogramma anomala*: ascospore adhesion, mode of penetration of immature shoots, and host response. *Phytopathology* 85, 1260-1268.

Pinkerton JN, Johnson KB, Mehlenbacher SA & Pescheidt J (1993) Susceptibility of European hazelnut clones to eastern filbert blight. *Plant Disease* 77, 261-266.

Revord RS, Lovell ST, Capik JM, Mehlenbacher SA & Molnar TJ (2020) Eastern filbert blight resistance in American and interspecific hybrid hazelnuts. *Journal of the American Society for Horticultural Science* 145:162–173.

Stone JK, Pinkerton JN & Johnson KB (1994). Axenic culture of *Anisogramma anomala*: evidence for self-inhibition of ascospore germination and colony growth. *Mycologia* 86, 674-683.

Yamaoka Y & Katsuya K (1984) Axenic culture of four *Melampsora* species parasitic on willows. *Transactions of the Mycological Society of Japan* 25, 85-92.

How to cite this article: EPPO (2024) PM 7/90 (2) *Anisogramma anomala*. *EPPO Bulletin*, 54, 114–123. Available from: https://doi.org/10.1111/epp.13001

APPENDIX 1 - ISOLATION OF *A. ANOMALA* FROM SPORULATING PERITHECIA

A. anomala exhibits characteristics of an obligate, biotrophic parasite and therefore does not grow on standard mycological media such as PDA (potato dextrose agar), MA (malt agar), MEA (malt extract agar), CMA (corn meal agar), OMA (oatmeal agar), etc. However, Stone et al. (1994) described a medium that allows the (very slow) culture of *A. anomala* from ascospores (see Appendix 2).

Based on Stone et al. (1994), perithecia should be dissected from the stromata, hydrated and crushed in a well-type glass slide with a few drops of water, containing 10 ppm rifampicin, to release the ascospores. Cai et al. (2013) first surface sterilize the samples taken from branches, then hydrate the stromata and expose the necks of the perithecia using a sterile razor blade before pushing out the white viscous material consisting of the asci and ascospores (2013). The ascospores can be diluted to 2–5 $\times 10^5$ ascospores per mL (in water with 10 ppm rifampicin and 100 ppm streptomycin) before transfer of 20 μL drops to the growing medium. The exact number of spores per drop is probably not so relevant for the establishment of a culture (versus the evaluation of germination rates as in Stone et al. (1994)). The plates are incubated at approximately 18°C in the dark (Stone et al., 1994).

Characteristics of colonies

Colony growth is very slow, producing micro-colonies with an average diameter of only 15–35 mm after 6 months of incubation on the medium described in Appendix 2. Ascospores germinate with a short germ tube, which develops into a spherical vesicle (primary vesicle) between 48 and 72 h of inoculation. A hypha emerges from this vesicle, initially unbranched, but with lobe-like projections. From these projections, small hyphal vesicles and later hyphal branches emerge. Often, additional primary vesicles and hyphae are produced from the germinated ascospore. Hyphal branches continue to proliferate from 10 to 14 days and highly-branched, mounded micro-colonies form by 14–21 days (Stone et al., 1994). Although colonies need several months to grow to reasonable proportions, one- to two-month-old microcolonies can be combined for DNA extraction and molecular identification via real-time PCR.

APPENDIX 2 - MEDIA

The medium allowing *A. anomala* growth was developed by Stone et al. (1994) and is a modification of the MMS medium[2] (Modified Murashige & Skoog medium; Yamaoka & Katsuya, 1984). Stone et al. (1994) also described a version of their medium based on a modification of Anagostakis' improved *Cryphonectria* (*Endothia*) *parasitica* medium (EMM, Anagnostakis, 1982). Only the MMS version is described here, as it worked better.

The main modifications by Stone et al. (1994) to the MMS medium are the addition of a chemical adsorbent such as Bovine Serum Albumin (BSA) (to remove self-inhibitory fungal compounds), the use of sucrose instead of glucose (which sustained growth beyond 28 days), and the replacement of KNO_3 and NH_4NO_3 by the alternative nitrogen source L-asparagine (allowing larger and more dense growth). The Stone et al. (1994) medium also contains a larger amount of solidifying agent than MMS (1.5% instead of 0.8%) and does not contain the peptone, soytone, and plant hormones which are in the MMS medium.

Cai et al. (2013) use a modification of the Stone medium and overlay it with sterile cellophane, allowing easier recovery of the cultures by rinsing the cellophane in sterile water.

Medium of Stone et al. (1994)

Macronutrients[a]	
$CaCl_2.2H_2O$	110 mg
$MgSO_4.7H_2O$	92.5 mg
KH_2PO_4	42.5 mg
Micronutrients[a]	
Na_2 EDTA	9.325 mg
$FeSO_4.7H_2O$	6.95 mg
$MnSO_4.4H_2O$	5.575 mg
$ZnSO_4.7H_2O$	2.15 mg
H_3BO_3	1.55 mg
KI	0.208 mg
$Na_2MoO_4.2H_2O$	0.063 mg
$CuSO_4.5H_2O$	0.006 mg
$CoCl_2.6H_2O$	0.006 mg
Vitamins and organics[a]	
Myo-inositol	25 mg
Nicotinic acid	0.125 mg
Pyridoxine HCl	0.125 mg
Thiamin-HCl	0.025 mg
Glycine	0.5 mg
L-asparagine[b]	925 mg
Sucrose	20 g
Chemical adsorbent[a]	
BSA	5 g (up to 8 g)
Solidifying agent and water[a]	
Microbiological grade agar	15 g
Distilled water to	1L

[a]Categories as used in Stone et al. (1994).

[b]Instead of L-asparagine, yeast extract can be used as a nitrogen source at 0.2% (w/v). The standard MMS medium with KNO_3 (475 mg/L) and NH_4NO_3 (412.5 mg/L) as nitrogen sources can also be used instead of L-asparagine or yeast extract but this results in less large and less dense colonies. It may however allow the assembly of the basal part of the medium from commercially available Murashige & Skoog medium mixes.

[2]The basal medium of MMS is ¼ diluted basal medium of MS.

The medium is prepared as follows (after Stone et al. (1994)):

- Prepare double strength (2×) medium without agar and without BSA in distilled water. Set the pH of the medium to 5.5 with 1N HCl[3] and filter-sterilize. It is not clear how crucial is filter sterilization compared to autoclaving the medium for 10 min.
- Prepare the solidifying agent as a separate solution at double strength (3% agar) and autoclave.
- Combine 500 mL of the 2× solidifying agent with 500 mL of the 2× basal medium and let the medium cool down to approximately 45–50°C.
- Add 25 mL of a BSA 20% filter-sterilized solution, just prior to pouring the plates to obtain a final concentration of 0.5% BSA.[4] It is crucial that BSA is not denatured (so not autoclaved). It is presumed that the addition of BSA has no significant diluting effect.

Medium of Cai et al. (2013)

Modified Murashige and Skoog basal salt mixture (without NH_4NO_3)	2.7 g
Sucrose	20 g
Yeast extract	2 g
L-Asparagine	2 g
Activated charcoal	0.25 g
Microbiological grade agar	15 g
Distilled water to	1 L

Add 10 mg of Rifampicin after autoclaving.

This medium is a modification of the Stone et al. (1994) medium. As in the Stone et al. (1994) medium, it includes a chemical absorbent and preferred carbon and nitrogen sources of *A. anomala*. However, based on the amount of basal salt mixture listed, it seems to have an unintentional difference with the Stone et al. (1994) medium, which possibly leads to suboptimal growth. The 2.7 g modified Murashige and Skoog basal salt medium they refer to is most likely commercially available (e.g. Sigma Aldrich M2909) regular strength Murashige and Skoog basal salts but without the NH_4NO_3 instead of the ¼ strength Murashige and Skoog basal salts (and excluding NH_4NO_3 and KNO_3) as described by Yamaoka and Katsuya (1984) and used by Stone et al. (1994). So there may have been confusions as to the nature of the modification of the Murashige and Skoog basal medium. Other differences with the Stone et al. (1994) medium include the omission of the vitamins, and the presence of L-Asparagine and yeast extract and KNO_3, instead of either L-Asparagine or yeast extract and no KNO_3.

The ascospores are said to germinate into small colonies and then die within 4 months on the Cai et al. (2013) medium (pers. comm. Alanna Cohen, Hillman lab, Rutgers University). However, as long as the colonies are of sufficient size to harvest enough DNA for molecular analysis, the medium may serve its purpose.

[3]The pH of MMS medium is set at 5.8–5.9 with 1 N HCl. In Stone et al. (1994) it was determined that a pH of 5.2 is optimal for *A. anomala* but no difference was observed between colonies grown on medium that was buffered at 5.2–5.5 with 10 mM MES and medium in which the standard pH setting of MMS medium was used. In this protocol, it is proposed to set the pH of the first part of the medium to 5.5 with 1N HCl before filter sterilization, and to not include an MES buffer.

[4]Instead of BSA, activated charcoal can also be used as chemical adsorbent, at 0.05% (w/v) final concentration. It is autoclaved separately and also added just before pouring the plates. BSA has the best activity though (Jeffrey Stone, Oregon State University, personal communication).

APPENDIX 3 - REAL-TIME PCR (MOLNAR 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 test can be used for the detection of *A. anomala* in symptomatic plant material or for the identification of isolates from pure cultures. The test can also be used for the identification of *A. anomala* directly from stromata although this matrix was not evaluated in the original publication.

1.2. This real-time PCR test was developed by Molnar et al. (2013).

1.3. This test targets the ribosomal DNA internal transcribed spacer 1 (ITS1).

1.4. Oligonucleotides

Type	Name	Sequence (5′–3′)	Amplicon size (bp)
Forward primer	AaITSF1	TTTGTGAATC TTCTCCGT TGC	135 bp
Reverse primer	AaITSR1	TCATTTCTGT CAAAGGC TCAGA	
Probe	AaITSprobe1	FAM-CGGCCCC ATAAACACT GCTCCTGTT-Iowa Black FQ	

1.5 The test was carried out on a StepOnePlus real-time PCR instrument (Applied Biosystems).

2. Methods

2.1. DNA extraction

2.1.1. From infected tissue plant

30 to 40 mg of fresh plant material was cut into 1-mm strips and homogenized using the Qiagen Tissue-Lyser for 10 to 15 min at 20 oscillations/second, adding both

7-mm stainless steel beads and sterile sand. Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol.

2.1.2. From fungal mycelium

Mycelium from isolates was ground with a mortar and pestle to a fine powder in liquid nitrogen. Total genomic DNA was extracted using a standard phenol/chloroform protocol, as described by Crouch et al. (2005).

Although not reported in the original publication, DNA can be extracted using commercially available DNA extraction kits, according to the manufacturer's instructions.

2.1.3. DNA should preferably be stored at approximately −20°C.

2.2. Real-time PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade Water		1.625	
iTaq Supermix with ROX (Bio-Rad)	2×	7.5	1×
Forward primer	10 µM	0.75	0.5 µM
Reverse primer	10 µM	0.75	0.5 µM
Probe	10 µM	0.375	0.25 µM
Subtotal		11	
Nucleic acid extract		4	
Total		15	

2.2.2 Amplification reactions

1 cycle of 95°C for 3 min; 45 cycles of 95°C for 15 s, and 60°C for 40 s. Note that 40 cycles may be used instead of 45 in particular if considering a Ct cut-off value of 35 as published by Molnar et al. (2013).

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 or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to 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). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the PIC which may be difficult to obtain because the pathogen is difficult to culture, internal positive controls (IPC) can be used to monitor each individual sample separately. These 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 (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA; e.g. Ioos et al., 2009).
- amplification or co-amplification of nucleic acid control from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid.

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Laboratories should take additional care to prevent risks of cross contamination when using high concentration positive controls (e.g. cloned products, gBlocks, and whole genome amplicons) directly or when preparing dilutions of them.

3.2 Interpretation of results

The Ct cut-off value given below is as established in Molnar et al. (2013). As a Ct cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.

Verification of the controls

- The PIC and PAC (and if relevant IPC) 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. Molnar et al. (2013) consider a sample positive if the Ct value is below 35. This is near the lowest concentration tested and detected in their assays (120 fg).
- 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, for example if a Ct value between 35 and 40 is observed in a sample.

4. **Performance characteristics available**

The validation data reported below are from Molnar et al. (2013). This 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 0.12 pg of *A. anomala* genomic DNA per PCR reaction. It is the lowest amount of DNA evaluated by Molnar et al. (2013).

4.2 Analytical specificity data

Inclusivity evaluated on 12 isolates of *A. anomala*: 100%.

Exclusivity evaluated on 15 isolates of other species that are either closely related to the genus *Anisogramma* or common inhabitants of the environment including *Alternaria alternata* (2 isolates), *Amphiporthe hranicensis*, *Apiognomonia errabunda*, *Cladosporium tenuissimum*, *Colletotrichum acutatum*, *Cryptosporella femoralis*, *Diaporthe eres*, *Discula destructiva* (2 isolates), *Gnomonia gnomon*, *Plagiostoma fraxini*, *Pleuroceras pleurostylum*, *Pythium* sp., *Waitea circinata*: 100%.

4.3 Diagnostic sensitivity and diagnostic specificity

Diagnostic sensitivity: 100% on symptomatic plant material from naturally infested plants, with Ct values ranging from 16.3 to 30.9. When evaluated on asymptomatic plant material from artificially inoculated plants (inoculation area): 79%, 90% and 100% at 6, 15 and 29 weeks after inoculation, respectively.

Diagnostic specificity (evaluated on non-symptomatic non-inoculated plants): 100%.

4.4 Data on Repeatability

Not available.

4.5 Data on Reproducibility

Not available.