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EUPHRESO III - Special Issue on Plant Health Research Priorities RESEARCH PAPERS

Quantitative real-time PCR assay for detection and quantification of *Anthostoma decipiens*, the cause of ‘mal dello stacco’ in hazelnut

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Summary. ‘Mal dello stacco’, a destructive fungal trunk disease of hazelnut, is caused by *Anthostoma decipiens*. This disease manifests as wedge-shaped cankers, vascular necroses, branch cracking, and reddish masses on bark surfaces. The disease has rapidly increased in incidence and spread, resulting in significant qualitative and quantitative yield reductions. Disease management strategies remain insufficient, without reliable tools for early pathogen detection in hazelnut nurseries or field plantations. This study developed and validated an SYBR Green quantitative real-time PCR (qPCR) assay for the detection and quantification of *A. decipiens* in hazelnut wood. Primers were designed on the nuclear ribosomal internal transcribed spacer (ITS) region. The assay was optimized and validated according to EPPO standard PM 7/98 (6), for analytical specificity and sensitivity, selectivity, repeatability, and reproducibility. The primer pair AD8_F/AD16_R successfully amplified a 154 bp fragment in all of 32 *A. decipiens* isolates, without amplification for 42 off-target species up to 35 cycles. The assay detected up to 100 fg of pathogen DNA in water and hazelnut DNA, with reaction efficiencies from 97% to 100%, and high reproducibility across operators and sessions. The assay was validated on artificially inoculated and naturally infected hazelnut plants from Italy, Spain, and Serbia, and from ten hazelnut cultivars. The qPCR assay developed is a reliable, rapid, and cost-effective diagnostic tool for early detection of *A. decipiens*, with potential applications in nursery certification, orchard monitoring and epidemiological studies.

Keywords: Fungal Trunk Diseases, Nut crop, molecular diagnostic, real-time PCR, pathogen detection.

INTRODUCTION

‘Mal dello stacco’ (*Cytospora* canker), is an important fungal trunk diseases (FTDs) affecting hazelnut (Martino *et al.*, 2025a). Symptoms include wedge-shaped cankers and internal dark brown vascular necroses in trunk tissues, which cause dieback and branch cracking and, in the severe cases, the death of affected plants. Reddish conidia masses occur on surface of symptomatic tree branches (Linaldeddu *et al.*, 2016; Martino *et al.*, 2025a). Although mal della stacco was reported in the main Italian hazelnut-producing regions in 1918, in recent years the disease has rapidly spread, irrespective of plant age and orchard management strategies (Sevastano, 1918; Trotter, 1933; Salerno, 1961; Tavella and Gianetti, 2006). Disease incidence is often exacerbated by abiotic stresses (late spring frosts, elevated temperatures, water deficit), together with the reduced availability of active ingredients for plant protection, and this has resulted in substantial yield losses and increased nut production costs (Lamichhane *et al.*, 2014; An *et al.*, 2020). For example, in the 2025 season, Italian hazelnut production declined by approx. 50%, despite the steady increase in orchard area by more than 30% during the last decade (ISTAT, 2026). Italy is one of the most important hazelnut producers, so these yield losses are significant concerns.

Historically, several species of *Cytospora*, including *C. corylicola*, were associated with ‘mal dello stacco’ (Trotter, 1933; Salerno, 1961; Houshyarfard, 2020). However, recent studies have identified *Anthostoma decipiens* (syn. *Eutypella decipiens*) as the causal agent of ‘mal dello stacco’ (Linaldeddu *et al.*, 2016; Martino *et al.*, 2025a; Piattino *et al.*, 2026). The morphological similarities between *A. decipiens* and *Cytospora* spp. (conidium shape and size, and colony characteristics) have led to misidentifications (Martino *et al.*, 2025a). Current disease management strategies for FTDs primarily rely on agronomic practices, including effective pruning timing, wound protection, and use of non-contaminated pruning tools (Martino *et al.*, 2025a). No biological or chemical products are currently registered for management of ‘mal dello stacco’ or other FTD symptoms in hazelnut (Martino *et al.*, 2025b).

Anthostoma decipiens (Diatrypaceae) is a pathogen historically known as a cause of cankers and dieback in woody plants in Europe, including *Alnus glutinosa*, *Betula pendula*, *Corylus avellana*, *Fagus sylvatica*, *Ostrya carpinifolia*, *Quercus robur*, and *Vitis vinifera* (Rocchi *et al.*, 2010; Luque *et al.*, 2012; Saracchi *et al.*, 2015; Linaldeddu *et al.*, 2016; Stoykov and Alvarado, 2023; Martino *et al.*, 2025a; Dissanayake *et al.*, 2025). This pathogen has also been identified as the cause of decline of European hornbeam (*Carpinus betulus*) in Austria, Hungary,

Italy and Iran (Tóth, 1967; 1994; Saracchi *et al.*, 2008; Rocchi *et al.*, 2010; Mirabolfathy *et al.*, 2018; Cech, 2019).

Symptoms on *A. decipiens* infections include external and internal alterations of woody tissues. Externally, the pathogen causes cortical cankers, longitudinal bark streaks, branch cracking or detachment, and twig dieback which is often associated with canopy thinning and leaf chloroses (Rocchi *et al.*, 2010; Saracchi *et al.*, 2015; Linaldeddu *et al.*, 2016; Martino *et al.*, 2025a). Internally, affected tissues have dark brown necrotic vascular discolouration and wedge-shaped friable cankers (Linaldeddu *et al.*, 2016; Martino *et al.*, 2025a). In spring and summer, *A. decipiens* produces orange to red conidia masses on the surface of symptomatic woody tissue, particularly in high humidity conditions (Rocchi *et al.*, 2010; Linaldeddu *et al.*, 2016).

Conidia of *A. decipiens* are mainly dispersed by rain and wind, and are the primary inoculum spread of the pathogen (Martino *et al.*, 2025a). In the sexual stage, black perithecia producing ascospores develop on necrotic tissues (Rocchi *et al.*, 2010). Infections occur through host wounds, including pruning cuts, and through natural openings (Martino *et al.*, 2025a).

Knowledge of pathogen etiology and epidemiology is limited. Identification of *A. decipiens* is polyphasic, combining micro- and macro- morphological observation of colonies and conidia in cultures (shape, size, colour, presence of septa), and on molecular analyses mainly of the nuclear ribosomal internal transcribed spacer (ITS) (Linaldeddu *et al.*, 2016; Martino *et al.*, 2025a).

Advances in molecular methods have provided sensitive and specific tools for the reliable identification and detection of the FTD agents, reducing time and costs associated with techniques based on isolation and DNA sequencing (Luchi *et al.*, 2020; Venbrux *et al.*, 2023; Martino *et al.*, 2025b). Several conventional PCR assays have been developed for FTD pathogens including *Botryosphaeriaceae*, *Diatrypaceae*, *Diaporthe* spp., and *Cystospora* spp. (Xu *et al.*, 2016; Zhang *et al.*, 2020; Avenot *et al.*, 2022; Ghaedi *et al.*, 2025). However, PCR-based tools do not provide pathogen quantification, and may give false negative results due to the presence of PCR inhibitors in plant tissues, or false positives from contamination during sample handling (Venbrux *et al.*, 2023).

Quantitative PCR (qPCR) is a widely used molecular identification technique, due to its high sensitivity and specificity for pathogen detection, where DNA concentrations are low, and across broad ranges of environmental samples, and without requirement for post-amplification processing steps (Venbrux *et al.*, 2023). qPCR assays have been developed to detect causal agents of FTDs, including *Botryosphaeriaceae* on avocado, blueberry,

grapevine, or nut crops, *Diaporthe* and *Cytospora* spp. on stone fruit and nut crops, and *Neonectria ditissima* on apple (Ghasemkhani *et al.*, 2016; Luo *et al.*, 2017; Jacobs *et al.*, 2024; Romero-Cuadrado *et al.*, 2024). To date, no molecular tools have been developed for the detection and quantification of *A. decipiens* in hazelnut.

Given the limited management strategies available for Mal dello Stacco, and the increasing international economic value of hazelnut, an early, rapid, and accurate diagnostic tool for the detection of *A. decipiens* is necessary. The present study aimed to develop a sensitive and specific qPCR assay using the SYBR Green method, for identification and quantification of *A. decipiens* in hazelnut wood. This research also aimed to validate the assay for DNA extracted from artificially inoculated and naturally infected hazelnut plants of different cultivars. The validated diagnostic tool could be used in nurseries and in orchards, for monitoring disease incidence to assist mitigation of damage caused by *A. decipiens*.

MATERIALS AND METHODS

Fungus isolates

A total of 30 *A. decipiens* isolates were obtained from symptomatic wood of seven *Corylus avellana* cultivars ('Daria', 'Negret', 'Pauetet', 'San Giovanni', 'Tombul', 'Tonda Gentile Trilobata', 'Tonda Giffoni'), that were cultivated in Italy and Spain. The isolates were previously identified through molecular and phylogenetic analyses, based on the ITS gene locus by Martino *et al.* (2025a) and Piattino *et al.* (2026). Two isolates of *A. decipiens* (ex-type strain CBS 133221, and CBS 231.51), and 11 off-target species (*Botryosphaeria dothidea* CBS 115476, *Cytospora leucostoma* CBS 122754, *Diaporthe cryptica* CBS 140348, *Diplodia sapinea* CBS 138184, *Dothiorella omnivora* CBS 140349, *Eutypella citricola* CBS 128330, *Eutypella leprosa* CBS 276.87, *Eutypella microtheca* CBS 128336, *Eutypella sorbi* CBS 280.87, *Gnomoniopsis smithogilvyi* CBS 130190, *Neofusicoccum parvum* CBS 138824) were provided by the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands). The isolate CBS 231.51 was reported as *C. corylicola* based on morphological characteristics, but was reclassified as *A. decipiens* through molecular characterization (Crous *et al.*, 2026). The 18 off-target species were obtained from the Turin University Culture Collection (TUCC) and the collection of the Department of Agricultural, Forest and Food Sciences (DISAFA), University of Turin, Italy. A complete list of the fungal isolates used in this study shown in Table 1.

All isolates were stored as mono-hyphal cultures in PDA slant tubes at 4 °C, in the fungal collection of the

Department of Agricultural, Forest and Food Sciences (DISAFA), University of Turin, Italy. Total genomic DNA from each isolate was extracted from 0.1 g of 10-d-old mycelium grown on potato dextrose agar (PDA, VWR Chemicals) amended with 25 mg L⁻¹ of streptomycin sulphate (Sigma-Aldrich) (PDA-S), using the E.Z.N.A fungal DNA Mini-Kit (Omega Bio-Tek) following manufacturer's instructions. Quantity and quality of DNA were verified using a Nanodrop 2000 spectrophotometer (Thermo Fischer Scientific), and the samples were diluted to concentrations of 10 ng µL⁻¹.

All the off-target isolates were identified by amplification and sequencing of different genomic regions: the nuclear ribosomal internal transcribed spacer (ITS) region using the primers ITS1/ITS4 (White *et al.*, 1990), the translation elongation factor 1- α (*tef1*) locus with the primers EF1-728F/EF1-986R (Carbone and Kohn, 1999), and the β -tubulin genomic region (*tub2*) with the primers T1/Bt2b (Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997). DNA sequences were compared with reference sequences of NCBI GenBank through the standard nucleotide Basic Local Alignment Search Tool (BLAST) (Boratyn *et al.*, 2013).

Primer design

ITS gene sequences of the 32 *A. decipiens* isolates and off-target species were sourced from the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>) of National Centre of Biotechnology information (NCBI; <https://www.ncbi.nlm.nih.gov/>). Sequence alignments were performed through the CLUSTALW algorithm of software MEGA version 7.0 (Kumar *et al.*, 2016). The alignments were analyzed to select regions of *A. decipiens* sequences with high polymorphism relative to off-target sequences. Four primers were manually designed using Primer3 software (<https://primer3.ut.ee/>), and were validated in-silico through Primer-Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and BLASTN of NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The OligoCalc tools (<https://www.biosyn.com/gizmo/tools/oligo/oligonucleotide%20properties%20calculator.htm>) were used to check potential secondary structures and hairpin formation. Primer sequences were synthesized by Invitrogen (Carlsbad, California, United States of America).

Optimization of end-point PCR and qPCR assay

The best performing primer pairs were selected through conventional endpoint PCR using DNA of *A.*

Table 1. List of isolates of target and off-target fungal species used for validation of qPCR assay analytical specificity, including host, source country, and results (mean Cq values $\pm$ standard deviations).

Species	Strain ID	Host	Country	Cq mean $\pm$ SD
<i>Alternaria alternata</i>	CVG 641	<i>Rubus idaeus</i>	Italy	Negative
<i>Alternaria</i> sp.	CVG 2481	<i>Corylus avellana</i>	Italy	Negative
<i>Anthostoma decipiens</i>	CBS 133221	<i>Carpinus betulus</i>	Austria	17.63 $\pm$ 0.28
	CBS 231.51	<i>Corylus avellana</i>	unknown	16.72 $\pm$ 0.11
	TUCC00001101	<i>Corylus avellana</i> ‘Tonda Gentile Trilobata’	Italy	18.54 $\pm$ 0.35
	TUCC00001102	<i>Corylus avellana</i> ‘Tonda Gentile Trilobata’	Italy	17.47 $\pm$ 0.35
	CVG 1381	<i>Corylus avellana</i> ‘Tonda Gentile Trilobata’	Italy	19.38 $\pm$ 0.13
	CVG 1382	<i>Corylus avellana</i> ‘Tonda Gentile Trilobata’	Italy	19.53 $\pm$ 0.06
	CVG 2197	<i>Corylus avellana</i> ‘Tonda Gentile Trilobata’	Italy	19.01 $\pm$ 0.10
	CVG 2198	<i>Corylus avellana</i> ‘Tonda Gentile Trilobata’	Italy	18.39 $\pm$ 0.17
	TUCC0001103	<i>Corylus avellana</i> ‘Tonda Gentile Trilobata’	Italy	16.92 $\pm$ 0.16
	CVG 2203	<i>Corylus avellana</i> ‘Tonda Gentile Trilobata’	Italy	18.93 $\pm$ 0.15
	CVG 2211	<i>Corylus avellana</i> ‘Tonda Gentile Trilobata’	Italy	17.81 $\pm$ 0.13
	TUCC00001105	<i>Corylus avellana</i> ‘Tonda Gentile Trilobata’	Italy	18.25 $\pm$ 0.06
	TUCC00001106	<i>Corylus avellana</i> ‘Tonda Gentile Trilobata’	Italy	16.62 $\pm$ 0.24
	TUCC00001104	<i>Corylus avellana</i> ‘Tonda Gentile Trilobata’	Italy	19.70 $\pm$ 0.41
	CVG2222	<i>Corylus avellana</i> ‘Tonda Gentile Trilobata’	Italy	19.96 $\pm$ 0.46
	CVG 2522	<i>Corylus avellana</i> ‘Tonda Giffoni’	Spain	18.00 $\pm$ 0.45
	CVG 2524	<i>Corylus avellana</i> ‘Tonda Giffoni’	Spain	18.06 $\pm$ 0.22
	CVG 2526	<i>Corylus avellana</i> ‘Negret’	Spain	18.30 $\pm$ 0.15
	CVG 2527	<i>Corylus avellana</i> ‘Negret’	Spain	18.07 $\pm$ 0.23
	CVG 2528	<i>Corylus avellana</i> ‘Tonda Giffoni’	Spain	19.05 $\pm$ 0.27
	CVG 2529	<i>Corylus avellana</i> ‘San giovanni’	Spain	19.67 $\pm$ 0.25
	CVG 2530	<i>Corylus avellana</i> ‘San giovanni’	Spain	18.16 $\pm$ 0.27
	CVG 2531	<i>Corylus avellana</i> ‘San giovanni’	Spain	18.38 $\pm$ 0.17
	CVG 2533	<i>Corylus avellana</i> ‘Pauetet’	Spain	18.75 $\pm$ 0.29
	CVG 2536	<i>Corylus avellana</i> ‘Tonda Giffoni’	Spain	18.49 $\pm$ 0.23
	CVG 2537	<i>Corylus avellana</i> ‘Daria’	Spain	18.24 $\pm$ 0.28
	CVG 2539	<i>Corylus avellana</i> ‘Daria’	Spain	19.24 $\pm$ 0.29
	CVG 2540	<i>Corylus avellana</i> ‘Daria’	Spain	18.40 $\pm$ 0.21
	CVG 2542	<i>Corylus avellana</i> ‘Tombul’	Spain	17.45 $\pm$ 0.06
	CVG 2543	<i>Corylus avellana</i> ‘Tombul’	Spain	18.71 $\pm$ 0.33
	CVG 2545	<i>Corylus avellana</i> ‘Pauetet’	Spain	17.94 $\pm$ 0.17
	CVG 2547	<i>Corylus avellana</i> ‘Pauetet’	Spain	18.51 $\pm$ 0.24
<i>Aspergillus</i> sp.	CVG2583	<i>Corylus avellana</i>	Italy	Negative
<i>Botryosphaeria dothidea</i>	TUCC00001099	<i>Corylus avellana</i> ‘Tonda Gentile Trilobata’	Italy	Negative
	TUCC00001100	<i>Corylus avellana</i> ‘Tonda Gentile Trilobata’	Italy	Negative
<i>Cytospora leucostoma</i>	CBS 122754	<i>Prunus dulcis</i>	Spain	Negative
<i>Diaporthe eres</i>	CVG1334	<i>Corylus avellana</i>	Italy	39.38 $\pm$ 0.41
	CVG1338	<i>Corylus avellana</i>	Italy	38.28 $\pm$ 0.35
	CVG1363	<i>Corylus avellana</i>	Italy	38.78 $\pm$ 0.58
	CVG1365	<i>Corylus avellana</i>	Italy	38.56 $\pm$ 0.40
	CVG1130	<i>Vaccinium corymbosum</i>	Italy	37.77 $\pm$ 0.42
	CVG1131	<i>Vaccinium corymbosum</i>	Italy	38.38 $\pm$ 0.20
<i>Diaporthe rudis</i>	CVG1333	<i>Corylus avellana</i>	Italy	Negative
	CVG661	<i>Vaccinium corymbosum</i>	Italy	Negative
<i>Diaportheella cryptica</i>	CBS140348	<i>Corylus avellana</i>	Italy	Negative

(Continued)

Table 1. (Continued).

Species	Strain ID	Host	Country	Cq mean $\pm$ SD
<i>Diplodia seriata</i>	CVG1405	<i>Corylus avellana</i>	Italy	38.33 $\pm$ 0.4
	CVG1406	<i>Corylus avellana</i>	Italy	38.09 $\pm$ 0.85
	CVG 1586	<i>Vitis vinifera</i> 'Barbera'	Italy	38.30 $\pm$ 0.32
	CVG 1755	<i>Vitis vinifera</i> 'Nebbiolo'	Italy	38.55 $\pm$ 0.40
	CVG 1838	<i>Vitis vinifera</i> 'Merlot'	Italy	38.60 $\pm$ 0.50
	TUCC00000734	<i>Vitis vinifera</i> 'Barbera'	Italy	38.62 $\pm$ 0.51
<i>Diplodia subglobosa</i>	CVG1367	<i>Corylus avellana</i>	Italy	37.16 $\pm$ 0.28
	CVG1368	<i>Corylus avellana</i>	Italy	37.58 $\pm$ 0.54
<i>Diplodia sapinea</i>	CBS 138184	<i>Pinus patula</i>	South Africa	39.68 $\pm$ 0.17
<i>Dothiorella iberica</i>	TUCC00000920	<i>Platanus hispanica</i>	Italy	Negative
<i>Dothiorella omnivora</i>	CBS 140349	<i>Corylus avellana</i>	Italy	Negative
<i>Dothiorella parva</i>	CVG 1414	<i>Corylus avellana</i>	Italy	Negative
	CVG 1415	<i>Corylus avellana</i>	Italy	Negative
<i>Epicoccum catenisorum</i>	TUCC00000695	<i>Oryza sativa</i>	Italy	Negative
<i>Eutypa lata</i>	CVG 500	<i>Malus domestica</i>	Italy	Negative
	CVG 504	<i>Malus domestica</i>	Italy	Negative
	CVG 1733	<i>Vitis vinifera</i>	Italy	Negative
<i>Eutypella leprosa</i>	CBS 276.87	<i>Tilia</i> sp.	Switzerland	35.82 $\pm$ 0.15
<i>Eutypella sorbi</i>	CBS 280.87	<i>Sorbus aucuparia</i>	Switzerland	Negative
<i>Eutypella citricola</i>	CBS 128330	<i>Vitis vinifera</i>	Australia	Negative
<i>Eutypella microtheca</i>	CBS 128336	<i>Citrus paradisi</i>	Australia	Negative
<i>Fusarium oxysporum</i>	TUCC00000714	<i>Oryza sativa</i>	Italy	Negative
<i>Gnomoniopsis smithogilvyi</i>	CBS 130190	<i>Castanea</i> sp.	Australia	Negative
<i>Neofusicoccum parvum</i>	TUCC00000490	<i>Corylus avellana</i>	Italy	Negative
	CVG 2574	<i>Corylus avellana</i> 'Pauetet'	Spain	Negative
	CVG 2576	<i>Corylus avellana</i> 'Negret'	Spain	Negative
<i>Nothophoma brennandiae</i>	CVG 1361	<i>Corylus avellana</i> 'Tonda Gentile Trilobata'	Italy	Negative
	CVG 1377	<i>Corylus avellana</i> 'Tonda Gentile Trilobata'	Italy	Negative
<i>Paraconiothyrium brasiliense</i>	TUCC00000394	<i>Malus domestica</i>	Italy	Negative
<i>Penicillium</i> sp.	CVG 2484	<i>Corylus avellana</i> 'Tonda Gentile Trilobata'	Italy	Negative
<i>Peroneutypa scoparia</i>	CVG 562	<i>Vaccinium corymbosum</i>	Italy	36.57 $\pm$ 0.18
	CVG 564	<i>Vaccinium corymbosum</i>	Italy	36.02 $\pm$ 0.22
	CVG 566	<i>Vaccinium corymbosum</i>	Italy	35.93 $\pm$ 0.35
	CVG 568	<i>Vaccinium corymbosum</i>	Italy	36.11 $\pm$ 0.30
	CVG 571	<i>Vaccinium corymbosum</i>	Italy	36.10 $\pm$ 0.20
	CVG 580	<i>Vaccinium corymbosum</i>	Italy	36.00 $\pm$ 0.18
<i>Trichoderma harzianum</i>	MUT00001420	<i>Solanum lycopersicum</i>	Italy	Negative
<i>Trichoderma</i> sp.	CVG 507	<i>Malus domestica</i>	Italy	Negative

decipiens and the off-target isolates (Table 1). Endpoint PCR reactions were each carried out in a total volume of 25 μ L, including 18.4 μ L of nuclease free water, 2.5 μ L of 10 $\times$ Qiagen Buffer, 1.4 μ L of MgCl₂, 0.5 μ L of dNTPs (10 mM), 0.5 μ L of each primer (10 μ M), 0.2 μ L of Taq DNA polymerase (Qiagen), and 10 ng of DNA. Thermal cycling conditions were optimized using the T100TM thermal Cycler (Bio-Rad) as follows: 5 min at 94 $^{\circ}$ C, followed by 34 cycles each of 94 $^{\circ}$ C for 30 s, 60 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 1 min and 15 s, and final extension at 72

$^{\circ}$ C for 7 min. Primer specificity was evaluated by electrophoresis on 1% agarose gels (VWR Life Science AMRES-COR biochemicals) that were stained with GelRedTM in Tris-acetate-EDTA (TAE) buffer. The primer pair showing greatest specificity for *A. decipiens* in endpoint PCR was selected for SYBR Green qPCR assays using the CFX Opus 96 Real-Time PCR System (Bio-Rad), with 96 well-plates (Bio-Rad) sealed with optical adhesive film (Bio-Rad). The qPCR assay was optimized by evaluating different primer concentrations (from 2 μ M to 10 μ M),

annealing temperatures (from 60 to 64 °C), and annealing times (from 2 to 15 s). The qPCR reactions were each carried out in total volumes of 10 µL, with 5 µL SsoAdvanced Universal SYBR Green Supermix 2× (Bio-Rad), 0.2 µL of each primer, and 1 µL of DNA (10 ng). Thermal conditions were 98 °C for 3 min, followed by 40 cycles each of 98 °C for 15 s, and annealing temperature for 7 s. The melting curves were acquired after each run by ramping the temperature from 65 to 95 °C with an increase of 0.5 °C s⁻¹. Each reaction was carried out in triplicate, including a negative control (sterile nuclease-free water) and a positive control (*A. decipiens* reference strain CBS 133221). Data were collected using Bio-Rad CFX maestro software. The quantification cycles (C_q) values obtained were interpolated into a standard calibration curve which was generated using serial dilutions of *A. decipiens* DNA.

Specificity, sensitivity, selectivity, repeatability, and reproducibility of the qPCR assay

Validation of the qPCR assay was carried out by evaluating analytical specificity, analytical sensitivity, selectivity, repeatability, and reproducibility, in accordance with the EPPO standard PM 7/98 (6) (EPPO, 2025). Analytical specificity was evaluated using a total 30 species, including 32 *A. decipiens* isolates and 45 off-target species isolates (Table 1). Off-target species included *Diatrypaceae* spp. phylogenetically close to the target (*Eutypa lata*, *Eutypella* spp., *Peroneutypa scoparia*), species commonly found in wood tissue (*Alternaria* spp., *Aspergillus* spp., *Epicoccum catenisporem*, *Fusarium oxysporum*, *Penicillium* spp., *Paraconiothyrium* spp.), and other hazelnut trunk pathogens (*B. dothidea*, *Cytospora* spp., *Diaporthe* spp., *Diaportheella cryptica*, *Diplodia* spp., *Dothiorella* spp., *G. smithogilyvi*, *N. parvum*, *Nothophoma brennandiae*), according to previous studies (Linaldeddu *et al.*, 2016; Guarnaccia *et al.*, 2020; Dardani *et al.*, 2023; Bosco *et al.*, 2024; Martino *et al.*, 2025a; Lione *et al.*, 2025; Sanna *et al.*, 2026). Additionally, multiple sequence alignments of the target amplicon and the AD8_F/AD16_R primer binding regions were performed using MAFFT v7.505 to compare these regions among all off-target species. Amplicon sequence identities were calculated as the proportion of identical nucleotides at aligned, non-gap positions. Primer-binding regions (AD8_F and AD16_R) were analyzed to assess primer-template complementarity, and identify mismatches potentially affecting amplification specificity. To determine the qPCR analytical sensitivity and limit of detection (LOD), a standard calibration curve was constructed based on seven ten-fold serial dilutions

from 10 ng to 1 fg of the CBS 133221 and TUC00001102 isolates. Dilutions were carried out in sterile nuclease-free water, each with two biological replicates and three technical replicates. The standard curve based on CBS 133221 was used to quantify *A. decipiens* DNA in different samples. Selectivity was assessed to establish the influence of host DNA on target detection by carrying out serial dilutions of the target DNA into DNA of *Co. avellana* ‘Tonda Gentile Trilobata’ at 10 ng µL⁻¹. One operator carried out three qPCRs on one day in the laboratory, to confirm repeatability, while two operators in the same laboratory on different days tested analysis reproducibility.

Validation of qPCR in naturally and artificially inoculated tissues

The qPCR assay was validated for symptomatic and asymptomatic hazelnut plants that were either artificially inoculated in a greenhouse or naturally infected in the field. Six 1-year-old potted plants of *Co. avellana* each of ‘Tonda Gentile Trilobata’ and ‘Tonda Giffoni’ were inoculated with *A. decipiens* TUC00001102. The plants were wounded with a sterile scalpel, and 7-d-old mycelium plugs (5 mm diam.) grown on PDA-S were placed with mycelium in contact with internal plant tissue (Piattino *et al.*, 2026). The inoculation points were then sealed with Parafilm. Two plants for each cultivar were inoculated with sterile agar plugs as inoculation control treatments. The plants were then placed in the greenhouse, and, after 4 months, internal necrotic lesions were observed on the inoculated plants, and the control plants showed no symptoms. Five small wood pieces (3 × 3 mm) were collected from necrotic areas to compare pathogen quantities.

A total of 24 naturally infected field plants were collected during summer of 2024 in Spain or summer of 2025 in Italy and Serbia. The sampling sites are reported in Table 2. Wood samples were collected from asymptomatic plants and symptomatic plants showed necrotic lesion and cankers. The samples originated from ten *Co. avellana* cultivars: ‘Barcelona’, ‘Daria’, ‘Negret’, ‘Nocchione’, ‘Pauetet’, ‘San Giovanni’, ‘Tombul’, ‘Tonda Gentile Romana’, ‘Tonda Gentile Trilobata’, ‘Tonda Giffoni’.

Total DNA was extracted from 20 mg of wood tissues, as reported for other woody crops by Dardani *et al.* (2025). The quality and quantity of DNA was checked using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific). Each wood sample was adjusted to a final DNA concentration of 10 ng µL⁻¹. DNA samples were then screened for presence of *A. decipiens* using the qPCR assay. Isolations and molecular identifications

Table 2. Results of qPCR assay validation for detection of *Anthostoma decipiens* in artificially inoculated or naturally infected *Corylus avellana* plants, including both asymptomatic and symptomatic plant tissues.

Infection status	Sample code	Origin	Cultivar	Plant status	Cq mean $\pm$ SD	DNA quantity (ng/ μ L)
Artificially infected	PT1	/	Tonda Giffoni	Inoculated	27.70 $\pm$ 0.41	4,88E+04
	PT2	/	Tonda Giffoni	Inoculated	24.66 $\pm$ 0.38	9,96E+04
	PT3	/	Tonda Giffoni	Inoculated	25.92 $\pm$ 0.34	4,22E+04
	PT4	/	Tonda Gentile Trilobata	Inoculated	31.55 $\pm$ 0.50	9,06E+02
	PT5	/	Tonda Gentile Trilobata	Inoculated	29.45 $\pm$ 0.60	3,78E+03
	PT6	/	Tonda Gentile Trilobata	Inoculated	33.87 $\pm$ 0.56	1,86E+02
	PT7	/	Tonda Giffoni	Control	Negative	0,00E+00
	PT8	/	Tonda Giffoni	Control	Negative	0,00E+00
	PT9	/	Tonda Gentile Trilobata	Control	Negative	0,00E+00
	PT10	/	Tonda Gentile Trilobata	Control	Negative	0,00E+00
Naturally infected	IT1	Italy, San Damiano (AT)	Tonda Gentile Trilobata	Symptomatic	32.73 $\pm$ 0.42	4,07E+02
	IT2	Italy, San Damiano (AT)	Tonda Gentile Trilobata	Symptomatic	27.40 $\pm$ 0.28	1,53E+04
	IT3	Italy, San Damiano (AT)	Tonda Gentile Trilobata	Symptomatic	28.15 $\pm$ 0.37	9,22E+03
	IT4	Italy, Cravanzana (CN)	Tonda Gentile Trilobata	Symptomatic	33.20 $\pm$ 0.59	2,94E+02
	IT5	Italy, Cravanzana (CN)	Tonda Gentile Trilobata	Symptomatic	34.37 $\pm$ 0.28	1,33E+02
	IT6	Italy, Vaccheria (CN)	Tonda Gentile Trilobata	Symptomatic	28.10 $\pm$ 0.20	9,51E+03
	IT7	Italy, Vaccheria (CN)	Tonda Gentile Trilobata	Symptomatic	26.57 $\pm$ 0.43	2,70E+04
	IT8	Italy, San Damiano (AT)	Tonda Gentile Trilobata	Asymptomatic	Negative	0,00E+00
	IT9	Italy, Cravanzana (CN)	Tonda Gentile Trilobata	Asymptomatic	Negative	0,00E+00
	IT10	Italy, Vaccheria (CN)	Tonda Gentile Trilobata	Asymptomatic	Negative	0,00E+00
	SP1	Spain, Selva del Camp (TGN)	Tonda Giffoni	Symptomatic	34.02 $\pm$ 0.61	1,69E+02
	SP2	Spain, Selva del Camp (TGN)	Pauetet	Symptomatic	31.07 $\pm$ 0.69	1,26E+03
	SP3	Spain, Vilaplana (TGN)	Negret	Symptomatic	33.61 $\pm$ 0.22	2,23E+02
	SP4	Spain, Vilaplana (TGN)	San Giovanni	Symptomatic	32.04 $\pm$ 0.74	6,50E+02
	SP5	Spain, Vilaplana (TGN)	Tonda Giffoni	Symptomatic	29.43 $\pm$ 0.39	3,85E+03
	SP6	Spain, Tarragona (TGN)	Tombul	Symptomatic	24.35 $\pm$ 0.46	1,23E+05
	SP7	Spain, Tarragona (TGN)	Daria	Symptomatic	26.16 $\pm$ 0.24	3,61E+04
	S1	Serbia, Aleksa Santic (ZB)	Barcelona	Symptomatic	25.40 $\pm$ 0.29	5,99E+04
	S2	Serbia, Gornja Rogatica (RS)	Tonda Giffoni	Symptomatic	27.55 $\pm$ 0.12	1,38E+04
	S3	Serbia, Velika Plana (PD)	Barcelona	Symptomatic	29.32 $\pm$ 0.33	4,15E+03
	S4	Serbia, Batrovci (SR)	Tonda Giffoni	Symptomatic	27.08 $\pm$ 0.35	1,90E+04
	S5	Serbia, Sivic (ZB)	Tonda Gentile Romana	Symptomatic	27.65 $\pm$ 0.47	1,29E+04
	S6	Serbia, Paljuvi (KB)	Nocchione	Symptomatic	27.13 $\pm$ 0.94	1,84E+04
	S7	Serbia, Kikinda (SB)	Tonda Gentile Romana	Symptomatic	32.36 $\pm$ 0.47	5,20E+02

were carried out on the wood samples as described by Martino *et al.* (2025a) to determine if *A. decipiens* was isolated.

Data analyses

Baseline range, calibration standard curve, and Cq values were automatically generated by Bio-Rad CFX maestro software (Bio-Rad). The Wilcoxon-Mann-Whitney test was used to analyze repeatability and reproducibility of the qPCR assay using R v.4.3.3.

RESULTS

Optimization of the SYBR Green qPCR assay

The ITS genomic region was selected for primer design, as it is currently the only reference gene locus for *A. decipiens* sequenced and deposited in public databases. Four primers were designed and three primer combinations were tested in conventional endpoint PCR (Table 3). The primer pair AD8_F/AD16_R showed the greatest sensitivity and specificity for detecting *A. decipiens* during endpoint PCR screening,

Table 3. Primer pairs designed on ITS gene locus for the detection of *Anthostoma decipiens*.

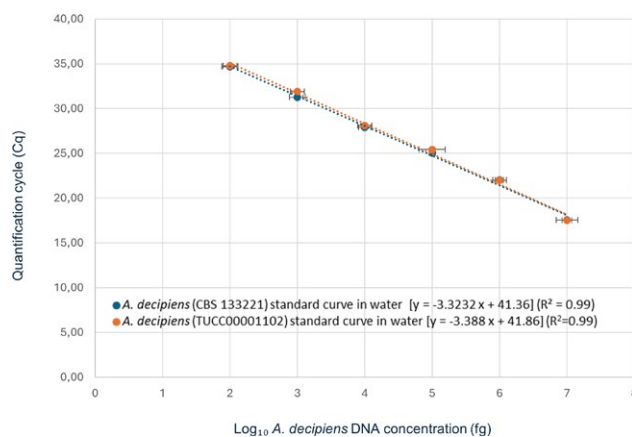
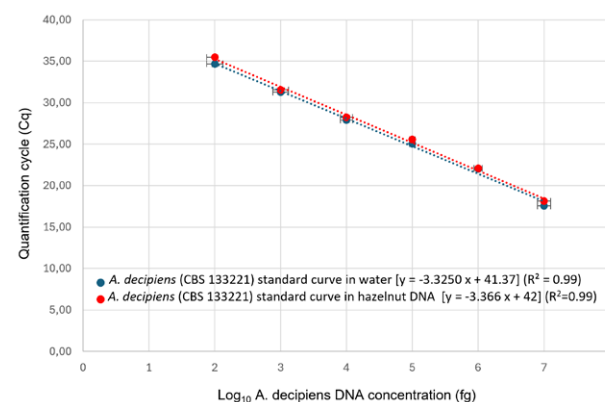
Primer name	Primer sequence (5' – 3')	Melting temperature (°C)	Product length (bp)
AD8_F	gtcatttcgaccatcaagtc	60	83
AD12_R	cgcaactccgccaataat	60	
AD8_F	gtcatttcgaccatcaagtc	60	154
AD16R	agagggtttaacggcta	60	
AD12_F	attattggcggagttgcg	60	89
AD16_R	agagggtttaacggcta	60	

and was validated in the SYBR Green qPCR assay. The reaction conditions were optimized to a primer concentration of 0.2 μ M and an annealing temperature of 63.2 °C.

Specificity, sensitivity, selectivity, repeatability, and reproducibility of the qPCR assay

The primer pair AD8_F/AD16_R successfully amplified a fragment of 154 bp of all 32 target isolates, with a mean Cq value of 18.38 ± 0.11 , and melt temperature of 81.5 °C (Table 1). No amplification was observed for the 42 off-target species up to 35 cycles (Table 1). Amplification was occasionally observed for the off-target species *Diaporthe eres* (mean Cq = 38.58 ± 0.40), *D. sapinea* (39.68 ± 0.17), *D. seriata* (38.47 ± 0.59), *D. subglobosa* (37.3 ± 0.41), *Eut. leprosa* (35.63 ± 0.15), and *P. scoparia* (36.20 ± 0.28) (Table 1).

To further investigate assay specificity, the target amplicons were aligned with the corresponding ITS regions of all off-target species included in the specificity panel. Sequence similarity ranged from 24 to 100% (Table S1), with the greatest values observed among phylogenetically related *Diatrypaceae* (Table S1). Analysis of the AD8_F and AD16_R primer-binding regions revealed mismatches in one or both primer-binding sites for all the off-target species (Table S1). Standard calibration curves were built based on seven serial dilutions in sterile nuclease-free water of two *A. decipiens* isolates CBS 133221 and TUC00001102, obtaining the equations $y = -3.3250x + 41.37$ ($R^2 = 0.99$) for CBS 133221, and $y = -3.389x + 41.88$ ($R^2 = 0.99$) for TUC00001102 (Figure 2). The reaction efficiencies were 100% for CBS 133221 and 97% for TUC00001102. For both *A. decipiens* isolates, DNA was detectable from 10 ng to 100 fg, with an average LOD Cq of 34.77 ± 0.24 (Figure 1). For selectivity, the standard curve obtained from the dilution of target DNA in the host plant DNA

**Figure 1.** Standard curves obtained with genomic DNA of two *Anthostoma decipiens* isolates CBS 133221 (blue) and TUC00001102 (orange). The curve shows the relationship between target DNA (from 10 ng to 100 fg), and the quantification cycle (Cq), from three qPCR runs performed in triplicate. Error bars indicate standard deviations of the Cq values between runs.**Figure 2.** Standard curves obtained with dilutions of *Anthostoma decipiens* isolate CBS 133221 in sterile nuclease-free water (blue) or hazelnut wood DNA concentrated at 10 ng (red). The relationships between ten-fold diluted target DNA and the quantification cycle (Cq) is shown. Error bars represent standard deviations between runs. The coefficient of determination (R^2), and standard deviation bars are shown in the graph.

gave an equation of $y = -3.3669x + 42$ ($R^2 = 0.99$), and a reaction efficiency of 98% (Figure 2). The LOD value was 100 fg with an average LOD Cq of 35.49 ± 0.80 (Figure 3). Target DNA dilutions in sterile nuclease-free water and hazelnut wood DNA showed no statistically significant differences ($P > 0.05$). No differences ($P > 0.05$) were found when evaluating the repeatability and reproducibility, with reliable amplification across both biological and technical replicates under constant or modified conditions.

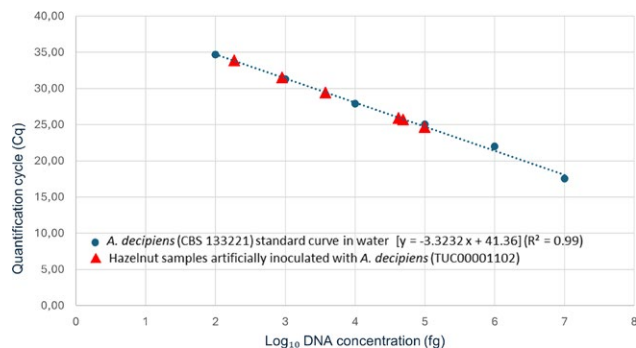


Figure 3. Detection and quantification of *Anthostoma decipiens* in artificially inoculated *Corylus avellana* plants with a qPCR assay. Runs were performed in triplicate. Error bars indicate standard deviations of Cq between runs.

Detection of *Anthostoma decipiens* in artificially or naturally inoculated plants

The qPCR assay was validated for detection of *A. decipiens* in naturally and artificially infected hazelnut plants using a total of 34 samples (Table 2). Detection was considered negative if the Cq value of samples exceeded the LOD Cq of 35.

For the artificially infected plants, six inoculated plants and four non-inoculated control plants were analyzed. The qPCR assay detected the target in all six inoculated plants with the Cq values ranging from 24.66 ± 0.37 (1.07×10^5 ng) to 33.87 ± 0.56 (1.80×10^2 ng) (Figure 3, Table 2). *Anthostoma decipiens* was not detected on the four control plants (Table 2).

For the naturally infected plants, 24 wood samples were considered, including seven symptomatic samples from Serbia, seven symptomatic samples from Spain, and ten samples (seven symptomatic, three asymptomatic) from Italy (Table 2). *Anthostoma decipiens* was detected in all the symptomatic samples with Cq values ranging from 24.35 ± 0.46 (1.32×10^5 ng) to 34.37 ± 0.56 (1.28×10^2 ng) (Figure 4, Table 2). The target DNA quantities ranged from $1.33E + 0.02$ ng to $2.70E + 0.4$ ng in the Serbian samples, from $1.69E + 02$ ng to $1.23E + 0.5$ in the Spanish samples, and from $5.20E + 02$ ng to $5.99E + 04$ ng in the Italian samples. No amplification was observed for the asymptomatic samples (Table 2).

Results obtained for naturally and artificially infected samples were confirmed through isolation and molecular identification. *Anthostoma decipiens* was isolated only from samples that had tested positive in the qPCR assays.

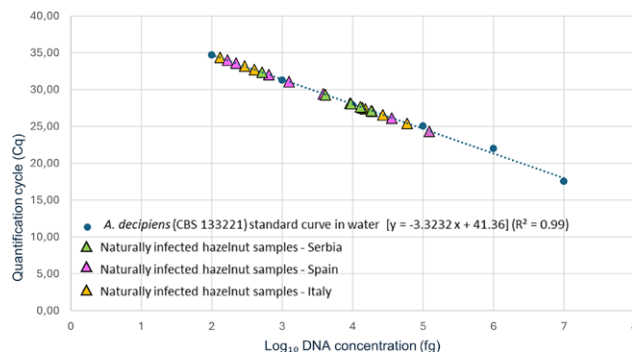


Figure 4. Detection and quantification of *Anthostoma decipiens* in naturally infected *Corylus avellana* plants cultivated in Italy, Serbia or Spain with qPCR assays. Runs were performed in triplicate. Error bars represent the standard deviation of Cq between runs.

DISCUSSION

‘Mal dello stacco’ is historically one of the most important FTDs reported on hazelnut, and has recently spread across traditional and emerging hazelnut production areas resulting in significant qualitative and quantitative losses for hazelnut production (Sevastano, 1918; Linaldeddu *et al.*, 2016; Martino *et al.*, 2025a; Piattino *et al.*, 2026). Effective management of ‘mal dello stacco’ is limited, due to the restricted number of registered active ingredients for hazelnuts, and the lack of knowledge on the disease etiology and epidemiology (Martino *et al.*, 2024). Identification of the causal agent has long been uncertain, with contradictory identifications in literature (Servazzi, 1950; Salerno, 1961; Linaldeddu *et al.*, 2016). Currently, *A. decipiens* is considered the pathogen responsible for ‘mal dello stacco’ symptoms (Linaldeddu *et al.*, 2016; Martino *et al.*, 2025a; Piattino *et al.*, 2026). In this scenario, development of a precise molecular diagnostic tool for *A. decipiens* is essential for pathogen identification, and for control of spread of the disease.

In this study, a SYBR Green qPCR assay was validated for the detection and quantification of *A. decipiens* on *Co. avellana* plants, in accord with EPPO standards PM 7/98 (6) (EPPO, 2025). This assay is the first reported species-specific diagnostic tool developed for *A. decipiens*. Previously, Molnar *et al.* (2013) validated a TaqMan qPCR for *Anisogramma anomala*, which causes Eastern Filbert Blight. Several diagnostic tools based on end-point PCR and qPCR have also been developed for different hazelnut pathogens, including *Erysiphe corylacearum* and *Phyllactinia guttata*, associated with

powdery mildew, and *Pseudomonas avellana* and *Xanthomonas arboricola* pv. *corylina*, the causal agents of bacterial blight (Gervasi and Scortichini, 2009; Baykal, 2020; Bilge *et al.*, 2023; Turco *et al.*, 2023; Minardi *et al.*, 2025; Rojas-Durán *et al.*, 2026). Akbaş *et al.* (2025) optimized a RT-qPCR assay for accurate detection of apple mosaic virus (ApMV) in hazelnut to support certification of virus-free plant material.

SYBR Green chemistry was selected for the present study due to its cost-efficiency and simple assay design, making it suitable for routine and large-scale pathogen screening (Tajadini *et al.*, 2014). Several studies have also reported comparable sensitivity and specificity between SYBR Green methods and TaqMan chemistry (Demitis *et al.*, 2008; Gonzalez-Salgado *et al.*, 2009; Tajadini *et al.*, 2014). The primers used in the present study were designed on the ITS region, the only *A. decipiens* genomic locus broadly sequenced and available in public sequence repositories.

High copy numbers and sequence variability of rDNA units in fungal genomes enable the reliable detection of small amounts of target DNA (Bridge and Spooner, 2001). Previous qPCR assays based on this gene sequence have been reported to successfully detect fungal pathogens in various plant matrices (Salamone and Okubara, 2020; Dharmaraj *et al.*, 2022; Campos *et al.*, 2023; Coronelli *et al.*, 2025). The qPCR assay for *A. decipiens* gave 100% efficacy and a LOD of 100 fg, which are acceptable values comparable with the other diagnostic tools that have been developed for FTD pathogens (Ghasemkhani *et al.*, 2016; Billones-Baaijens *et al.*, 2018; Romero-Cuadrado *et al.*, 2023; Jacobs *et al.*, 2024; Dardani *et al.*, 2025).

A broad panel of *A. decipiens* isolates, fungal trunk pathogens, and pathogen species phylogenetically close to *A. decipiens* were assessed, to confirm the analytical specificity of the qPCR assay. Positive amplification was registered for all the *A. decipiens* isolates, while no signals were observed for off-target species for up to 35 cycles. Beyond 35 cycles, *Dia. eres*, *D. sapinea*, *D. seriata*, *D. subglobosa*, *Eut. leprosa* and *P. scoparia* occasionally showed non-specific amplifications. Based on these results, the Ct 35 was chosen as the limit value to identify false positive results, in accordance with the recommended cycle range of 35-40 cycles in EPPO guidelines (McMullen and Petter, 2014).

The dilution of target DNA in hazelnut wood DNA did not show any significant differences, confirming the selectivity of the assay. Validation on artificially inoculated and naturally infected hazelnut plants confirmed the applicability of the assay under *in planta* conditions.

Wood is a complex matrix containing cellulose, lignin, and numerous secondary metabolites including tannins, phenolics, and polysaccharides, which can inhibit DNA amplifications by interfering with polymerase activity and primer binding (Rossi *et al.*, 2021). Presence of co-infections, often common in trunk diseases and in latent phases, can make pathogen detection inefficient (Abdullah *et al.*, 2018; Negahban *et al.*, 2024; Dardani *et al.*, 2025). The qPCR assay developed in the present research overcame these limitations, allowing direct detection of *A. decipiens* in woody host tissues without interference from the host matrix or other fungal species. In addition, repeatability and reproducibility analyses showed minimal variation among repeated assays performed by one operator or between different operators, demonstrating that the method is robust and reliable for routine diagnostic applications. Overall, these results confirm the suitability of the qPCR assay for detection of *A. decipiens* directly in hazelnut wood under controlled and field conditions, representing an important advance for diagnosis of trunk diseases of hazelnut.

In conclusion, a specific and sensitive SYBR Green qPCR assay has been developed for the detection and quantification (even at low DNA concentrations) of *A. decipiens* in hazelnut wood. To date, no validated species-specific primers were available for this pathogen, highlighting the diagnostic relevance of the developed tool. The assay enables accurate and rapid detection of *A. decipiens* from woody host tissues, and is an important advance for diagnosis and monitoring of this pathogen in hazelnut orchards. Considering the complexity of woody matrices, and the frequent occurrence of co-infections in trunk diseases, ability to specifically detect this pathogen *in planta* can support reliable disease assessments, and improve investigations of pathogen distribution in the field. Nursery propagation material is probably an important pathway for spread of *A. decipiens*, as has been reported for several woody crops, including grapevines, blueberry, apple and almond trees (Retief *et al.*, 2006; Havenga *et al.*, 2019; Stack *et al.*, 2022; Carbone *et al.*, 2022; Luo *et al.*, 2024). Although further validation on propagation material is required, the qPCR assay developed in this study is a promising tool for screening of nursery plants and mother stocks, potentially contributing to reduction of pathogen spread in newly established orchards. Future optimization of this molecular tool could support epidemiological and etiological investigations, including studies on spore production and dispersal, inoculum persistence on pruning residues, and identification of additional inoculum sources.

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