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To cite this article: Seyed Mahyar Mirmajlessi, Inmaculada Larena, Marika Mänd & Evelin Loit (2016) A rapid diagnostic assay for detection and quantification of the causal agent of strawberry wilt from field samples, Acta Agriculturae Scandinavica, Section B — Soil & Plant Science, 66:7, 619-629, DOI: 10.1080/09064710.2016.1205656

To link to this article: https://doi.org/10.1080/09064710.2016.1205656

Published online: 10 Jul 2016.
A rapid diagnostic assay for detection and quantification of the causal agent of strawberry wilt from field samples

Seyed Mahyar Mirmajlessia, Inmaculada Larenab, Marika Mändc and Evelin Loita

ABSTRACT
Verticillium dahliae Kleb., the cause of Verticillium wilt disease, is a destructive pathogen that leads to severe yield losses in strawberry fields and thus considerable economic damages. Although rapid identification and detection methods are becoming available more, pathogen quantification remains one of the main challenges in the disease management. In this study, a real-time polymerase chain reaction (rtPCR) assay was developed to quantitatively assess V. dahliae abundance directly from affected roots and soil collected from different areas in Estonia. A specific primer pair based on the ribosomal DNA (rDNA) internally transcribed spacer was designed for SYBR Green-based assay. Strawberry plant and soil samples were randomly collected from different areas in Estonia and analyzed for V. dahliae by soil plating technique and rtPCR assay. The assay was specific for V. dahliae so that the minimum detection limit was 0.93 pg µl\(^{-1}\) of pathogen DNA and the lowest amount of V. dahliae detected in soil was 10.48 pg µl\(^{-1}\) of target DNA corresponding to one microsclerotia per gram of soil. This technique allowed rapid detection and quantification of the pathogen DNA at the picogram level in soils and even in symptomless plants, facilitating the screening of the pathogen in diverse areas. This is the first study about the rtPCR technique being used successfully to assess populations of V. dahliae with high specificity and sensitivity in Estonia strawberry fields. Results of this research can be useful for growers and agricultural organizations to improve available disease management strategies against Verticillium wilt.

ARTICLE HISTORY
Received 5 April 2016
Accepted 21 June 2016

KEYWORDS
Microsclerotia; quantitative detection; strawberry; SYBR Green-based assay; Verticillium wilt

Introduction
Strawberry (Fragaria \(\times\) ananassa) is one of the most commercially important fruit crops in European and Scandinavian countries, and its cultivation has been developed in Estonia in recent years. Strawberry diseases caused by soil-borne fungi are a major limiting factor that severely impacts plant performance and leads to economic losses (De Cal et al. 2004). Verticillium dahliae Kleb., the cause of Verticillium wilt disease, is an economically important soil-borne pathogen worldwide that can cause significant strawberry crop losses, even at low inoculation densities (Bhat & Subbarao 1999; Mirmajlessi et al. 2015a). The fungus can survive independent of its proper host plant for prolonged periods in the soil by producing multicellular and melanized structures (Perry & Evert 1982; Pérez-Jiménez et al. 2012) known as microsclerotia (MS). Since inoculum densities of as few as 2 MS g\(^{-1}\) soil can result in plant contamination with latent infection (Harris & Yang 1996), information about the existence and amount of the pathogen in the soil can be an important factor in determining suitable control strategies. So, there is a need for a quick and precise method to assess the inoculum densities of V. dahliae in the field.

The conventional methods, which can take several days, to identify the pathogen have often relied on the symptoms and culturing of pathogen followed by morphological observations, which are time consuming and laborious (Lievens et al. 2006). Polymerase chain reaction (PCR)-based technology using specific primers have been described for the detection and identification of V. dahliae in plant tissue (Hu et al. 1993; Li et al. 1999; Dan et al. 2001) and soil (Nazar et al. 1991; Platt & Mahuku 2000; Mahuku & Platt 2002; Kageyama et al. 2003). Generally, these methods are more sensitive, accurate, and faster than conventional methods. In addition, ribosomal DNA (rDNA) internally transcribed spacer (ITS) regions encompassing the 5.8S rDNA are the regions most

Accurate quantification of the target DNA is not possible using conventional PCR (cPCR). However, techniques using real-time PCR (rtPCR) will provide a more accurate quantification of pathogen with a higher level of sensitivity (Bilodeau et al. 2012). rtPCR is an ideal system that allows accurate and sensitive detection and quantification of targets that cannot be extracted or cultured easily from tissue, or are present at low inoculum load in samples (Bustin et al. 2009). This technology has even multipurpose applications in phytopathology and provides conclusive results as it can even discriminate between closely related microorganisms (Cooke et al. 2007; Schena et al. 2013; Mirmajlessi et al. 2015b). Increasingly, rtPCR is being used for the detection and/or quantification of V. dahliae on different hosts (Fradin & Thomma 2006; Lievens et al. 2006; Atallah et al. 2007; Gayoso et al. 2007; Banno et al. 2011; Debode et al. 2011; Bilodeau et al. 2012; Wang et al. 2013).

As the necessity for a fast, sensitive, and specific method to quantify V. dahliae is critical for disease management, rtPCR has been considered the gold standard technique for pathogen quantification in recent years. To date, although several articles have already been published on PCR-based methods for the detection of this fungus, there are no data about the accurate detection and quantification of V. dahliae using rtPCR in Estonia strawberry fields. Therefore, the main objectives of the current research were to (i) develop a sensitive and specific real-time PCR assay (based on SYBR Green chemistry) for the detection and quantification of V. dahliae directly from field-grown strawberries and soil in Estonia and (ii) enumerate the abundance of V. dahliae in the soil using the traditional soil plating technique and compare these estimates with data from quantitative rtPCR.

**Materials and methods**

**Sample collection**

Plant and soil samples were collected at random from several strawberry fields located in different production sites of Estonia (Vasula, Rohu, Unipha, Utsu, and Marjamaa) during 2014–2015. Sampling areas were suspected of being infested with wilt diseases and plant samples were mostly collected based on visible symptoms of Verticillium wilt such as drying, and marginal and interveinal browning on outer leaves. Soil samples (500 g each) were also collected at random from different points (depth of 12–15 cm) in each field and placed in plastic bags until use. Plant and soil samples were then divided into two parts. The first part was used for pathogen isolation by culturing methods, and the second part was subjected to molecular experiments in order to detect and quantify the pathogen as described below.

**Fungal cultures**

The fungal isolates used in this research were saprobic and pathogenic fungi that are usually found in soil and causing similar wilt symptoms on strawberry (i.e. V. dahliae, Verticillium albo-atrum, Verticillium longisporum, Fusarium oxysporum f.sp. fragariae, Fusarium solani, Rhizoctonia spp., Rhizopus spp., and Pythium spp.). Isolates of V. dahliae (VD9, VD12a, and VD4), V. albo-atrum, and F. oxysporum f.sp. fragariae were kindly provided by Agricultural National Research Institute (INIA), Madrid, Spain. Isolates from other genera in this study were also obtained from North Dakota State University (Department of Plant Pathology), USA. Isolates from other genera in this study were also obtained from North Dakota State University (Department of Plant Pathology), USA. Isolates from other genera in this study were also obtained from North Dakota State University (Department of Plant Pathology), USA.

**Isolation of fungi**

To isolate fungi from strawberry fields, 1 g soil per each sample was diluted in 9 ml of sterile water and then six 1:10 serial dilutions were prepared. Each dilution (200 μl) was spread on plates of PDA-rose-bengal medium containing tetracycline (30 μg ml⁻¹) as described by Dhingra and Sinclair (1985), and incubated at 26°C for 8 days. To isolate fungal pathogens from strawberry plants, parts of root were washed in 75% ethanol, disinfected superficially with sodium hypochlorite for 3 min, rinsed several times in sterile water, and dried. Then, cross-sections were made under an aseptic condition and transferred onto acidified potato-dextrose agar (APDA). The APDA contained 1.5 ml of 25% lactic acid per liter. All plates were incubated at 26°C for two weeks. Totally, morphologically dissimilar colonies were removed to fresh PDA
Table 1. Fungal isolates used in this study to evaluate primer specificity for the specific detection of V. dahliae.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Abundance</th>
<th>Source</th>
<th>PCR amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. oxysporum f.sp. fragariae</td>
<td>3</td>
<td>Strawberry plant</td>
<td>+</td>
</tr>
<tr>
<td>F. solani</td>
<td>2</td>
<td>Tomato plant</td>
<td>+</td>
</tr>
<tr>
<td>Pythium spp.</td>
<td>2</td>
<td>Unknown</td>
<td>+</td>
</tr>
<tr>
<td>Rhizoctonia spp.</td>
<td>4</td>
<td>Soil</td>
<td>+</td>
</tr>
<tr>
<td>Rhizopus spp.</td>
<td>1</td>
<td>Soil</td>
<td>+</td>
</tr>
<tr>
<td>Unknown</td>
<td>6</td>
<td>Strawberry plant and soil</td>
<td>+</td>
</tr>
<tr>
<td>V. dahliae, VD9</td>
<td>1</td>
<td>Tomato plant</td>
<td>+</td>
</tr>
<tr>
<td>V. dahliae, VD12ab</td>
<td>2</td>
<td>Tomato plant</td>
<td>+</td>
</tr>
<tr>
<td>V. dahliae, VD4ab</td>
<td>2</td>
<td>Tomato plant</td>
<td>+</td>
</tr>
<tr>
<td>V. dahliae</td>
<td>8</td>
<td>Strawberry plant</td>
<td>+</td>
</tr>
<tr>
<td>V. albo-atrum</td>
<td>22</td>
<td>Soil</td>
<td>+</td>
</tr>
<tr>
<td>V. longisorum</td>
<td>1</td>
<td>Canola plant</td>
<td>+</td>
</tr>
</tbody>
</table>

Notes: +, amplification of expected PCR product; −, no PCR band on the electrophoretic gel. ND, not done.

aAbundance of isolates.
bCodes proceeded by VD9, 12, and 4 are accession numbers of isolates obtained from INIA, Madrid, Spain.
cIsolates collected from strawberry fields in this study.
dIsolates provided by Department of Plant Pathology, North Dakota State University, USA.

DNA extraction

Genomic DNA of fungal pure cultures shown in Table 1 was extracted from 50 mg of lyophilized fungal mycelium using DNasey Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. Double-distilled water (40 µl) was added to dissolve extracted DNA and then stored at −20°C until use. To extract DNA from strawberry plants, root pieces were placed in liquid nitrogen and severely pulverized with a mortar and pestle. The total DNA was then extracted using DNasey Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. To extract DNA directly from soil, 150 g soil per sample (depth of 12–15 cm) was taken from selected points of each field and placed in plastic bags before DNA extraction. Then, around 2 g soil was subjected to total DNA extraction using a PowerSoil® DNA Isolation Kit (MoBio), according to the manufacturer’s instructions. DNA quality was determined by agarose gel electrophoresis and DNA quantity was measured by NanoDrop 2000 Spectrophotometer (Thermo Scientific) at 260 nm. The extracted DNA was adjusted to a final concentration of 20 ng µl⁻¹ with Tris-EDTA buffer and stored at −20°C until further use.

Primer design

Total genomic DNA was initially amplified via cPCR using ITS1 (TCCGTAGGTAACCTGCGG) and ITS4 (TCCTCCGCTATTGATATGC) primers to confirm the existence of amplifiable DNA (White et al. 1990; Kernaghan et al. 2007). Amplification was done in 25 µl reaction volume containing 10 ng of DNA template, 2U Taq DNA polymerase (Thermo Scientific, ABgene®, UK), 0.1 mM dNTP solution (Eurogentec, Germany), 1.5 mM MgCl₂, 1× PCR buffer, and 0.1 µM of each primer. PCR mixtures were run on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, USA) using the following conditions: initially 95°C for 3 min to denature the DNA, followed by 40 cycles of 1.30 min at 95°C, 1 min at 55°C, 2 min at 72°C, followed by 10 min at 72°C. Control reactions, in which no DNA template was present, were performed with fungal DNA to test for possible contamination of the reagents. rtPCR primer set was designed based on the ITS1 and ITS2 regions. For this, the region between the small and large subunits of the rRNA gene of different Verticillium spp. was amplified and sequenced by Illumina MiSeq sequencer (Genome Center of Tartu University). Following sequence alignment by the ClustalW algorithm with related ITS sequences found in GenBank, species-specific primer was designed using the web-based program Primer3 and checked to discover similar sequences for the amplicons using basic local alignment search tool (BLAST) analysis (Altschul et al. 1990). Specificity of this primer pair was assessed using cPCR against different isolates of the strawberry pathogens as listed in Table 1. The PCR mixtures of 25 µl contained 2 mM MgCl₂, 200 µM dNTP solution (Eurogentec, Germany), 0.2 µM of each primer, 50 ng of DNA, and 1U Taq DNA polymerase (Thermo Scientific, ABgene®, UK), with the following amplification program: a denaturation step for 5 min at 95°C, followed by 35 amplification cycles consisting of 30 s at 95°C, 30 s at 63°C, and 30 s at 73°C. A final extension step was added for 7 min at 72°C. Amplified DNA fragments were visualized on 1% agarose gel in 1× Tris/Borate/EDTA (Ethylene diamine tetra acetic acid) buffer stained with GelRed™ under UV light. A 100-bp DNA ladder was used as a size marker. All PCRs were repeated at least twice.

Real-time PCR assay

Real-time PCR was carried out on DNA extracted from soil and plant samples using SYBR Green chemistry on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Each rtPCR was performed in triplicate in MicroAmp® optical
96-well reaction plates, which were sealed with a transparent sticky cover (Applied Biosystems, Foster City, CA, USA) in 25 μl reaction mixture containing 0.4 μl of forward (VD-rtPCR-F ACAGTCCGATGGATAATTCTC) and reverse (VD-rtPCR-R GATCTGGGCGCAAGGCAG) primers, with reference to the ITS sequences of \textit{V. dahliae}, at concentrations of 200 nM each, 5 μl of template DNA (5 ng), 10 μl of 1× IQ SYBR Green Master-Mix (Bio-Rad), and 4.6 μl of sterile RNase-free water. The amplification was performed under the following conditions: 95°C for 5 min; 40 cycles of 95°C for 10 s and 65°C for 35 s to calculate the cycle threshold (Ct) values; followed by 95°C for 15 s, 67°C for 1 min, and then heating to 97°C at a rate of 1°C/5 s to obtain the melting curves. The threshold cycle (Ct) value for each rtPCR was automatically calculated using the ABI Prism sequence detection software (version 3.0).

\textbf{Spike assay}

The efficiency of the pathogen-specific primer VD-rtPCR-F/VD-rtPCR-R to detect target DNA among high concentrations of nontarget DNA was also examined by a spiking test. Different amounts of \textit{V. dahliae} DNA \((1 \times 10^{-2}, 1 \times 10^{-3}, \text{and } 1 \text{ ng})\) were spiked into stable plant DNA amounts (1, 5, and 20 ng) and utilized in the SYBR Green rtPCR as templates. The reaction mixture and amplification conditions were the same as described previously, and fluorescence readings were obtained at the end of each cycle. Afterward, the correlation between the concentration of input amounts and the Ct values was established. In order to assess whether SYBR Green dye has produced a single and specific product, an analysis of the melting curve was accomplished by heating the PCR product from 65°C to 95°C, with monitoring by fluorescence spectroscopy every 0.3°C (data not shown).

\textbf{Standard curve and amplification efficiency}

Two standard curves were created by means of plotting the logarithm of known initial DNA concentrations of \textit{V. dahliae} isolates VD12a \((0.022 \mu g \text{ ml}^{-1})\) and VD4 \((0.018 \mu g \text{ ml}^{-1})\) over at least five orders of magnitude \((10^{-2} - 10^{-6})\) versus Ct values. The data were separately analyzed and described as the cycle number at which a significant increase in the reported fluorescence can be detected. Then, the results were combined together in order to establish a unique standard curve with more accuracy. Amplification efficiency was computed via \(E = (10^{−1/slope}) − 1) \times 100\), where \(E\) is the amplification efficiency and the slope is the log of template concentrations versus Ct. Also, the minimum detection limit was assessed as minimum amount of target DNA detected when the Ct was reached up to 30 cycles. In fact, Ct value is inversely related to the log of initial DNA concentration at which the higher initial concentration has a lower Ct value. Each sample used to calculate the standard curve was amplified three times and from at least two different DNA concentrations to control the consistency of the curve.

\textbf{Detection and quantification of \textit{V. dahliae} in strawberry field samples}

Fifty-six samples from plant and soil were collected from different strawberry production areas as described previously. Detection and quantification of \textit{V. dahliae} were achieved by SYBR Green rtPCR using DNA extracted from each sample as unknown target. Each sample was analyzed in triplicate. Plant DNA and \textit{V. dahliae} genomic DNA were used as negative and positive controls, respectively.

\textbf{Enumeration of \textit{V. dahliae} in field soil}

Inoculum density of \textit{V. dahliae} in strawberry field soils was also assessed using a wet-sieving plating method, as previously described by Harris et al. (1993) with some modifications. Soil samples were air-dried for two weeks at room conditions, mixed, and ground with a mortar and pestle. Then, the samples were sifted using a 20-mesh sieve to eliminate large and unbreakable debris. Twenty gram of each sieved soil sample was shaken (at 250 rpm) and dispersed in distilled water for 1 h. Then, the soil was wet-sieved through 60- and 400-mesh sieves, respectively, and the residue retained in the 400-mesh sieve was suspended in 100 ml distilled water. Aliquots of 1 ml from each suspension after wet-sieving were scattered onto 90-mm petri plates of modified sodium polypectate agar medium (Butterfield & DeVay 1977) and incubated in the dark at 26°C. After two weeks of incubation, the plates were gradually washed under running water in order to remove residual soils. Afterward, the drained plates were scanned for the existence of typical star-shaped colonies of \textit{V. dahliae} by checking the plates using a stereomicroscope (Olympus, SZX10). In fact, identification of \textit{V. dahliae} was based on the shape of cultures, conidia morphology, and production of globose to elongate dark MS without melanized mycelium. Inoculum density in each soil sample was assessed by the number of \textit{V. dahliae} colonies and indicated as MS g\(^{-1}\) soil. To compare the results of MS counts with rtPCR assay, Pearson correlation coefficient was calculated using R program (version 3.2.2).
null hypothesis ($P < .05$) rejection demonstrates a lack of
significant difference between quantities.

**Results**

*Isolation of fungi*

In this study, eight isolates of *V. dahliae* were totally
obtained from strawberry plants from different investi-
gated districts, including four isolates from Vasula,
three from Marjamaa, and only one isolate from Utsu
area (Table 1). Twenty-two isolates of *V. dahliae* were
identified from soil samples, including eight isolates
from Vasula, four from Rohu, seven from Marjamaa,
and two from Utsu area. Only one *V. dahliae* was iso-
lated from Unipiha district. Some more pathogens
including *F. solani* causing similar wilt symptoms
along with *Rhizopus* spp. and *Rhizoctonia* spp. were iso-
lated from the soil of strawberry fields (Table 1).

*Primer design and testing*

The primer pairs were examined using cPCR against
genomic DNA from pure fungal cultures in this study
(Table 1). PCR products were generated from all DNA
extracted using nonspecific primers, demonstrating
the existence of amplifiable template (data not shown).
BLAST searches of the GenBank database showed that the primer pair would be specific to
amplify the region of 5.8S rDNA-ITS in *V. dahliae* and
this was practically confirmed using DNA of different
fungal isolates as shown in Table 1. The amplified
specific DNA fragment from *V. dahliae* isolates was
approximately 100 bp that confirmed the specificity
of this primer set. No DNA fragment was amplified
from other isolates using these primers (Figure 1).

*Spike assay*

In this assay, the plant DNA concentration was, respect-
vively, 100, 500, and 2000 times larger than the lowest
amount of spiked genomic DNA of *V. dahliae* ($1 \times
10^{-2}\text{ng}$). The Ct values were significantly correlated
with the target DNA concentration, with a regression
coefficient of 0.96, demonstrating the suitability of the
assay for quantitative and qualitative tests. In fact,
the primer pair showed a high sensitivity in detecting
the target of interest among varying different genes
(Table 2). This confirms that strawberry DNA had no sig-
nificant effect on quantifying *V. dahliae* in strawberry
plants.

*Standard curve*

A wide range of concentrations was subjected to rtPCR
analysis. Standard curves were initially developed by
plotting the Ct values against known serial dilutions
of quantified DNA from *V. dahliae* isolates (VD12a and
VD4) (Figure 2(a)). Finally, a combined standard curve
between the log of DNA concentration and Ct value
was constructed, and then a linear relationship with a
slope of $-3.43$ and a strong correlation coefficient
0.989 was obtained. The amplification efficiency of
the assay was calculated as 95.67% over at least five orders of magnitude (Figure 2(b)) based on 100% effi-
ciency, corresponding to $-3.32$ as the ideal optimum
value (Selim et al. 2005; Taylor et al. 2010). The ampli-
faction was observed in the presence of *V. dahliae* as the
template, but no amplification was detected by using
sterile RNase-free water as the negative control. The
lowest amount of DNA amplified was 0.93 pg µl$^{-1}$, con-
firming that this procedure represents a rapid, sensitive
rtPCR procedure for *V. dahliae* detection.

*Detection and quantification of V. dahliae in field samples*

Based on the morphological characteristics of the cul-
tures, most strawberry plants had evidence of
*V. dahliae* colonization on their roots. Pathogen MS
were also enumerated using the plate count technique.
and then concentrations of target DNA were assessed using rtPCR. In some plates, identification of *V. dahliae* was difficult and so single cPCR experiments using the specific primer VD-rtPCR-F/VD-rtPCR-R were performed to confirm the presence of *V. dahliae* in the culture medium before counting. No false-negative/positive results were obtained in the experiments, which indicates the specificity of the primer pair to detect *V. dahliae* with high sensitivity and accuracy. In line with our experimental design, rtPCR was developed to detect and quantify *V. dahliae* directly from strawberry plant and soil samples as described in the section ‘Materials and Methods’. The target DNA concentration available in each reaction was measured by comparing known amounts of *V. dahliae* DNA with Ct values of the unknown samples. Generally, all sampling areas were shown to be infested with *V. dahliae* when assessed with the rtPCR technique. However, most samples from Unipiha were negative, which means no MS g$^{-1}$ soil was obtained in the corresponding plate technique except in one isolate (Table 3). Amounts of *V. dahliae* varied in a range from 1 to 13 MS g$^{-1}$ soil from different strawberry fields, and results of corresponding rtPCR showed a diverse range of DNA concentrations from 10.48 to 313.05 pg µl$^{-1}$ (Table 3). Indeed, the lowest amount of *V. dahliae* population detected in soil was 10.48 pg µl$^{-1}$, which was equal to 1 MS g$^{-1}$ soil obtained from the soil plating technique (Table 3). Based on the Pearson correlation coefficient used, there was a significant correlation ($R^2 = 0.92$ and $P < .0001$) between the quantity of MS g$^{-1}$ soil and the Ct value of the rtPCR assay (data not shown). Basically, an amount $\leq$ 1 MS g$^{-1}$ soil indicated a mean Ct value of approximately 30 in soil.

Using the SYBR Green rtPCR assay, fluorescent signals were produced from most samples collected from strawberry fields. The mean Ct values for the DNA samples from *V. dahliae*-infected root/soil varied from 24.95 ± 0.04 to 19.89 ± 0.44. However, five plants had no *V. dahliae* symptoms but returned positive results using rtPCR, in which the lowest target DNA concentration (11.05 pg µl$^{-1}$) was measured from sample S55 (Table 3). Overall, the presence of *V. dahliae* in strawberry production areas showed considerable variation, being high in samples from Vasula and Marjamaa, moderate in Rohu and Utsu, and low in Unipiha. These experiments verified the sensitivity and accuracy of the rtPCR assay for the quantification of *V. dahliae* at a very low density.

**Discussion**

Early detection and identification of plant pathogens have extensively increased with the enhancement of molecular methods to improve disease management decisions (Babu et al. 2011). Quantitative rtPCR technology allows the rapid screening of suspected samples for the detection of pathogens that cannot be extracted easily from host tissue, or are present at low inoculum loads in soils. The main objective of the current study was mainly on direct and real-time

![Figure 2](image-url)

**Table 2.** Detection of *V. dahliae* in strawberry plants by the spike test analysis.

<table>
<thead>
<tr>
<th>Strawberry DNA (ng)</th>
<th>Verticillium DNA (ng)</th>
<th>Mean Ct</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 x 10^{-2}</td>
<td>28.36</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>1 x 10^{-1}</td>
<td>24.89</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>21.56</td>
<td>0.21</td>
</tr>
<tr>
<td>5</td>
<td>1 x 10^{-2}</td>
<td>28.23</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>1 x 10^{-1}</td>
<td>25.01</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>21.15</td>
<td>0.25</td>
</tr>
<tr>
<td>20</td>
<td>1 x 10^{-2}</td>
<td>29.12</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>1 x 10^{-1}</td>
<td>26.37</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>22.35</td>
<td>0.44</td>
</tr>
</tbody>
</table>

*a*Mean cycle threshold (Ct) of three rtPCR replicates.  
*b*Standard deviation based on three replicates.
detection and quantification of *V. dahliae*, the cause of Verticillium wilt disease, suited for field applications in Estonia.

As a pre-analysis step, DNA was directly obtained from different sources, such as mycelia, soil, and plant samples, using commercial kits in this study, because direct extraction of DNA was more efficient than former laborious and time-consuming cultivation-based procedures in this study. Then, the real-time detection assay was developed to detect and quantify

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Location</th>
<th>Source</th>
<th>Symptoms on field</th>
<th>Mean Ct</th>
<th>SD</th>
<th>DNA concentration (pg µl⁻¹)</th>
<th><em>V. dahliae</em> (MS g⁻¹)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>S01</td>
<td>Vasula</td>
<td>Soil</td>
<td>–</td>
<td>22.22</td>
<td>0.15</td>
<td>65.51</td>
<td>9</td>
</tr>
<tr>
<td>S02</td>
<td>Soil</td>
<td>Soil</td>
<td>–</td>
<td>19.89</td>
<td>0.04</td>
<td>313.05</td>
<td>13</td>
</tr>
<tr>
<td>S03</td>
<td>Soil</td>
<td>Soil</td>
<td>–</td>
<td>21.79</td>
<td>0.11</td>
<td>87.43</td>
<td>10</td>
</tr>
<tr>
<td>S04</td>
<td>Soil</td>
<td>Soil</td>
<td>–</td>
<td>23.35</td>
<td>0.05</td>
<td>30.68</td>
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<tr>
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<td>Soil</td>
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<tr>
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<td>Soil</td>
<td>Soil</td>
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<td>S07</td>
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</tr>
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</tr>
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<td>0.06</td>
<td>30.47</td>
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</tr>
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<td>24.42</td>
<td>0.05</td>
<td>14.95</td>
<td>+</td>
</tr>
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<td>S11</td>
<td>Plant</td>
<td>Plant</td>
<td>Plant collapse and death</td>
<td>21.75</td>
<td>0.08</td>
<td>89.81</td>
<td>+</td>
</tr>
<tr>
<td>S12</td>
<td>Plant</td>
<td>Plant</td>
<td>Poor growth and stunting</td>
<td>22.35</td>
<td>0.22</td>
<td>52.49</td>
<td>+</td>
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<tr>
<td>S13</td>
<td>Plant</td>
<td>Plant</td>
<td>Green dry necrotic</td>
<td>24.75</td>
<td>0.33</td>
<td>11.98</td>
<td>N</td>
</tr>
<tr>
<td>S14</td>
<td>Rohu</td>
<td>Soil</td>
<td>–</td>
<td>24.46</td>
<td>0.15</td>
<td>14.56</td>
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</tr>
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<td>Soil</td>
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<td>S16</td>
<td>Soil</td>
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<td>0.07</td>
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</tr>
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<td>S18</td>
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<td>0</td>
</tr>
<tr>
<td>S19</td>
<td>Soil</td>
<td>Soil</td>
<td>–</td>
<td>23.53</td>
<td>0.45</td>
<td>27.18</td>
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<tr>
<td>S20</td>
<td>Plant</td>
<td>Plant</td>
<td>Yellow wilt</td>
<td>N/A</td>
<td>–</td>
<td>–</td>
<td>N</td>
</tr>
<tr>
<td>S21</td>
<td>Plant</td>
<td>Plant</td>
<td>Wilting, initially older leaves only</td>
<td>24.25</td>
<td>0.38</td>
<td>16.76</td>
<td>N</td>
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<td>Plant</td>
<td>Plant</td>
<td>No symptom</td>
<td>24.77</td>
<td>0.12</td>
<td>11.82</td>
<td>N</td>
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<td>24.25</td>
<td>0.2</td>
<td>16.76</td>
<td>N</td>
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<td>No symptom</td>
<td>N/A</td>
<td>–</td>
<td>–</td>
<td>N</td>
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<tr>
<td>S25</td>
<td>Marjamaa</td>
<td>Soil</td>
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<td>24.68</td>
<td>0.07</td>
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<td>Soil</td>
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<td>Soil</td>
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<td>0.55</td>
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<td>Soil</td>
<td>Soil</td>
<td>–</td>
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<td>Soil</td>
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<td>0.22</td>
<td>20.50</td>
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<td>Soil</td>
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<td>24.65</td>
<td>0.08</td>
<td>12.81</td>
<td>5</td>
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<td>S32</td>
<td>Plant</td>
<td>Plant</td>
<td>Plant collapse and death</td>
<td>23.33</td>
<td>0.46</td>
<td>31.09</td>
<td>+</td>
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<td>Plant</td>
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<td>0.15</td>
<td>28.68</td>
<td>+</td>
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<td>0.24</td>
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<td>13.52</td>
<td>N</td>
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<td>0.33</td>
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<td>S37</td>
<td>Unipiha</td>
<td>Soil</td>
<td>–</td>
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<tr>
<td>S38</td>
<td>Soil</td>
<td>Soil</td>
<td>–</td>
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<tr>
<td>S39</td>
<td>Soil</td>
<td>Soil</td>
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<td>N/A</td>
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<tr>
<td>S40</td>
<td>Soil</td>
<td>Soil</td>
<td>–</td>
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<tr>
<td>S41</td>
<td>Soil</td>
<td>Soil</td>
<td>–</td>
<td>N/A</td>
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<tr>
<td>S42</td>
<td>Soil</td>
<td>Soil</td>
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<td>0.44</td>
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<td>Plant</td>
<td>No symptom</td>
<td>24.57</td>
<td>0.15</td>
<td>13.52</td>
<td>N</td>
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<td>No symptom</td>
<td>N/A</td>
<td>–</td>
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<td>N</td>
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<tr>
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<td>Plant</td>
<td>No symptom</td>
<td>N/A</td>
<td>–</td>
<td>–</td>
<td>N</td>
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<tr>
<td>S46</td>
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<td>Plant</td>
<td>No symptom</td>
<td>N/A</td>
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<tr>
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<td>Utsu</td>
<td>Soil</td>
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<td>0.07</td>
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<tr>
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<td>–</td>
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<td>Soil</td>
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<td>–</td>
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<tr>
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<td>Soil</td>
<td>Soil</td>
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<tr>
<td>S52</td>
<td>Plant</td>
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<td>No symptom</td>
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<td>0.63</td>
<td>26.82</td>
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<td>Plant</td>
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<td>N/A</td>
<td>–</td>
<td>–</td>
<td>N</td>
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<tr>
<td>S54</td>
<td>Plant</td>
<td>Plant</td>
<td>Yellow wilt</td>
<td>24.11</td>
<td>0.35</td>
<td>18.42</td>
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<td>Plant</td>
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<td>24.87</td>
<td>0.22</td>
<td>11.05</td>
<td>N</td>
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<tr>
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<td>Plant</td>
<td>Plant</td>
<td>Yellow wilt</td>
<td>23.77</td>
<td>0.17</td>
<td>23.14</td>
<td>N</td>
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</table>

Notes: N/A, high Ct > 25, was considered negative. +, identified as *V. dahliae* presence by the plate culture assay; N, not detected; -, no symptom on plant or no signal detected (rtPCR).

*Mean cycle threshold (Ct) based on three PCR replicates.

Standard deviation based on three replicates.

Microsclerotia per gram of soil.
V. dahliae in strawberry plants (with and without symptoms of infection) and field soils. However, many studies have been reported for the detection and quantification of Verticillium species in plant and soil using rtPCR from various hosts and geographical areas (Li et al. 1994; Mercado-Blanco et al. 2003; Larsen et al. 2007; Cubero et al. 2009; Markakis et al. 2009; Duressa et al. 2012). Finally, we compared the results of the plate assay regarding MS counts in strawberry field soils with rtPCR data.

Since there may be a number of soil-borne fungi along with Verticillium present in natural samples which will be isolated, a specific primer pair is needed for a reliable and accurate assay. This study established a species-specific primer set designed for the target sequence for the SYBR Green rtPCR assay. From our results, the described primer pair (VD-rtPCR-F/VD-rtPCR-R) was species specific when tested against a range of other Verticillium spp. as well as other common soil-borne fungi that were collected from different geographic regions. As shown in Table 1, none of the fungal strains showed positive results with the primer pair used in cPCR. On the basis of Inderbitzin et al. (2011) studies, V. longisporum is a hybrid fungus containing a copy of V. dahliae DNA sequences which could limit the use of these primers for species-specific detection. But, since the pathogenicity of V. longisporum is restricted to cruciferous hosts, relationship of this species with strawberry plants is not anticipated (Steventon et al. 2002; Eynck et al. 2007; Duressa et al. 2012). Primers VD-rtPCR-F and VD-rtPCR-R indicated the best specific amplification for V. dahliae, and gel electrophoresis confirmed the amplification of a 100-bp target (Figure 1). In fact, specific primers developed based on the ITS regions have been deployed as a fruitful strategy regarding diagnostic tests for many fungal pathogens (Bates et al. 2001; Heuser & Zimmer 2002; Lees et al. 2002; Luchi et al. 2005). Nazar et al. (1991) demonstrated the effective use of intergenic sequences for V. dahliae detection and suggested that these sequences can be applied for the differentiation of closely related species even when the mature rRNAs are too homologous. In a similar study, Lievens et al. (2006) used a SYBR Green assay based on ITS regions I and II to quantitatively assess the presence of a number of important fungal and oomycete tomato pathogens including F. solani, R. solani, Verticillium species, and Pythium ultimum in biological samples.

Spike assay, as the initial assay, was performed using SYBR Green rtPCR in order to evaluate the specificity of the assay by considering nonspecific primer dimers at the end of thermocycling. As shown in Table 2, the pathogen-specific primer could specifically detect V. dahliae among a varying different genes. For assay validation, a unique standard curve was then developed with known concentrations of serially diluted genomic DNA extracted from V. dahliae in which the minimum detection limit was 0.93 pg µl$^{-1}$ DNA (Figure 2). rtPCR allowed an accurate detection and quantification of V. dahliae in strawberry plants that revealed typically different symptoms. Since there were differences between amounts of V. dahliae with different symptoms, the assay was strongly capable of quantifying 11.05 pg µl$^{-1}$ of pathogen DNA even in symptomless plants (Table 3). This shows that many strawberry plants in the fields may have latent infection without any symptoms. Result of our study is almost in accordance with Markakis et al. (2009) who reported that symptomatology is associated with the amount of V. dahliae DNA in plant tissues. Hence, this assay should be able to identify all stages of V. dahliae infection in plant tissue prior to and during the appearance of disease symptoms. On the other hand, similar symptoms such as wilting and stunting might be due to root damage caused by pests or other soil-borne pathogens instead of Verticillium infections (Maurer et al. 2013). Moreover, field diagnosis of V. dahliae is made somewhat difficult by the fact that F. oxysporum causes similar early symptoms on strawberry (Koike & Gordon 2015), and so foliar disease symptoms are not a reliable indicator of Verticillium infection (Pernezny et al. 2003). Overall, knowing about the minimum amount of V. dahliae in the soil and plant is a very important factor in the strawberry industry as choosing pathogen-free plants before planting is fundamentally essential.

In this study, we observed a relationship between the concentration of V. dahliae DNA and the number of MS g$^{-1}$ of soil, ranging from 1 up to 13 MS g$^{-1}$ of soil (Table 3). Also, DNA concentrations of V. dahliae were found in most samples analyzed, whereas no pathogen was detected in some corresponding samples using the plate technique, indicating the probability of dead propagules. So, comparing these methods confirmed that rtPCR is not only more accurate and specific, but also requires less time. Our results explicitly showed that a mean Ct value of approximately 26 in the samples demonstrates the presence of 10.48 pg µl$^{-1}$ of pathogen DNA and at least one (≥1) V. dahliae MS g$^{-1}$ of soil, which shows a high quantification level in comparison with other studies. For instance, a detection limit of 6.7 MS g$^{-1}$ of soil for V. dahliae using the SYBR Green rtPCR assay was reported by Lievens et al. (2006). More recently, Bilodeau et al. (2012) demonstrated a
significant correlation between the development of the disease and the V. dahliae MS counts by the use of a TaqMan rTPCR assay with an internal control probe for each amplification and could quantify the pathogen presence as low as 1–2 MS g⁻¹ of soil with a high level of sensitivity.

In conclusion, the rTPCR assay described herein was able to identify and quantify the presence of V. dahliae in symptomless strawberry plants and soil samples without culturing. Recently, advances in related technologies such as next-generation sequencing enable the simultaneous detection of microbial communities at a much higher resolution and so may improve the interpretation of current estimates.

Acknowledgements
The authors specially thank Prof. Peter Harley (National Center for Atmospheric Research, Colorado, USA) for the critical reviewing of this manuscript and giving valuable comments. We also thank Raquel Castillo and Yolanda Herranz (INIA, Madrid, Spain) for their great help regarding rTPCR assays.

Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
This work was supported by the European Union through the European Regional Development Fund (Contract #10.1-9/14/471), institutional research funding (IUT36-2) of the Estonian Ministry of Education and Estonian Science Foundation (grant number #9450).

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