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First report on the detection and quantification of *Verticillium dahliae* from Estonian strawberry fields using quantitative real-time PCR

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Abstract

The main aim of this investigation was to develop a SYBRGreen real-time polymerase chain reaction (PCR) assay for detection and quantification of *Verticillium dahliae* directly from affected strawberry roots and soils. The proposed assay utilizes a specifically designed primer pair on the basis of an internal transcribed spacer (ITS) sequence. During 2014–2015, plant and soil samples were randomly collected from different areas including Vasula, Rohu, Unipiha, Utsu and Marjamaa in Estonia and analyzed for *V. dahliae*. Real-time PCR technique using primers designed to the rDNA ITS1 was highly sensitive and accurate and so allowed reliable quantification of the pathogen DNA at low inoculation in soils (3×10^{-1} pg μl^{-1}) and even in root of symptomless plants (5×10^{-1} pg μl^{-1}). This is the first study of using this technique to quantify the population of *V. dahliae* in strawberry fields from Estonia.

Key words: quantitative detection, real-time PCR.

Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is one of the most popular berry fruits in the world due to its high nutritional value (Rugienius et al., 2015). It is one of the most important fruits in European and Baltic countries, and its cultivation in Estonia has increased over the past few years. Verticillium wilt, caused by *Verticillium dahliae* Kleb., is an economically important disease that can cause extensive strawberry plant and fruit damage, even at low inoculum load (Pegg, Brady, 2002). The pathogen can survive for long periods by producing black microsclerotia that persist in the soil for years (Klosterman et al., 2009). Moreover, Verticillium wilt is difficult to completely control even with soil fumigation. This disease was historically controlled by soil fumigation with methyl bromide but it was phased out by 2005 (Pérez-Jiménez et al., 2012). In fact, early detection of this pathogen on strawberry became more important as well as considerable in recent years in order to work with pathogen free plants. So, the aim of this study was to establish a sensitive real-time PCR (rtPCR) method for the detection and quantification of the pathogen directly from strawberry root and soil.

Materials and methods

Sampling and fungal isolation. During 2014–2015, a study was conducted in order to detect and quantify *Verticillium dahliae* directly from open field grown strawberries and soil to estimate the incidence of Verticillium wilt outbreak in different regions of Estonia. Sampling areas (Vasula, Rohu, Unipiha, Utsu and Marjamaa) were suspected of being infected with wilt diseases. Strawberry plant (cv. 'Sonata') and soil samples (78 samples in total) were randomly collected from different points in each field and culturing methods were used to isolate fungi (Dhingra, Sinclair, 1985). Pure cultures of *V. dahliae* (VD12a and VD4), *V. albo-atrum* and *F. oxysporum* f. sp. *fragariae* isolates were provided by National Institute for Agricultural and Food Research and Technology (INIA), Madrid, Spain. Isolates from other genera were isolated from plant roots and soil of strawberry fields in Estonia (Table).

Extraction of DNA. Genomic DNA of pure fungal cultures and strawberry plants were extracted using a DNeasy Plant Mini Kit ("Qiagen", USA) according to the manufacturer's instructions. Also, soil genomic DNA was extracted using a PowerSoil DNA Isolation

Table. Isolates used for the primer pairs specificity

Isolates	Host	Polymerase chain reaction (PCR)	
		ITS1 / ITS4	VD-rtPCR-F / VD-rtPCR-R
Verticillium sp.**	soil	+	−
V. dahliae, VD12a*	tomato plant	+	+
V. dahliae, VD4*	strawberry plant	+	+
V. albo-atrum	strawberry plant	+	−
*V. dahliae***	strawberry plant	+	+
*V. dahliae***	soil	+	+
Fusarium oxysporum f.sp. fragariae	strawberry plant	+	−
*Fusarium solani***	soil	+	−
*Rhizoctonia solani***	soil	+	−
Rhizopus sp.**	soil	+	−

Notes. * – isolates obtained from National Institute for Agricultural and Food Research and Technology (INIA); ** – isolates were collected from strawberry fields of Vasula area. + amplification of expected PCR product; − no PCR band on electrophoretic gel.

Kit ("MoBio", USA), according to the manufacturer's instructions. Then, DNA quality was determined by agarose gel electrophoresis and DNA was quantified by a spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific, USA) at 260 nm.

Conventional and real-time polymerase chain reactions (PCRs). Extracts of genomic DNA were initially amplified via conventional PCR (cPCR) using ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers to confirm the presence of amplifiable DNA (Kernaghan et al., 2007). The web-based program *Primer3* was applied to design the primers VD-rtPCR-F (ACTTTTAACAACGGATCTCT) and VD-rtPCR-R (AGCGTCGTTTCAACCCTCGAG). BLAST searches of the GenBank (http://www.ncbi.nlm.nih.gov/nuccore/?term=Verticillium+dahliae) showed that the primer pair would be specific to amplify the region of 5.8S rDNA-ITS in *V. dahliae*. Primer specificity was assessed using cPCR against different isolates of the strawberry pathogens as shown in Table. Samples were then analysed by rtPCR assay using a specifically designed primer pair. Real-time PCR was performed using SYBRGreen chemistry on ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA) in 25 µl reaction volume containing: 0.4 µl of forward (VD-rtPCR-F) and reverse (VD-rtPCR-R) primers at concentrations of 200 nM each, 5 µl of template DNA, 10 µl of 1 × IQ SYBRGreen MasterMix and 4.6 µl of sterile RNase-free water. Reactions were 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 threshold cycle (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 per 5 s to obtain the melting curves. For assay validation, a unique standard curve was developed by plotting the logarithm of known concentrations of serially diluted genomic DNA extracted from *V. dahliae* isolates (VD12a and VD4) over at least five orders of magnitude (10^{-2}–10^{-6}) against values of threshold cycle. Amplification efficiency was computed via $E = (10^{(-1/slope)} - 1) \times 100$ and the sensitivity of assay was measured based on minimum quantity of target DNA when the threshold cycle was reached up to 40 cycles.

Results and discussions

Identification of *V. dahliae* from strawberry plants and soils was based on morphology of cultures such as production of globose to elongate black microsclerotia with hyaline and septate mycelium (Fig. 1).

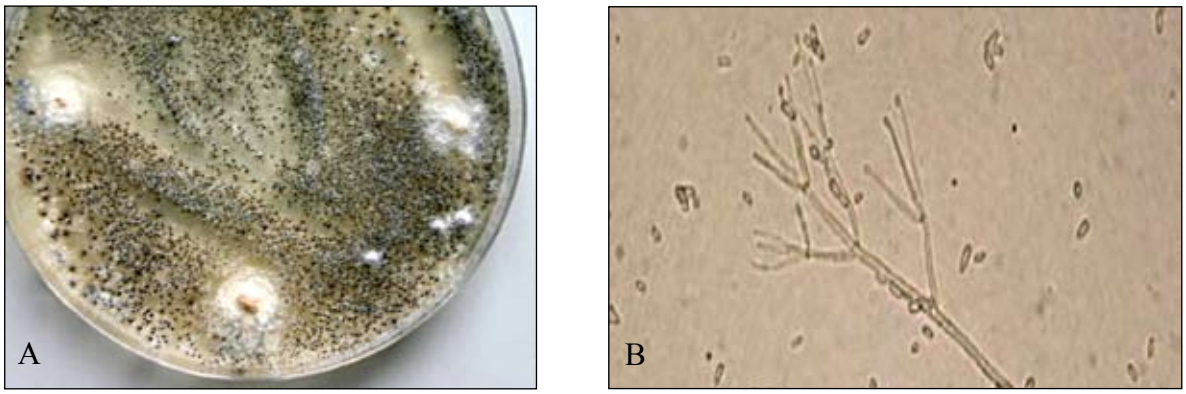

Figure 1. Dark microsclerotia (A) and microscopic view of *Verticillium dahliae* with hyaline and septate mycelium (B)

The pathogens including *F. solani* and *Verticillium* sp. causing similar wilt symptoms along with *Rhizopus* sp. and *R. solani* were isolated from soil of strawberry fields (Table). The specific primer pair was examined using cPCR against genomic DNA of fungal pure cultures to confirm the specificity of the primer. The amplified specific DNA fragment was approximately 100 bp. Blast analysis of the GenBank showed that the primer pair would be specific to amplify the region of 5.8S rDNA-ITS in *V. dahliae*. The application of specific primers based on the ITS regions has been confirmed as a proper strategy regarding diagnostic tests for many pathogens (Lees et al., 2002; Luchi et al., 2005).

Using standard curves analysis, the amplification efficiency of assay was estimated 95.67% over at least five orders of magnitude based on 100% efficiency (Fig. 2), in which the minimum detection limit of assay was 0.93×10^{-6} pg μl^{-1} DNA, confirming sensitivity of method for *V. dahliae* detection.

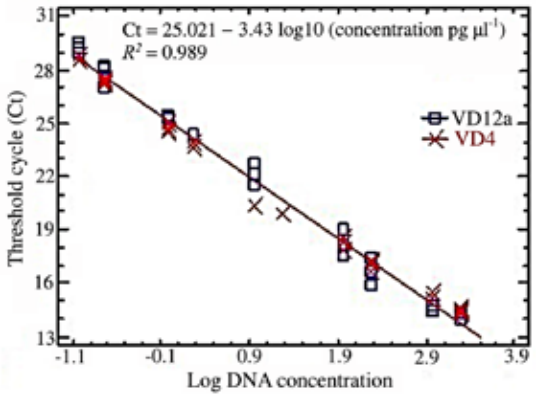

Figure 2. Combined standard curve using specific primer pair from different dilutions of pure genomic DNA of two *Verticillium dahliae* isolates (VD12a and VD4)

Real-time PCR was established to detect and quantify *V. dahliae* directly from strawberry plants and soil samples as described in materials and methods. Using SYBRGreen rtPCR assay, fluorescent signals were produced from all soil samples and suspicious plants collected from different sampling areas. The ITS region within prokaryotic and eukaryotic rDNA operons has been described as a stable genetic marker and actually this region is the most widely sequenced for strawberry pathogens (Mirmajlessi et al., 2015 a). The mean threshold cycle (Ct) values from *V. dahliae*-infected root/soil varied from 29.97 ± 0.21 to 21.39 ± 0.11. However, five plants had no common *V. dahliae* symptoms, but rtPCR results showed positive results with the lowest DNA concentration (5×10^{-1} pg μl^{-1}). Also, the lowest amount of *V. dahliae* DNA detected in soil was 3×10^{-1} pg μl^{-1}. Overall, the presence of *V. dahliae* in strawberry production areas showed wide variation, being high in samples from Vasula and Marjamaa, moderate in Rohu and Utsu, and low in Unipiha. Since there were differences between amounts of *V. dahliae* with different symptoms, the assay was strongly able to detect *V. dahliae* DNA with high accuracy and specificity in symptomless plants. In fact, rtPCR is an ideal method, which allows accurate and sensitive detection and quantification of pathogens in various environmental samples that cannot be isolated easily or are present at low inoculum load (Bustin et al., 2009; Mirmajlessi et al., 2015 b).

Conclusion

Many PCR-based detection methods are able to detect *Verticillium* spp. on plant tissues and soil, but no study has been found to quantify populations of *V. dahliae* using real-time PCR technique in Estonia strawberry fields. The developed assay was able to identify the presence of *V. dahliae* in symptomless strawberry plants and soil samples without culturing, facilitating the screening of the pathogen in diverse areas in real time. This is the first report of using SYBRGreen-based real-time PCR in order to detect and quantify populations of *V. dahliae* with high sensitivity from strawberry fields in Estonia. So, the results of this study may provide growers a means to enhance available disease management strategies against Verticillium wilt.

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Pirmasis pranešimas apie *Verticillium dahliae* identifikavimą ir nustatymą Estijos braškynuose, taikant kiekybinę realaus laiko polimerazės grandininę reakciją

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Santrauka

Tyrimo tikslas – taikant *SYBRGreen* realaus laiko polimerazės grandininės reakcijos (PGR) metodą, identifikuoti *Verticillium dahliae* ir nustatyti jų kiekį tiesiogiai tiriant pažeistas braškių šaknis bei dirvožemį. Tyrimo metu naudota speciali pradmenų pora sukurta, remiantis vidinio transkribuojamo tarpiklio (ITS) seka.
2014–2015 m. dirvožemio ėminiai buvo surinkti iš įvairių Estijos regionų – Vasula, Rohu, Unipiha, Utsu bei Marjamaa – ir analizuoti siekiant nustatyti *V. dahliae*. Realaus laiko PGR metodas naudojant specifinius rDNA ITS pradmenis buvo tikslus, jautrus ir leido patikimai nustatyti patogeno DNR kiekį, esant nedideliam patogeno kiekiui dirvožemyje (3×10^{-1} pg μl^{-1}) ir besimptomių augalų šaknyse (5×10^{-1} pg μl^{-1}).
Šis tyrimas yra pirmasis, kuriame taikytas šis metodas siekiant nustatyti *V. dahliae* populiacijos kiekį Estijos braškynuose.

Reikšminiai žodžiai: kiekio nustatymas, realaus laiko PGR.

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