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Disease risk assessment of sugar beet root rot using quantitative real-time PCR analysis of *Aphanomyces cochlioides* in naturally infested soil samples

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Abstract Sugar beet root rot, caused by the oomycete *Aphanomyces cochlioides*, is a serious and economically important disease of sugar beets world-wide. Today, disease risk assessment consists of a time-consuming greenhouse bioassay using bait plants. In the present study, a real-time quantitative PCR (qPCR) assay for determination of *A. cochlioides* DNA in field-infested soil samples was developed and validated using the standard bioassay. The qPCR assay proved to be species-specific and was optimized to give high amplification efficiency suitable for target copy quantification. A high correlation ($R^2 > 0.98$, $p < 0.001$) with pathogen inoculum density was shown, demonstrating the suitability for monitoring soil samples. The limit of detection (LOD) was evaluated in several different soil types and varied between 1 and 50 oospores/g soil, depending on clay content. Soils with a high LOD were characterised as having a low clay content and high content of sand. Varying levels of the *A. cochlioides* target sequence were detected in 20 of the 61 naturally infested soil samples. Discrepancies between the bioassay and the qPCR assay were found in soils from low- and medium-risk fields. However, the qPCR diagnostic assay provides a potentially valuable new tool in disease risk assessment, enabling sugar beet growers to identify high-risk fields.

Keywords *Aphanomyces cochlioides* · Real-time PCR · Diagnostics · Soil-borne plant pathogen · Sugar beet root rot

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Introduction

Aphanomyces root rot, caused by the oomycete *Aphanomyces cochlioides* Drechs., is a serious and economically important disease of sugar beet present world-wide in sugar beet growing countries such as USA, Germany, Poland, UK and Sweden (Rush 1988; Payne et al. 1994; Olsson and Olsson 2004; Piszczek 2004; Amein 2006; Windels et al. 2007; Moliszewska and Piszczek 2008; Nechwatal et al. 2012). In southern Sweden sugar beet is grown in 4 to 6 year crop rotations. *A. cochlioides* is one of the most severe pathogens of sugar beets in Sweden and in fields that are highly infested; sugar yield can be reduced by 27 % or more (Olsson et al. 2011).

A. cochlioides causes damping-off and root rot on sugar beet seedlings. The seedling is infected shortly after emergence with early symptoms including brown, water-soaked roots and hypocotyls that turn black and thread-like (Windels and Lamey 1998; Windels 2000). Later in the season, a chronic phase of the disease may occur and cause malformed and scarred roots. The infection is favoured by high temperatures (20–28 °C) and wet soils (Papavizas and Ayers 1974; Windels 2000; Amein 2006), and the disease is more prevalent in soils with low pH and low content of calcium (Payne et al. 1994; Olsson et al. 2011). Oospores of *A. cochlioides* are formed in diseased roots and can remain viable in the soil for up to 20 years (Rowntree and Windels 2003). Previous studies in Sweden confirm that the infestation level can remain high despite long crop rotations (Persson and Olsson 2010). The plant disease can partly be controlled by using tolerant varieties, early planting, proper soil drainage, seed treatment and application of lime (Harveson 2007; Persson and Olsson 2010; Persson and Olsson 2014; Olsson and Persson 2014). However, in fields with high infestation levels, the only practical mean of disease control is to avoid growing sugar beet. Specific, reliable and fast diagnostic methods are therefore needed to identify fields with medium to high levels of *A. cochlioides*.

Real-time quantitative PCR (qPCR) is a specific and reproducible technique suitable for pathogen diagnostics and quantification. Several studies presenting real-time PCR assays for detection of plant pathogens in soil samples have been published (e.g. Cullen et al. 2002; Lees et al. 2002: Ratti et al. 2004: Schroeder et al. 2006; Bilodeau et al. 2012; Wallenhammar et al. 2012). Time from DNA extraction to qPCR analysis is short, less than one working day. Despite the suitability of this technique, only a few methods are commercially used as diagnostic tools to quantify the soil inoculum of plant pathogens and to determine the disease risk potential. In Sweden, it is now possible to analyse soils for the clubroot pathogen *Plasmodiophora brassicae* (Wallenhammar et al. 2012), a service that has received high interest. The demand of commercially available diagnostic tests using qPCR will most likely increase as the farmers become more aware of the potential of this technique as a decision making tool. However, it is important that the methods are thoroughly validated and that guidelines for the interpretation of results are available to both extension services and farmers.

Traditional PCR methods for detection of specific DNA-sequences using gel electrophoresis were previously developed for *A. cochlioides* (Vandemark et al. 2000; Weiland and Rundsbak 2000). However, the primers developed by Weiland and Rundsbak (2000) targeting the actin coding sequence of *A. cochlioides* were not able to distinguish *A. cochlioides* from the closely related species *A. euteiches*. Also, none of the previously reported methods were tested on soil samples and none of them are quantitative.

The main aim of the present study was to improve disease risk assessment of Aphanomyces root rot on sugar beet. The specific objectives were to develop and validate an *A. cochlioides* specific real-time PCR assay for direct detection and quantification of *A. cochlioides* in soil samples infested during field conditions.

Materials and methods

Fungal species and isolates

The plant pathogenic species used are listed in Table 1. They are either soil-borne pathogens and/or pathogens that are commonly found in Sweden. A total of 18 different species were tested, including five different *A. cochlioides* isolates. The strains were grown on oatmeal agar (Sigma), potato dextrose agar (Acumedia) or malt extract agar (Merck). DNA was extracted from cultures grown in peptone-glucose broth (20 g peptone and 5 g glucose per l distilled water) or from material collected directly from agar plates by scraping. DNA of *Sclerotinia sclerotiorum* was extracted from apothecia.

Sampling of field soils

Plots measuring 20 m × 20 m were established in commercial sugar beet fields. In late March and April at the time of sowing, a soil sample consisting of approximately 10 subsamples to a depth of 20 cm was taken in a W-pattern from each plot. These samples were taken to the laboratory, mixed by hand to gain a homogenous sample and exclude larger stones and used in the bioassay within a short period. In 2011 and 2014, a total of 42 and 19 samples, respectively, were collected in fields in Southern Sweden.

Determination of soil properties

Particle size distribution was assessed by sieving and hydrometer methods (Gandahl 1952). The contents of

Table 1 Specificity of the primers and probe used in the *Aphanomyces cochlioides*-specific qPCR assay. Origin of the fungal isolates is stated when known

Species	Origin	*A. cochlioides* primers/probe
Aphanomyces cochlioides JstrK3	Sweden	+
Aphanomyces cochlioides BramsS4	Sweden	+
Aphanomyces cochlioides Wadb.K2	Sweden	+
Aphanomyces cochlioides MollS2	Sweden	+
Aphanomyces cochlioides GretelK2	Sweden	+
Aphanomyces euteiches #5	Sweden	-
Aphanomyces euteiches #9	Sweden	-
Aphanomyces cladogamus	Sweden	-
Aphanomyces sp.	Sweden	-
Botrytis cinerea CBS 676.89		-
Botrytis fabae CBS 108.57	UK	-
Cylindrocarpon destructans var. destructans CBS 940.97		-
Drechslera tritici-repentis CBS 265.80	Germany	-
Fusarium avenaceum CBS 408.86		-
Fusarium culmorum	Sweden	-
Fusarium graminearum	Sweden	-
Gaeumannomyces graminis var. tritici	France	-
Phoma exigua var. exigua CBS 119.94		-
Phoma medicaginis var. medicaginis CBS 316.90		-
Sclerotinia sclerotiorum (apothecia)	Sweden	-
Septoria tritici CBS 292.69	Germany	-
Stagonospora nodorum	Sweden	-
Verticillium dahliae	Sweden	-

organic and inorganic carbon were determined by heating dried and homogenised soil samples (fraction <2 mm) from 100 °C to 1000 °C in a Leco furnace (Carbon-Analyzer RC 412) and recording the amount of carbon dioxide released (Persson and Olsson 2000). Soluble phosphate, K, Ca and Mg were determined by extraction with acid ammonium lactate (AL; pH 3.75), (SIS 1993). The pH of the soil was determined in water using a conventional pH metre (1.0:2.5, soil: water, w/w).

Artificially infested soil samples

A non-infested soil (clay 11 %, sand 66 %, organic matter 2.5 %, pH 5.3) was chosen for a dilution series experiment with *A. cochlioides* oospores. The soil was air-dried in room temperature and passed through a 2 mm sieve. A dilution series was made by inoculating the soil with dried oospore-talcum inoculum produced from mycelia cultures prepared and quantified as described by Persson et al. (1999). Two parallel series were made using oospores from two different Swedish strains, Wadb K2 and Brams S4, containing 893 690 oospores/g talcum powder and 725 208 oospores/g talcum powder respectively. The serial diluted soil samples of 25 g soil contained 0, 1, 10, 10^2, 10^3 and 10^4 oospores/g soil (d.w.).

To determine the limit of detection (LOD) in different soil types, six different soils were artificially infested with *A. cochlioides* oospores of strain Wadb K2. The serial diluted soil samples of 10 g soil contained 0, 1, 2, 5, 10, 20, 50 and 100 oospores/g soil (d.w.). Soil characteristics of the six soils are presented in Table 2. Two soils with high organic matter content (>10 %) were included as well as four soils with clay content in the

Table 2 Characteristics of six different soils artificially infested with 0, 1, 2, 5, 10, 20, 50 and 100 oospores/g soil (d.w.) analysed by real-time quantitative PCR. Detection of target sequence (+), or not (−), for two biological replications of DNA extraction

Soil sample	pH	Clay %	Sand %	Organic matter %	Oospores[a, b]							
					0	1	2	5	10	20	50	100
1	6.7	3	89	4.1	−	−	−	−	−	−	++	++
2	5.8	14	41	3.2	−	−	−	−	++	++	++	++
3	6.0	36	10	4.7	−	−	++	++	++	+−	++	++
4	7.1	54	5	3.6	−	++	++	++	++	++	++	++
5	7.8	5	75	12.0	−	−	−	−	−	++	++	++
6	5.2	51	2	12.9	−	−	−	+−	++	++	++	++

[a] Oospores/g soil (d.w)

[b] ++ = amplification in both replicates, + − = amplification in one replicate, − = no amplification

range 3 to 54 %. The soils were dried in 35 °C for >24 h, milled and passed through a 2 mm sieve before oospores were added.

Repeatability

The inter-sample repeatability was evaluated on separate extractions of nine soil subsamples quantified in a single real-time PCR assay. An artificially infested soil sample (clay 11 %, sand 66 %, organic matter 2.5 %, pH 5.3) containing 10^3 oospores/g soil (d.w.) was chosen for this experiment.

DNA extraction from fungal material

DNA was extracted according to Wallenhammar et al. (2012) or using the FastDNA SPIN Kit (MP Biomedicals) according to the manufacturer's recommendations for fungal material. All DNA extracts were stored at −20 °C until qPCR analysis.

DNA extraction from soil

The subsamples of field soils to be analysed by qPCR were air-dried at room temperature and 350 mg soil was used for the DNA extraction. A few selected naturally infested soil samples were also extracted using a second protocol starting with 10 g soil to investigate if an increased starting amount could improve the limit of detection. In addition, 10 soil samples (both artificially and naturally infested) were also extracted using the first protocol with a pre-treatment included.

In the first protocol (referred to as protocol A), DNA was extracted from 350 mg soil samples (two replicates per sample) using the FastDNA 2 mL SPIN Kit for Soil (MP Biomedicals) with modifications according to Wallenhammar et al. (2012). Further purification of the eluate was performed using Wizard DNA Clean-Up System (Promega Corporation) and Illustra MicroSpin S-300 HR Columns (GE Healthcare) according to Wallenhammar et al. (2012). In the second protocol (referred to as protocol B), DNA was extracted from 10 g soil samples (two replicates per sample) using the FastDNA 50 mL SPIN Kit for Soil (MP Biomedicals). The eluates were purified according to the same routine as described for the first protocol. All DNA extracts were stored at −20 °C until qPCR analysis.

The pre-treatments of protocol A that were evaluated included heating of the soil and lysis buffers for 10 min at 65 °C (just before the bead beating) or the addition of 15 mg skim milk powder to the Lysing Matrix E tubes before the addition of soil and lysis buffers.

Primers and probes

Primers and a TaqMan MGB probe with putative specificity to *A. cochlioides* (Table 3) were designed using a ribosomal RNA gene sequence available in the GenBank (AY353911) and amplified a 96 base pair fragment. Primers were purchased from Eurofins MWG Operon and the probe was purchased from Life Technologies. The 5′ terminal reporter dye of the probe was FAM (Applied Biosystems).

Primer and probe sequences were checked for identity against other known sequences using the BLASTN programme (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Real-time PCR

Real-time quantitative PCR was performed using the 7300 Real Time PCR System (Applied Biosystems) in a total volume of 25 μL. The reaction mixture included 1× TaqMan Universal PCR Master Mix II No AmpErase UNG (Applied Biosystems/Life Technologies), 0.6 μM AcF, 0.6 μM AcR, 0.2 μM AcP and 5 μL template DNA. Soil DNA extracts were diluted five-fold before analysis and were analysed in duplicate reactions. The thermal cycling conditions consisted of an initial denaturation for 10 min at 95 °C followed by 45 cycles at 95 °C for 15 s and 60 °C for 60 s.

Table 3 Primer and probe sequences used for the amplification and detection of the *Aphanomyces cochlioides* target sequence

Primer/probe	Sequence
Primer forward (AcF)	5′-TCC GGG CTA GCC GAA GGT T-3′
Primer reverse (AcR)	5′-ACA AGC AAT CAT TTC TGA TGC TAG ATA G-3′
Prob (AcP)	5′-CGA AAG GAA CCG ATG TAT-3′

Quantification

The amount of pathogen DNA was quantified using a standard curve generated by including reactions containing different amounts of a plasmid carrying the *A. cochlioides* target sequence. The qPCR standard was ordered as plasmids carrying a synthetic gene (Eurofins MWG Operon). A 10-fold dilution series of the plasmid standard was analysed in triplicate using the real-time PCR assay and Ct values were then plotted against the amount of target copies in the plasmid standard to create a standard curve. Soil samples with low levels of *A. cochlioides* DNA outside the range of the standard curve were quantified by extrapolating the standard curve to facilitate analysis of the results. However, these values should be considered as estimates. The target copy number in soil samples extracted using protocol A was expressed as number of target copies/g soil, taking into account that DNA was extracted from 350 mg soil samples and that 1 μL of the original DNA extract was analysed. The amplification efficiency (E) was calculated as a percentage using the equation:

$$E = 100\ \left(10^{-1/k} - 1\right) \quad (1)$$

Where k is the slope of the equation describing the plot of Ct versus the logarithm of the DNA amount.

Evaluation of inhibition

Inhibition of the PCR reaction by contaminants in the DNA extracts was assessed for the soil samples by analysing a separate PCR reaction containing the soil DNA extract and 2 μL of the *A. cochlioides* plasmid standard containing approximately 130 gene copies. The effect of inhibition was evaluated according to Wallenhammar et al. (2012).

Bioassay

Aphanomyces root rot potential in each soil sample was assessed in a bioassay conducted in greenhouse by sowing 10 seeds of the sugar beet cultivar Rasta (Syngenta seeds, Landskrona, Sweden) in 10 cm diameter polyethylene pots, with six replicates per soil. The seed was not treated with fungicides or insecticides. The cultivar Rasta is tolerant to Aphanomyces root rot and was a commercial variety until 2011 in Sweden. Pots were watered daily to maintain high soil moisture and optimal conditions for infection. The plants were grown with 16 h day/8 h night cycle, with a day-time temperature of 24 °C, a night-time temperature of 19 °C and with extra light (Osram, HQI-T 400 W) supplied for the 16 h of day-time. After four weeks, the seedlings were removed from the pots and washed in water. Each seedling was examined and classified into one of six classes according to discolouration of the root system and hypocotyls: 0 = healthy plant without symptoms; 10 = around 10 % of the root system discoloured; 25 = 50 % of the root system discoloured; 50 = 100 % of the root system discoloured; 75 = 100 % of the root system and the hypocotyl discoloured; and 100 = dead plant (Larsson and Gerhardson 1990; Olsson et al. 2011). The number of seedlings was counted in each class. An average disease severity index (DSI) over the six replicate pots was calculated for each soil according to the formula:

$$DSI = \frac{\sum_{\text{class no.}} [(\text{class no.}) \times (\text{no.of plants in each class})]}{(\text{total no.of plants})} \quad (2)$$

Subsamples of approximately 100 g soil were dried and stored at room temperature until DNA extraction.

Pathogen isolation from roots

Fungi and oomycetes were isolated from 15 symptomatic plants per soil sample. The roots were washed in tap water until most soil was removed and sections of about 20 mm were cut and placed on different agar media. The media used were potato dextrose agar (PDA; Fluka) and corn meal agar (CMA; Fluka). During incubation at 20–25 °C, developing fungi and oomycetes were identified as far as possible directly on the media or after transferring to new plates. Fungal and oomycete isolates were identified through microscopic observation of mycelial characteristics and colours, the presence, shape and size of conidia and oospores.

Statistical analysis

Statistical analyses were performed using the Analysis ToolPak in Microsoft Excel (Microsoft Office Standard 2010, Microsoft Corporation).

Results

Specificity and sensitivity of the real-time PCR assay

The specificity of primers AcF and AcR and of the probe AcP was assessed using DNA extracted from *A. cochlioides*, as well as DNA extracted from several other common plant pathogens, including the closely related species *A. euteiches* and *A. cladogamus* (Table 1). DNA amplification was observed in all five isolates of *A. cochlioides* tested, whereas no amplification of DNA from other plant pathogenic species occurred. Searches using BLASTN (www.ncbi.nlm.nih.gov/blast/Blast.cgi) confirmed the specificity of the primers and probe.

The standard curves used to calculate the starting quantity of *A. cochlioides* DNA in soil samples had high amplification efficiencies (93–100 %). The correlation was linear, with a correlation coefficient of $R^2 > 0.99$. The target sequence was regularly detected even at the highest template dilution of the standard curve, representing three copies of the target sequence. The assay was linear over at least six orders of magnitude.

Artificially infested soil samples

Linear regression analysis of the detected target copies and the number of added *A. cochlioides* oospores demonstrated a significant linear relationship for both isolates (Fig. 1; $R^2 > 0.98$; $p < 0.001$). *A. cochlioides* DNA was consistently detected at levels of 10 oospores/g soil. The estimated copy numbers of the target sequence per oospore calculated from the soil dilutions series were 90 (SD = 16) and 141 (SD = 61) for the isolates Wadb K2 and Brams S4, respectively, and these estimates were not significantly different from each other ($p < 0.05$).

The limit of detection was determined in six different soils and varied between 1 and 50 oospores/g soil depending on soil type (Table 3). In general, low LODs were correlated with high clay content (Fig. 2a) and low sand content (Fig. 2b). The lowest LODs, 1–5 oospores/g soil, were observed in soils with clay content ranging from 36 to 54 %, whereas LODs in soils with clay content below 10 % ranged between 20 and 50 oospores/g soil. The opposite correlation was found with sand, as the lowest

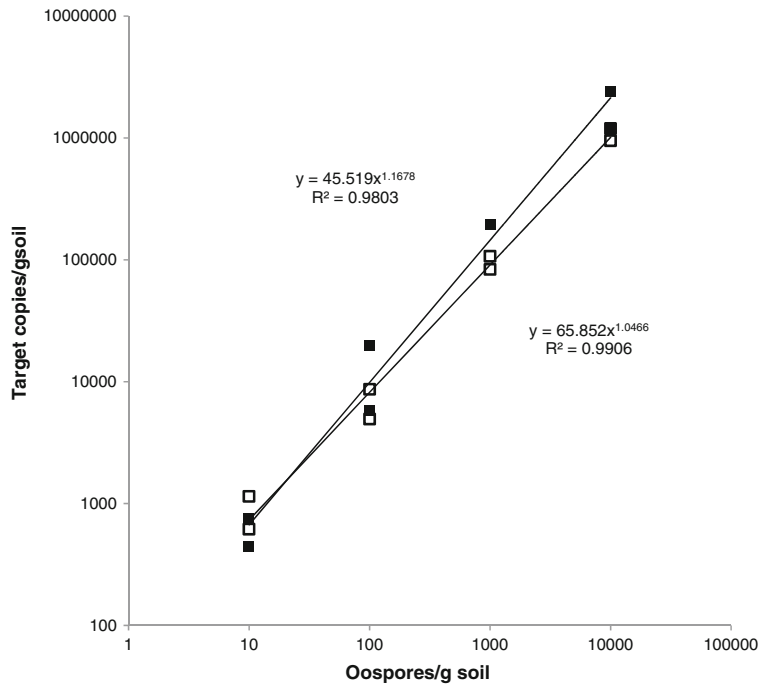

Fig. 1 Linear regression between real-time PCR results (target copies/g soil) and number of oospores/g soil in the artificially inoculated soil with oospores of *Aphanomyces cochlioides* in the range 10–10^4 oospores/g soil. Analysis was performed using oospores from two different *A. cochlioides* isolates; Wadb K2 (empty boxes) and Brams S4 (filled boxes) ($n = 2$)

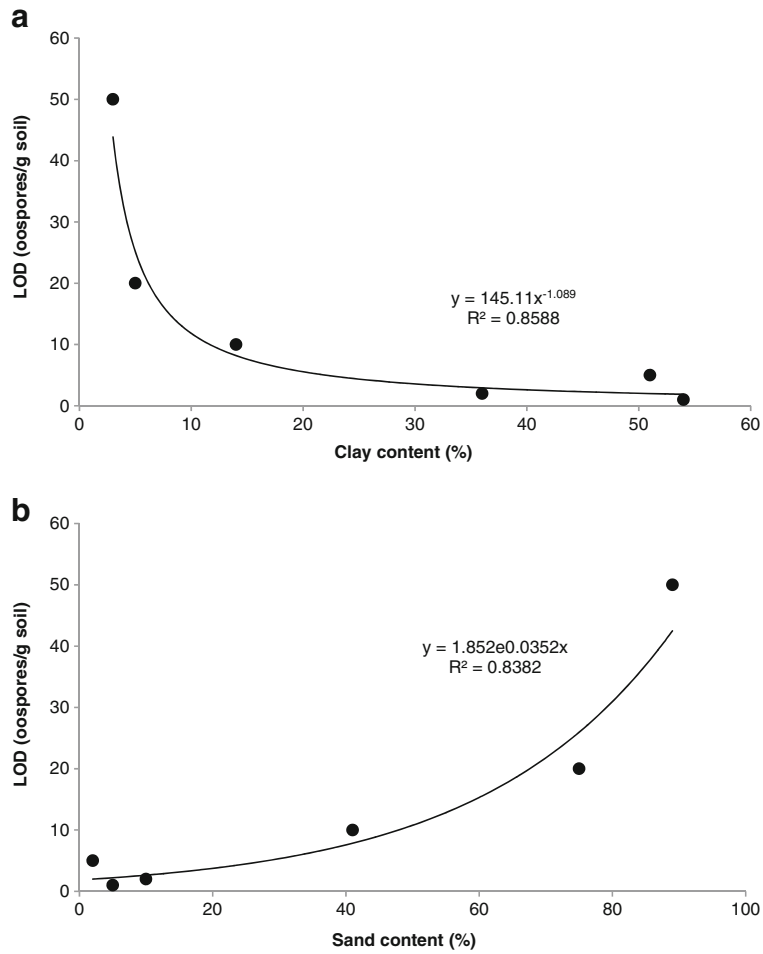

Fig. 2 Relationship between LOD (limit of detection, oospores/g soil) and soil physiochemical characteristics: clay content (**a**) and sand content (**b**)

LODs were observed in soils with low sand content (<10 %) and the highest LODs were observed in soils with high sand content (>75 %). The relationships between LOD and clay content as well as LOD and sand content is shown in Fig. 2. No *A. cochlioides* DNA was detected in the un-spiked soil samples.

The results from the inter-sample repeatability test, i.e. repeatability between separate extractions of subsamples, showed that the relative standard deviation for the nine subsamples analysed was 25 % (Table 4).

Field-infested soil samples

The relationship between DSI and real-time qPCR (target copies/g soil) for 61 naturally infested soil samples is presented in Fig. 3. The disease severity indices ranged from 29 to 85. The *A. cochlioides* target sequence was detected by qPCR in 20 soil samples with disease severity indices ranging from 50 to 85.

In addition to the bioassay, an agar test was made to evaluate whether *A. cochlioides* could be isolated from sugar beet roots grown in the field-infested samples. 80 % of the soils tested positive in the agar assay. All samples that tested positive using the qPCR assay were also positive using the agar test. In contrast, *A. cochlioides* was not detected by the qPCR assay in 29 soils although *A. cochlioides* was successfully isolated from roots of sugar beets grown in these soils (DSI ranged from 36 to 74). In the remaining 12 soils, *A. cochlioides* was detected neither by qPCR nor by isolation on agar. The indices ranged from 29 to 59 with six soils having indices above 50.

Three false negative soil samples with high disease severity indices were chosen for repeated extractions using protocol A. Five or six parallel extractions were analysed. The target sequence was only detected in one of six sub-samples in one of the false negative soils tested (DSI = 59) (Table 5). The qPCR test results of

Table 4 Inter-sample repeatability test (standard deviation between separate extractions of subsamples) of nine separate soil DNA extracts of *Aphanomyces cochlioides* from one soil sample analysed in the same real-time PCR run. The soil sample (clay 11 %, sand 66 %, organic matter 2.5 %, pH 5.3) was artificially infested with 10^3 oospores/g soil (d.w)

Sub-sample	Ct-value[a]	Target copies/g soil /1000
1	32.1	153
2	32.5	119
3	32.1	161
4	31.7	204
5	31.9	175
6	32.0	167
7	32.3	143
8	32.1	160
9	33.3	71
Mean		150
Standard deviation		38
Relative standard deviation		25 %

[a] Ct-values represent the mean of two replicates

Table 5 DNA extractions (n = 5–6) from three different soils with natural infestation of *Aphanomyces cochlioides*

Soil	Disease severity index	Sub-sample	Target copies/g soil
664	59	1	610
		2	Not detected
		3	1073[a]
		4	Not detected
		5	660[a]
		6	Not detected
682	73	1	Not detected
		2	Not detected
		3	Not detected
		4	Not detected
		5	Not detected
686	65	1	Not detected
		2	Not detected
		3	Not detected
		4	Not detected
		5	3600[a]
		6	Not detected

[a] Target sequence detected in one of two qPCR replicates

the other two soils were still negative despite repeated extractions.

A second DNA extraction protocol, protocol B, was used to evaluate whether the ratio of false negative samples could be reduced by increasing the amount of soil used for DNA extraction from 350 mg to 10 g. Nine naturally infested soil samples were selected; six samples with indices ranging from 45 to 73, but falsely negative using protocol A and three samples with indices ranging from 65 to 79 and positive using the qPCR assay on extracts from protocol A. *A. cochlioides* was successfully isolated from sugar beets grown in all nine soil samples. Using protocol B, the *A. cochlioides* target sequence was only detected in the three soils that were also positive using protocol A based on 350 mg soil. Pre-treatments, including heating or addition of skim milk powder, were also tested to evaluate whether the LOD of both artificially and naturally infested soil samples could be improved. However, none of the two modifications improved the LOD (data not shown).

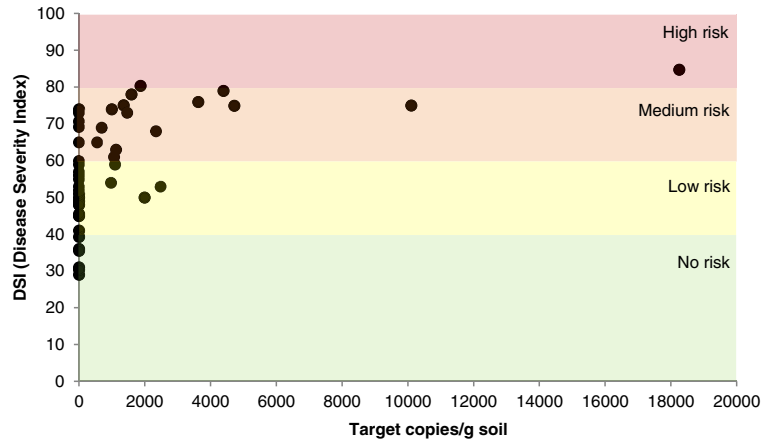

Fig. 3 Relationship between Disease Severity Index (DSI) according to the bioassay for *Aphanomyces cochlioides* and real-time quantitative PCR analysis (target copies/g soil) based on naturally infested soil samples from Swedish farms sampled in 2011 (n = 42) and 2014 (n = 19). One sample is not included in the diagram (37026;51). Aphanomyces root rot disease risk classes (no/low/medium/high) according to Olsson et al. (2011)

The level of inhibition was evaluated for all the soil samples analysed in this study. The lowest value for the effect of the inhibition (largest inhibition) was −29 % and therefore, no soil samples were regarded as inhibited.

Discussion

Previous reported *A. cochlioides* detection methods have in large been non-quantitative and based on detection in samples derived from plant material. Here we report the validation of a real-time PCR assay for quantification of *A. cochlioides* in naturally infested soils. The presented qPCR assay proved to be specific and was optimized to give high amplification efficiency suitable for target copy quantification. Standard curves demonstrated this linearity and showed that as few as three copies of the target sequence could be accurately quantified using the qPCR assay. Since soil samples with low levels of *A. cochlioides* DNA outside the range of the standard curve were quantified by extrapolating the standard, results below approximately 1000 target copies/g soil should be considered as estimates.

The assay showed a strong correlation ($R^2 > 0.98$, $p < 0.001$) of detected target copies and pathogen inoculum density ranging from 10 up to 10 000 oospores/g soil (Fig. 1), demonstrating its suitability for application to soil samples. Results from this experiment were also used to make a rough estimate of the copy number of the target sequence in the two *A. cochlioides* isolates studied. Our results clearly demonstrate that the copy number was similar between the two isolates tested ($p < 0.05$), indicating that the assay is suitable for quantification of the target sequence of different isolates. The estimated copy number of the target sequence per oospore was at a similar level as for the closely related species *A. euteiches* (Heyman 2008; Gangneux et al. 2014). However, a previous study of *Plasmodiophora brassicae* showed that the DNA yield of the extraction and purification protocol used in the present study probably means that this copy number should be multiplied by more than five to result in a more realistic copy number estimation (Wallenhammar et al. 2012). On the other hand, the DNA yield could vary for different types of spores. Further tests are therefore needed to determine the exact copy number of the ribosomal RNA genes in different isolates of *A. cochlioides*.

The limit of detection (LOD) was thoroughly evaluated in different soil types and varied between 1 and 50 oospores/g soil (Table 3). The LOD in soils with low/medium organic matter content (3.2–4.7 %) varied with different clay content and the lowest LOD was observed in the soil with the highest clay content. This correlation to clay content was also true for the two soils with high organic matter content (12–13 %). These results were unexpected since DNA extraction is often especially difficult for soils with high clay content (Frostegård et al. 1999) considering the fact that nucleic acids can easily be adsorbed to clay particles in the soil and thus reduce the DNA recovery (Cai et al. 2006). However, when comparing six different commercial DNA extraction kits, Dineen et al. (2010) demonstrated that the FastDNA SPIN kit yielded the highest amounts of DNA and the recovery of DNA from a clay soil was at a similar level as for a sand soil. All other kits tested had very low or no DNA yield from the clay soil. The LOD for soils with similar soil type as the naturally infested soils evaluated in this study (mean values of 15.3 ± 7.0 % clay and 3.4 ± 1.5 % OM) was approximately 10 oospores/g soil, which is very similar to LODs reported in other papers studying oomycetes using PCR (Hussain et al. 2005; Schroeder et al. 2006; Sauvage et al. 2007; Gangneux et al. 2014).

None of the DNA extracts from the samples included in the present study were regarded as inhibitory of the qPCR assay, since all values were above the set threshold value. The threshold was set at −40 %, since both positive values below 40 % and negative values above −40 % can be the result of technical and handling errors, such as pipetting errors, rather than showing a true effect of inhibition. As an example, for an ideal assay with an amplification efficiency of 100 %, a value of −50 % of the effect of inhibition for a sample containing no target copies corresponds to a Ct difference of 1 in the spiked sample compared with the spiked water control. Also, further purification of a moderately inhibited sample may not always give an improved result since each purification step also entails the loss of DNA. In addition, the effect of inhibition did not seem to be affected by the soil type since none of the artificially infested soils were regarded as inhibitory.

The precision between replicates in the same real-time PCR run as well as over separate runs is often very high (Valsesia et al. 2005). However, the inter-sample

repeatability between separate extractions of subsamples can have an effect on the precision of the result. The repeatability of the assay presented in this paper was shown to be similar to that of assays for other plant pathogens quantified in soil samples (Cullen et al. 2002; Atkins et al. 2003; Atkins et al. 2005; Zang et al. 2006; Wallenhammar et al. 2012). On the other hand, the repeatability may be dependent on soil type. Therefore, the sample chosen for the inter-sample repeatability test was selected carefully and had soil characteristics (clay and OM) in the same range as the naturally infested soil samples evaluated in this study.

In order to study if the detection limit could be further improved and whether the ratio of false negative samples could be reduced, a second DNA extraction procedure (protocol B) was evaluated. The basic principles of this extraction kit were the same as for the first protocol used, e.g. same procedure for oospore lysis (bead beating) and same type of chemicals, but the amount of starting material was several factors larger; 10 g instead of 350 mg. The detection limit of the analysis was not improved using this protocol, probably due to the fact that even though the initial soil sample was larger, the final eluate volume (DNA solution volume) was also larger compared to protocol A. On the other hand, DNA extraction from a larger soil sample could give more reproducible results and a more accurate quantification in soils with low inoculum levels. Nevertheless, protocol B is more expensive and the increased costs of this kit would probably not motivate the possible benefits. Instead, another approach to improve the sensitivity was tested; repeated DNA extractions. Three false negative soil samples with high indices were chosen for repeated extractions using protocol A. The target sequence was only detected in one of six sub-samples in one of the soils tested. Therefore, this strategy was also not considered as a suitable or useful alternative. A pretreatment of soil samples consisting of freezing followed by heating was successfully applied in a diagnostic assay for detection of *Verticillium dahliae* in soil (Bilodeau et al. 2012). When heating alone was tested in this study no significant improvement of the LOD was obtained. Nor the addition of skim milk powder improved the LOD. Other possibilities for improving the LOD of this assay could be to include different strategies for enrichment or recovery of the oospsores from the soil before the DNA extraction, e.g. wet sieving (Wang et al. 2006) or density flotation (Debode et al. 2011).

Bioassays using bait plants have been the most reliable and widely used methods for assessing soils for the presence and risk evaluation of *A. cochlioides* (Payne et al. 1994; Beale et al. 2002; Amein 2006; Olsson et al. 2011). Currently, in addition to standard and compulsory soil tests, farmers can also do biological soil mapping of soil borne pathogens including e.g. beet cyst nematodes (*Heterodera schachtii*) and *A. cochlioides*. In Sweden, following the results of the Aphanomyces root rot bioassay, soils are classified into one of four different risk categories according to their DSI: 0–39 = no risk; 40–59 = low risk; 60–79 = medium risk and >80 = high risk (Olsson et al. 2011). Based on the results of the bioassay the grower can choose the most suitable sugar beet variety for the field. For indices above 60 the advice is to use a tolerant variety. Until now, the bioassay has been used sparsely but with a possibility to do several analyses on the same soil sample, qPCR will aid in a disease support system to control many pathogens, including *A. cochlioides*. The quantitative real-time PCR assay presented in the present study is cost efficient, fast and highly reproducible and can give growers the possibility to assess the risk of *A. cochlioides* on whole or parts of their fields.

In the present study we compared a qPCR based detection assay with an established bioassay where a disease severity index is estimated based on visual assessment of disease symptoms followed by classical isolation of pathogens. Approximately one third of the naturally infested soil samples tested positive using the newly developed qPCR assay. Notably, the *A. cochlioides* target sequence was successfully detected in all soil samples with disease severity indices above 75. On the other hand, *A. cochlioides* was only detected in approximately 50 % of the soil samples with DSI between 60 and 74. Given the soil composition in the field infested soil samples, an LOD of approximately 10 oospores/g soil is expected. The exact concentration of *A. cochlioides* oospores needed for infection is not known. However, a level of 10 oospores/g soil will probably result in a DSI below 60, assuming a similar correlation between the concentration of oospores and DSI as for *A. euteiches* causing pea root rot (Persson et al. 1999). It is not clear that the discrepancy between the PCR assay and the bioassay is caused by low sensitivity due to the composition of individual soils or difficulties in extracting sufficient DNA. One alternative explanation could be that discoloration of the root system may be caused by other soil-organisms such as free-

living nematodes and secondary infections by fungi. As an example, the closely related *A. cladogamus*, which mostly affect spinach, can also cause similar symptoms of the root system in sugar beet (Larsson and Olofsson 1994) and is not, due to primer specificity, detected by the PCR assay developed here. In order to resolve the observed differences between the bioassay and PCR assay developed specifically for *A. cochlioides*, multiplex PCR methods that allow simultaneous detection of more than one pathogen could be used. Since pathogen pressure differs among regions unbiased detection methods based on large scales next generation sequencing methods could be used to identify possible candidate pathogens to include in such multiplex PCR detection methods.

In summary, this paper presents the development and validation of a potentially robust and reliable real-time PCR method for detection and quantification of *A. cochlioides* in naturally infested soil samples. The suitability and potential use of this assay as a tool in disease risk assessment has been demonstrated for fields with high risk of infection (DSI >80). The validation of this real-time PCR method for fields with medium risk of infection (DSI = 60–79) remains to be investigated.

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