Journal of Plant Diseases and Protection
https://doi.org/10.1007/s41348-022-00594-8

ORIGINAL ARTICLE

# PCR diagnostics for rapid detection of fungi associated with black root rot of strawberries

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Received: 8 February 2022 / Accepted: 24 February 2022


## Abstract

Black root rot is a disease of the strawberry with increasing importance for Germany. Until the early years of the twenty-first century, very different pathogens, including fungi and nematodes, were thought to cause this disease. However, based on substantial research between 2007 and 2014 at the fruit-growing center Jork (Esteburg, Germany) fungi with *Cylindrocarpon*-like anamorphs, i.e., *Dactylonectria torresensis* and *Ilyonectria* spp. were identified as causal agents. Both fungi are soil-borne pathogens and able to infect young strawberry plants in nurseries. Therefore, it is crucial to test plants prior to further propagation for the presence of these organisms. Although speed of diagnosis is a well-known and important factor, until today only time-consuming microbiological tests are available. To improve the situation, we aimed at developing a rapid, PCR-based, assay for the detection of fungi associated with black root rot. Most challenging was the design of specific primers that recognize the pathogen(s) in question while being unspecific for other fungi and Oomycetes routinely found on strawberries. Here, we report on a pair of primers that fulfill these rigorous criteria and present data on their specificity and sensitivity. A concentration of as low as 1.25 pg $\mu l^{-1}$ template DNA from target pathogens could be detected in infected tissue. Significantly, the same primers were successfully used in standard and quantitative PCR assays. Based on our results, diagnostic laboratories are now enabled to rapidly survey lots of strawberry plants and provide growers with precise information on the presence of fungi associated with black root rot even in asymptomatic plants.

**Keywords** Soft fruit · Orcharding · *Cylindrocarpon* spp.

## Introduction

Strawberry is one of the most popular soft fruits with an annual production of almost nine million tones worldwide on approx. 400,000 ha in the year 2019 (FAO 2021). In Europe, Germany was ranked fourth after Spain, the Russian Federation and Poland in the same year, regarding the production of strawberries with about 144,000 tones on 13,200 ha (FAO 2021). Success for growers is threatened by a whole range of different pathogens, among which black root rot is becoming increasingly important. For long it was thought that black root rot was caused by a complex of different fungal and oomycetal species and also root-infecting nematodes were considered. In sum, the disease affects plant growth which in consequence can result in losses at about 30–50%. The disease was reported from the US to be prevalent in Massachusetts, Michigan and New York in 1902–1908 (LaMondia 2004). Also in Germany, the disease has been on the rise since the last quarter of a century (Weber and Entrop 2017). Typical disease symptoms of plants affected by black root rot are stunted growth and, as the name suggests, a brownish to black coloring of the roots. At heavily infected parts of the root system, feeder roots may die or become less fibrous, why they are named "rat-tails".

Propagation of strawberry plants is done vegetatively, which means that nursery plantations are established from selected mother ("elite" class) material. A well-established method for commercialization of such nursery plants is the production of "frigo plants", for which plantlets are dug out in autumn, stored at − 1.5 °C, and then, being sold thereafter during a period of up to 9 months (Capocasa et al. 2019). Usually for plants generated this way plant health certificates have to be provided (EPPO Bulletin 2008).

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Published online: 01 April 2022

Since the disease can spread from contaminated soil, fumigation was practiced in the past, especially in fields where strawberry was cultivated repeatedly (Martin and Bull 2002). Additionally, management strategies were considered which can be applied post-planting and which were effective in slowing down the disease (Miles et al. 2018). Another important source for inoculum is diseased plantlets which are used for different cultivation systems such as open field, tunnel or greenhouse. Because plantlets may be infected by the black root rot fungus asymptomatically, diagnostics to detect pathogens are recommended. This is done by classical microbiological methods, encompassing isolation of the pathogen from infected parts of plants and their cultivation on culture media. Based on morphological characteristics and/or sequencing specimens are identified. This procedure is time-consuming, not suitable for high-throughput and requires well-trained examiners. To overcome the aforementioned shortfalls, a PCR-based assay was established in the frame of this study specifically targeting the fungi causing black root rot while being insensitive to other microorganisms found in association with strawberry plants.

## Materials and methods

### Plant material

Strawberry plants were obtained from KRAEGE Beerenpflanzen GmbH & Co. KG (Telgte, Germany). The plants were grown in ED73 substrate (Balster Einheitserdewerk GmbH, Fröndenberg, Germany) under controlled environmental conditions (day/night cycle: 16 h light, 210 µmol $m^{-2}$ $s^{-1}$ PAR, 20 °C, 65% RH; 8 h dark, 18 °C, 80% RH). Plants for root inoculation experiments were planted in a vermiculite substrate (see below) after washing the roots with tap water.

### Fungal isolates

Isolates of different fungi and oomycetes were provided by Dr. Monika Heupel (Pflanzenschutzdienst Landwirtschaftskammer NRW). This collection encompassed several species from *Dactylonectria* spec. and *Ilyonectria* spec. as target organisms (black root rot causing pathogens), as well as different necrotrophic fungi and oomycetes as non-target organisms (Table 1). For cultivation, all fungal isolates were grown on potato dextrose agar (PDA) containing 50 mg $l^{-1}$ streptomycin and 50 mg $l^{-1}$ penicillin at 22 °C. Oomycetes were plated on vegetable juice agar (V8A) with the aforementioned antibiotics and incubated at 18 °C. Every two weeks all organisms were transferred to a fresh medium.

### Inoculation assay

For optimization of the PCR-assay, we set on an artificial inoculation assay of strawberries with the target organisms. The inoculation protocol was based on the method described by Weber et al. (2017) with some modifications. Bare-rooted plants were washed and directly potted in vermiculite (type "Palabora" 3–6 mm, Deutsche Vermiculite Dämmstoff GmbH, Sprockhövel, Deutschland). The vermiculite was mixed with fungal mycelium from PDA agar plates at 14 d after sub-culturing (mycelium of two fungal colonies per 1000 ml vermiculite substrate). These plants were watered by soil drench application approximately every 2–3 days by bottom watering, carefully avoiding waterlogging.

**Table 1** Overview of fungi associated with black root rot in strawberries (target organisms) and other pathogenic fungi associated with strawberries (non-target organisms)

| ID | Target organisms | Non-target organisms |
|---|---|---|
| #03 | *Ilyonectria* spec. 19-4385 W | |
| #05 | *Ilyonectria* spec. 19-3357 | |
| #09 | *Ilyonectria* spec. 19-3237 | |
| #13 | *Dactylonectria* spec. 19-3352 | |
| #14 | *Ilyonectria* spec. 18-4283 | |
| Cyl | *D. torresensis* (Weber et al., 2017) | |
| Fus | | *Fusarium* spec. |
| Bot | | *Botrytis* spec. 16-8363 |
| Rhiz | | *Rhizoctonia* spec. |
| Scler | | *Sclerotinia* spec. |
| Ver | | *Verticillium spec.* |
| #P1 | | *Pythium ultimum* |
| #P2 | | *Phytophthora cactorum* 18-3008 |
| #P3 | | *Phytopythium* spec. 15-3466 |
| #P4 | | *Phytophthora citricola* 14-6257 |
| #P5 | | *Phytophthora cinnamomi* 17-5022 |

Fertilization was done once with 50 ml 0.2% Wuxal Super (Hauert Manna Düngewerke GmbH, Nünberg, Germany).

### DNA extraction

DNA was extracted from plants or fungal tissue after freezing by grinding in liquid nitrogen. Then, the resulting powder was subjected to a cetyltrimethylammonium bromide (CTAB)-buffer-based protocol as described in Primiano et al. (2019). Additionally, DNA extraction kits were tested as they are more suitable for a routine use in diagnosis. Finally, the DNeasy Plant Pro Kit (QIAGEN GmbH, Hilden, Germany) was selected because it performed best for strawberry leaves and roots, when used with modifications indicated for strawberry plants in the manufacturer's manual.

### Primer design, PCR and qRT-PCR assays

The end-point PCR was performed on a peqSTAR thermocycler (PEQLAB/VWR international GmbH, Erlangen, Germany) using the SilverStar Taq-DNA-Polymerase (Kaneka Eurogentec S.A., Seraing, Belgium) according to the manufacturer's instructions. The identity of fungal and oomycete isolates, and simultaneous check for their integrity, was verified by PCR at 50 °C annealing temperature with the primer pair ITS1: 5'-CCGTAGGTGAACCTGCGG-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3' (White et al. 1990). To confirm the integrity of plant DNA, a short fragment of the plant-specific *rbcL* gene was amplified at 52 °C annealing temperature as described in Primiano et al., 2019 (plant_fw_1: 5'-CTTCTACTGGTACATGGAC-3', plant_rev_1: 5'-GAAGTAAACATGTTAGTAACAGA-3'). A primer pair, specific for the black root rot-associated fungi, was designed based on an educated guess using sequences of single-copy genes for *Ilyonectria* spp. and *Dactylonectria* spp. Primers were compared to sequences of non-target organisms to avoid the chance of binding. A conserved domain search (with the full-length primer sequences (https://www.ncbi.nlm.nih.gov/cdd/) revealed a conserved domain identified as a metallo-dependent hydrolase belonging to the amidohydrolase superfamily (accession cl00281, cd01298) as a binding target for the primers. Hence, the primer pair was named "amdhyd". Based on the sequence information of available *Ilyonectria* spec. and *Dactylonectria* spec. sequences from JGI Mycocosm (https://mycocosm.jgi.doe.gov) the primer pair was first designed with ambiguous bases (amdhyd_fw_1: 5'-GCTTGCA(Y = C/T)ATCCACCTTTGCG-3', amdhyd_rev_1: 5'-TCGAA(R = A/G)GCGTCGATTCCCAT-3') and later optimized without the ambiguous bases (amdhyd_fw_2: 5'-GCTTGCATATCCACCTTTGCG-3', amdhyd_rev_2: 5'-TCGAAAGCGTCGATTCCCAT-3'). The PCR was conducted at 60 °C annealing temperature (initial denaturation phase 5', 95 °C; 36x [denaturation for 20'' at 95 °C; annealing for 10'' at 60 °C; elongation for 10'' at 72 °C]; final extension for 5' at 72 °C). The quantitative real time-PCR (qRT-PCR) was performed on a CFX384 Real-Time System using the iTAQ Universal SYBR Green Supermix (both Bio-Rad Laboratories GmbH, Feldkirchen, Germany) according to the manufacturer's instructions. The parameters were set as follow: 60 °C annealing temperature (initial denaturation phase 5', 95 °C; 40× [denaturation for 15'' at 95 °C; annealing and elongation for 1' at 60 °C]. Melt curves were analysed from 65 °C to 95 °C in 0.5 °C increments).

## Results

A basic requirement for a molecular assay in diagnostics is its specificity for the targeted organisms. Therefore, we firstly collected a range of different pathogens which are known to cause disease on, or found in association with, strawberries. Most of these pathogens were obtained from the diagnosis lab from the Landwirtschaftskammer North Rhine-Westphalia (Cologne, Germany) (Table 1). Our collection encompassed isolates of *Ilyonectria*- and *Dactylonectria* species which, according to the actual literature, are most likely the causal agents of black root rot of strawberries (Weber and Entrop 2017). In addition, isolates of *Pythium* spp. different *Phytophthora* spp., *Rhizoctonia* spp., *Sclerotinia* spp., *Fusarium* spp., *Verticillium* spp., and *Botrytis* spp. were present in the stock (Table 1). While *Ilyonectria*- and *Dactylonectria* species were referred to as target organisms, the other isolates are termed non-target organisms in this study.

At first, we verified that *Ilyonectria*- and *Dactylonectria* species from our collection are capable to cause typical black root rot disease symptoms on strawberries like stunted growth and black roots (Fig. 1A, B). Therefore, an artificial inoculation procedure was established using vermiculite as a substrate which was mixed with crushed agar overgrown with respective isolates. After 21 days all target isolates were able to cause blackening of roots to some degree (Fig. 1E, F), while stunting of the above-ground parts of the plants was not always visible (Fig. 1C, D). A single isolate from our collection (# 3) led to pronounced blackening of the roots and severe stunting of the seedling in comparison to plants inoculated with other isolates from the group of target organisms (Fig. 1D, F).

Next, a gene sequence was selected which is present in the target organism(s) while being not present or sufficiently different in the host or other microbes associated with the host. This candidate gene encodes for a 1353 bp transcript (protein id: 1,518,754 in the *Ilyonectria* sp. Assembly v. 1.0; see https://mycocosm.jgi.doe.gov/Ilysp1/Ilysp1.info.html). The deduced protein was predicted to contain an amidohydrolase


**Fig. 1** Symptoms of black root rot of strawberry. Strawberry plants affected by black root rot have smaller or discolored leaves (**A**) and a reduced root system with partially black roots (**B**). Isolates of Ilyonectria spp. and Dactylonectria spp. used in this study were inoculated on strawberry plants via a mixture of mycelium and vermiculite substrate and evaluated at 21 days after inoculation. Varying degrees of disease symptoms, ranging from almost no disease symptoms on above-ground parts (**C**) and only some blackish roots (**E**) were found as well as plants showing severe stunting (**D**) and completely black roots (**F**). Plants shown in (**D**) and (**F**) were inoculated with isolate #3. Scale bars in (**D**) and (**F**) correspond to 5 cm

domain (InterPro ID IPR006680) (Liao et al. 2019). Further analyses revealed that a part of this gene is nearly identical in several *Ilyonectria*- and *Dactylonectria* species, and hence, a degenerated primer pair targeting this fragment of the gene was developed (primer pair_ amdhyd_fw_1 and amdhyd_rev_1). To test whether this primer pair could be used for the differentiation of target and non-target organisms, genomic DNA was extracted from pure cultures of all organisms (Table 1). Quality and quantity of the extracted DNA were analyzed spectrophotometrically. In addition, the suitability of the DNA for PCR was verified by amplifying a part of the internal transcribed spacer (ITS) region with the ITS1 and ITS4 primer pair. The distinct PCR products obtained, confirmed DNA quality (Fig. 2A). A PCR was performed with

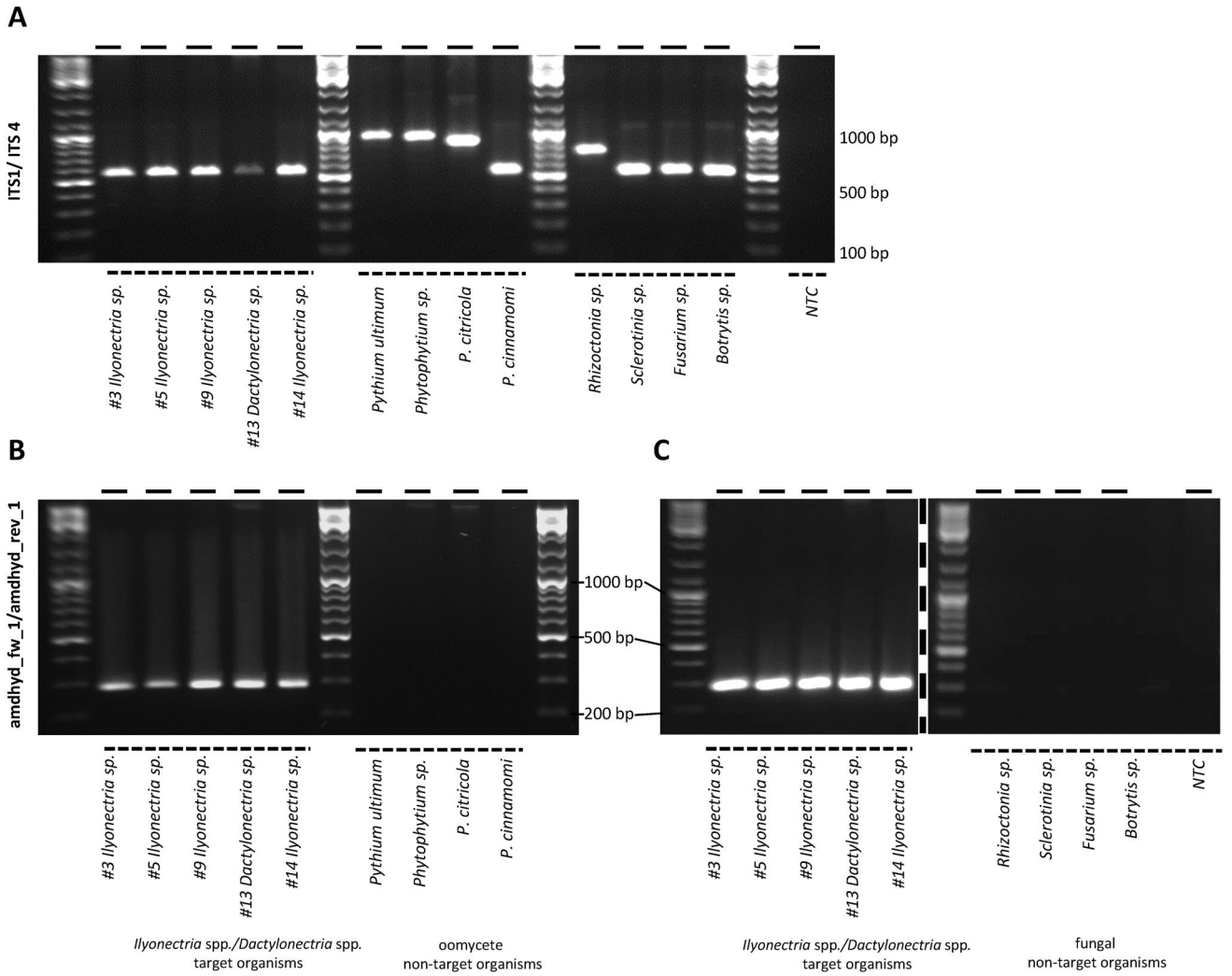



**Fig. 2** Analysis of target species specificity of PCR reactions. Genomic DNA was extracted from pure cultures of oomycete- and fungal pathogens used in this study. **A** PCR with primer pair ITS1 and ITS4. **B** The same DNA samples were used in a PCR with the primer pair amdhyd_fw_1 and amdhyd_rev_1. PCR was performed at 61 °C and with 1.25 mM $MgCl_2$. **C** PCR with the same primers used in (**B**) but at 63 °C and with 2 mM $MgCl_2$. PCR products with target gene primers were obtained only for *Ilyonectria*- and *Dactylonectria* species, and no product was obtained for oomycete- (**B**) and fungal non-target organisms (**C**). "NTC" = no template control

the same DNA samples and the primer pair amdhyd_fw_1 and amdhyd_rev_1. Applying different PCR conditions, a gene fragment of 286 bp size was generated only when DNA from the *Ilyonectria*- and *Dactylonectria* target species were used (Fig. 2B, C). Conversely, no PCR product was detected for DNA from the non-target organisms. Additionally, we tested whether this was also valid for *Verticillium* spec. and *Colletotrichum* spec., both of which are non-target organisms, which we received during the course of experiments. DNA from both of the latter mentioned fungi failed to yield a PCR product with the target primer pair, further confirming their specificity (not shown).

All PCR products, either generated by using the ITS primer pair ITS1 and ITS4 or with primer pair amdhyd_fw_1 and amdhyd_rev_1, were purified from agarose gels and sequenced. Based on the combined sequence data from both PCRs, a phylogenetic analysis was performed. In addition, corresponding sequences from five *Ilyonectria*- and *Dactylonectria* isolates obtained from genome databases, identified by BLAST analyses, were fed into the analysis (Supplemental data file 1). After alignment of all sequences with ClustalW, a Maximum Likelihood analysis was conducted using the program MEGA X (Kumar et al. 2018). The analysis revealed, that isolate #13-19-3352 clustered together with the *D. torresensis* and *D. macrodidyma* isolates mentioned in the study by Weber and Entrop (2017) and obtained from public databases, respectively (Fig. 2). This confirmed that our isolate #13-19-3352 belongs to the genus *Dactylonectia*. By contrast, all other isolates (#03, 09, 05, and 14) were assigned to the genus *Ilyonectria* based

on their clustering with confirmed *Ilyonectria* species from literature (Liao et al. 2019, Fig. 3).

So far, our results have confirmed the specificity of the target gene primer pair for pathogens causing black root rot of strawberry. The next series of experiments were designed to assess the sensitivity of the PCR-assay, i.e. determine the detection limit in mixed samples of fungal and plant DNA. Therefore, the same DNA from pure fungal isolates was used as in the experiments shown in Fig. 2. Firstly, the DNA was diluted with pure water resulting in samples containing 25, 2.5, 0.25, 0.025, or 0.0025 ng $\mu l^{-1}$ DNA of *Dactylonectria* sp. isolate #13 in PCR reactions (Fig. 4A). The highest dilution corresponding to 2.5 pg $\mu l^{-1}$ DNA still yielded a clearly visible PCR product on the agarose gel. Next, we simulated the situation for infected strawberry plants by diluting fungal DNA with strawberry DNA. For all PCR reactions, the total amount of DNA, composed of fungal and plant DNA, was consistently set to 50 ng $\mu l^{-1}$. Also in this case, the highest dilution, containing only 2.5 pg $\mu l^{-1}$ fungal DNA, yielded a definite PCR product (Fig. 4B). As a control, the same samples used in the latter assay were subjected to a PCR with primers specific for a plant gene (*rbcL* gene). The concentration of plant DNA in these samples ranged from 25 to nearly 50 ng $\mu l^{-1}$, which was in any case high enough to yield a pronounced PCR product, and wherefore, no differences between dilution steps were detectable (Fig. 4C).

The usefulness of any diagnostic assay is related to the accuracy of the results. In this regard, end point PCR reactions have to be handled with care because samples with very low amounts of target species DNA, which nonetheless yield clear PCR products, are prone to be over-interpreted. A way to overcome this drawback is the use of a quantitative real-time PCR (qRT-PCR) in which the increase of PCR products over time, which is directly correlated with the amount of target DNA in the sample reaction, can be monitored. The primer pairs established in this study have been designed right from the beginning to possibly work in qRT-PCR assays. The qRT-PCR was exclusively done with the primer pairs without ambiguous bases, amdhyd_fw_2 and amdhyd_rev_2. To confirm performance, primer pairs were tested on dilution series of pure fungal or strawberry DNA (Fig. 5A + D). Consistent with the results shown above, fungal and strawberry DNA were both detectable at a concentration as low as 0.125 pg $\mu l^{-1}$ DNA. Standard curves calculated from the results established a primer efficiency for the fungal target primer pair at 101% and the plant-specific primer pair at 115% (Fig. 5B + E). The uniform melting points in each of the underlying reactions confirmed that no side products were formed during qRT-PCR which further substantiated the accuracy of PCR conditions used (Fig. 5C + F). Finally, we tested whether these promising results would also withstand if field samples of infected strawberry roots were used. A respective sample, which was confirmed by re-isolation of the fungal pathogen(s) to be infected with black root rot, was used to extract DNA which then was channeled to qRT-PCR analyses. Comparing pure fungal, pure strawberry and field sample DNA, respectively, in a single PCR run verified the suitability of the assay for the filed sample (Fig. 5G + H). Results obtained further confirmed that the same qRT-PCR conditions can be used for the detection of fungal and strawberry DNA which makes the assay very user-friendly for diagnostic labs.

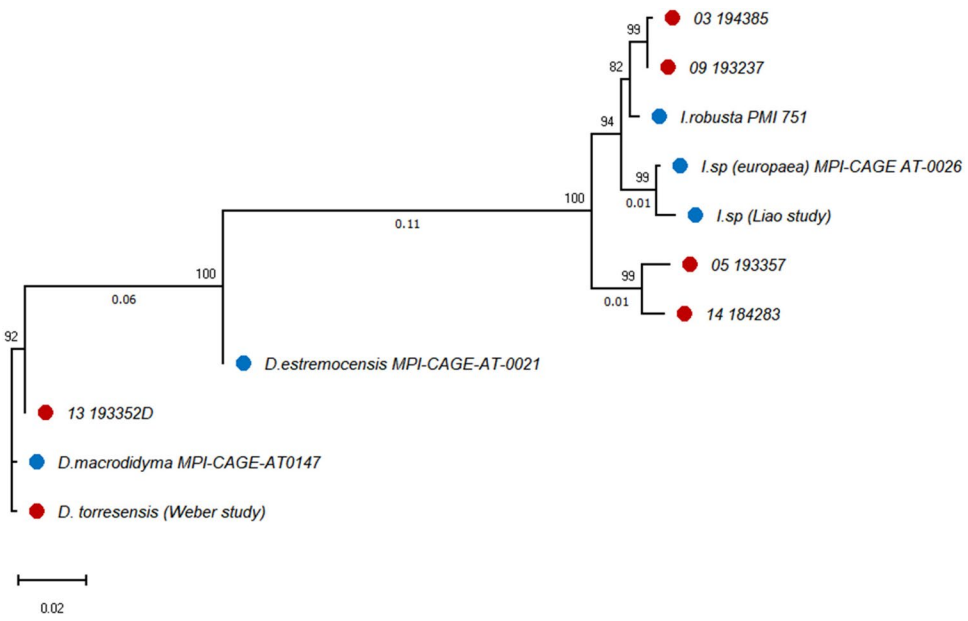



**Fig. 3** Phylogenetic Maximum Likelihood-tree based on ITS- and target gene sequences. The evolutionary history was inferred by using the Maximum Likelihood method on a ClustalW alignment of the concatenated sequences of the PCR products amplified with ITS1/ITS4 or amdhyd_fw_1/amdhyd_rev_1 primer pairs. The fungal isolates from strawberry used in this study are marked with red dots and isolates from literature and public databases are indicated by blue dots. Evolutionary analyses were conducted in MEGA X

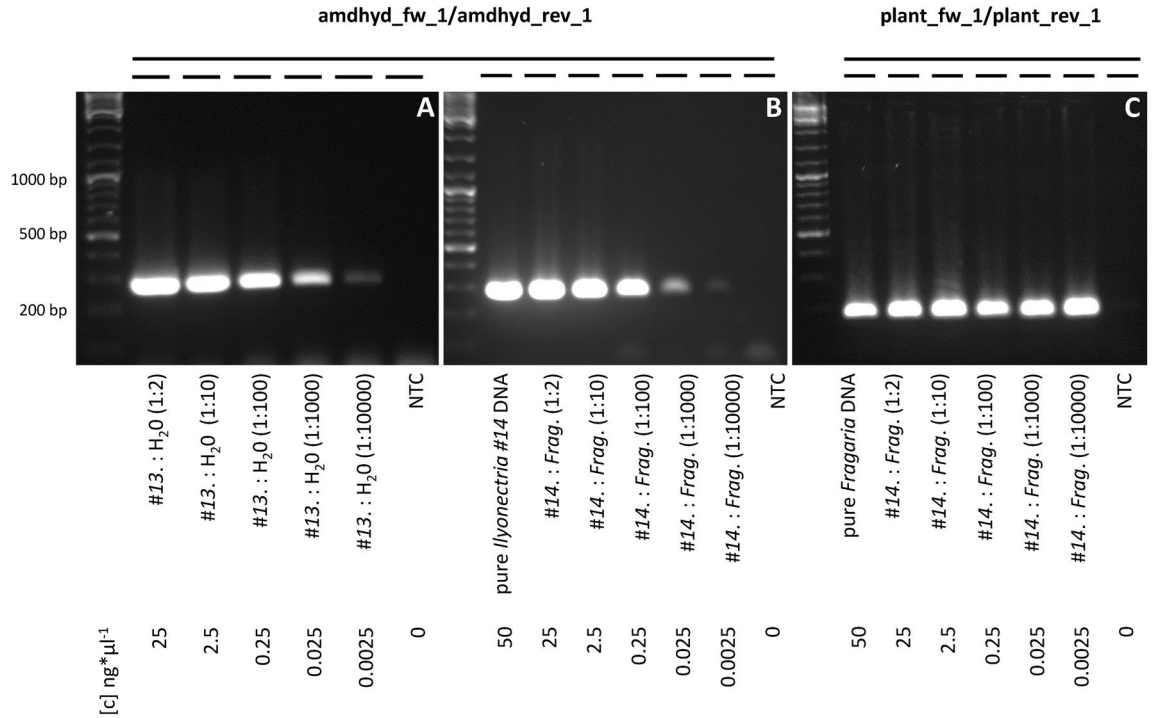
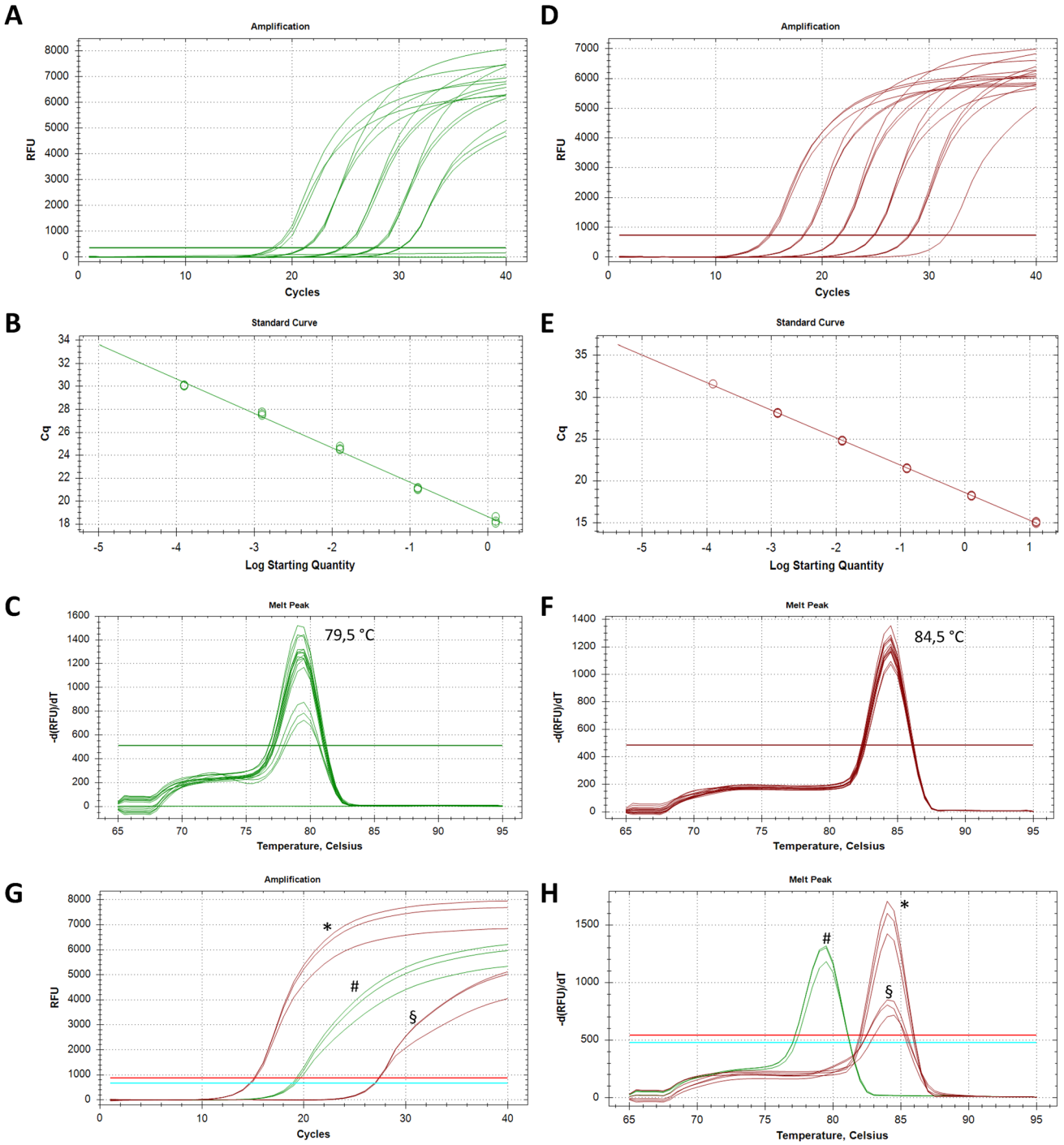



**Fig. 4** Analysis of detection sensitivity of target primer pair. DNA from pure cultures of the *Dactylonectria* sp. isolate #13 and the *Ilyonectria* sp. isolate #14 as well as the host plant *Fragaria* x *ananassa* cv. Rumba were used in dilution series as template for PCR reactions. In **A** the results of a target gene primer PCR with DNA of isolate #13 diluted with pure water is shown. The dilutions from 1:2 to 1:10,000 correspond to 25, 2.5, 0.25, 0.025, and 0.0025 ng*µl$^{-1}$ DNA of which 1 µl was as template in 20 µl PCR reactions. **B** and **C** depict the results of PCRs with similar template dilution series. In these experiments, fungal DNA was diluted with plant DNA in order to simulate samples of infected plant tissue. The total amount of DNA in all dilution steps was kept identical (50 ng*µl$^{-1}$), starting at 25 ng*µl$^{-1}$ fungal DNA mixed with 25 ng*µl $^{-1}$ plant DNA (1:2 dilution). One microliter of the highest dilution contains 0.0025 ng fungal DNA and 49.9975 ng plant DNA. **B** shows the results of the PCR reaction with the target gene primer pair and **C** shows the results of PCR reactions with the plant specific primer pair, using the same samples as in (**B**) as a control. "NTC" = no template control

## Discussion

Worldwide, plant diseases are on the rise and considerably threaten food production. This is especially true for soil-borne diseases which are increasingly important, e.g. in Finnish strawberry production (Parikka and Latvala 2021). Precise monitoring of the presence or absence of certain pathogens and advice on appropriate countermeasures are mandatory to avoid unnecessary use of fungicides. In this respect, PCR-based methods in combination with sequencing have become popular for accurate plant disease diagnostics (Hariharan and Prasannath 2021). Black root rot of strawberry is a disease known for a long time but relatively new to Europe as a whole and Germany in particular. This disease is caused by pathogens classified as *Ilyonectria* spp. and *Dactylonectria* spp. (Weber and Entrop 2017). In the course of this study, a PCR-based method was established which guaranteed the specific detection of the before-mentioned pathogens while being inert for other filamentous plant pathogens which might also occur on strawberries in fields. Until now, standard diagnostic techniques, such as isolation, culturing and microscopic investigations, were routinely used for the detection of black root rot pathogens on strawberries. While these methods need their time to yield reliable results, farmers are under the pressure to decide whether plant material is healthy and can be sold or used in commercial cultivation. The latter is especially true for plants infected asymptomatically. The before-mentioned circumstances prompted us for the development of a novel PCR-based assay for the rapid and accurate detection of black root rot pathogens on strawberries.

In cooperation with the plant protection service in North Rhine-Westphalia, a collection of different filamentous plant pathogens known to be found routinely on strawberries has been established (Table 1). In accordance with results published by Weber and Entrop (2017), it was verified that isolates belonging to *Ilyonectria* spp. and *Dactylonectria* spp. are capable to infect strawberries and produce disease symptoms typical for black root rot (Fig. 1). DNA from all organisms listed in Table 1 was used in a PCR reaction with a pair of primers targeting a gene fragment which is nearly identical in *Ilyonectria* spp. and *Dactylonectria* spp. While




these PCR reactions yielded a clearly detectable product for target organisms no product was found for the non-target organisms (Fig. 2), which pointed to the usability of methods established so far. Sequencing of these PCR products and PCR-fragments generated by the use of an ITS primer pair, enabled the construction of a phylogenetic tree which further substantiated the identity of isolates used in this study (Fig. 3). Next, the robustness and sensitivity of the PCR detection assay were verified in experiments where mixtures of fungal and plant DNA were used as templates (Fig. 4). As low as 2.5 pg*µl$^{-1}$ DNA was sufficient to produce a definite result regardless of whether water or strawberry DNA was used for generating serial dilutions. With these promising results in hand, with went the final step and converted the end-point PCR to a qRT-PCR. The advantage of the latter method lies in its sensitivity, the accuracy and the potential of improvement to a medium or high throughput assay. As expected from the way in which we designed

◂**Fig. 5** Establishment of a diagnostic qRT-PCR assay for black root rot of strawberry. DNA from pure cultures of *Fragaria* x *ananassa* cv. Rumba and the *Ilyonectria* sp. isolate #3 were used in dilution series as template for qRT-PCR reactions. In **A** the results of qRT-PCR reactions with plant specific primers on strawberry DNA are shown. DNA was stepwise diluted ranging from 1:10 to 1:100,000 which corresponded to 1.25, 0.125, 0.0125, 0.00125, and 0.000125 ng*µl$^{-1}$ DNA of which 2 µl were used as template in a 10 µl PCR reaction. **B** depicts the standard curve corresponding to **A** which allows to calculate the primer efficiency at 115% ($R^2$ 0.996, slope −3.002) and **C** shows the respective melting point analysis with an uniform melting point of 79.5 °C. In **D** the results of qRT-PCR with primers specific for *Ilyonectria* sp. are shown. DNA was diluted similarly as shown in A) ranging from 1:2 to 1:100,000 which corresponded to 12.5, 1.25, 0.125, 0.0125, 0.00125, and 0.000125 ng*µl$^{-1}$ DNA of which 2 µl were used as template in 10 µl PCR reaction. **E** depicts the corresponding standard curve (primer efficiency 101%, $R^2$ 1.0, slope −3288) and in (**F**) the results of the melting point analysis are shown (uniform melting point of 84.5 °C). A field sample of infected root material was used to test qRT-PCR with both primer pairs in a sample composed of DNA from both organisms (**G**, **H**). **G** Amplification curves correspond to pure fungal DNA (*) amplified with the fungal target primer pair and DNA from the infected root sample amplified with either the plant specific primer pair (#) or the fungal target primer pair (§). **H** Melting curves depicted correspond to samples shown in (**G**)

the before-mentioned primer pairs, they could be transferred smoothly to qRT-PCR conditions. Quality testing of each primer pair has been performed by running qRT-PCR reactions on serial dilutions of pure fungal or strawberry DNA. Thus, primer efficiency calculations and melting point analyses underlined the suitability of this new tool. This was finally confirmed by the successful application on a field sample from an infected plant in which we definitively detected the black root rot pathogen (Fig. 5).

Based on the results presented, a novel PCR-based assay is made available to the community which enables the accurate detection of black root rot pathogens on strawberries. This provides a handy assay for routine checking of potential infections in breeding material, which is a prerequisite to guarantee propagation of healthy strawberries.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s41348-022-00594-8.

**Acknowledgements** Marcus Staden from Kraege Beerenpflanzen GmbH & Co.KG, Telgte, Germany is kindly acknowledged for financial support and helpful discussion on strawberry cultivation practice.

**Authors' contribution** ML, LV, SPDS, and SE performed experiments and evaluated results. ML and MH were involved in the experimental design and finalizing of the manuscript. US designed the study, accompanied the experiments, drafted and finalized the manuscript.

**Funding** Open Access funding enabled and organized by Projekt DEAL.

**Data availability** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Conflict of interest** This work was funded in part by Kraege Beerenpflanzen GmbH & Co.KG, Telgte, Germany.

**Ethical approval** In the frame of this study, no experiments have been conducted on animals or humans.



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