DNA-based detection of *Aphanomyces cochlioides* in soil and sugar beet plants

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Abstract

Aphanomyces cochlioides, the causal agent of seedling damping-off and Aphanomyces root rot (ARR) of sugar beet, causes yield losses in major sugar beet growing regions. Currently, a 4-week soil bioassay and a 2-day culture-based assay are used to diagnose presence of *A. cochlioides.* However, these assays can be time-consuming and lack sensitivity. In this study we developed a sensitive, specific, and rapid assay to detect and quantify DNA of *A. cochlioides*. We developed a TaqMan qPCR assay targeting a region of the mitochondrial genome of *A. cochlioides* representing a unique gene order for *Aphanomyces* with genus-specific primers and a species-specific probe. The qPCR assay detected *A. cochlioides* in 12 naturally infested field soil samples with disease severity index (DSI) values of 48-100, in sugar beet seedlings 5-7 days after planting, and with as little as 1 fg of pure *A. cochlioides* DNA. Adult sugar beet roots with ARR symptoms were sampled to further validate this qPCR assay. *Aphanomyces cochlioides* was detected in 95% of these samples using this qPCR assay, while only 23% of the same samples were positive using a culture-based assay. This shows the improved sensitivity of this qPCR assay for disease diagnosis and could provide growers with ARR risk of a field, which would help them make informed disease management decisions. However, further research is required to translate the results of this study to growers' fields to quantify *A. cochlioides* with a high degree of accuracy.

Introduction

Sugar beet (*Beta vulgaris* L.) is an economically important crop that is central to the multi-billion dollar sugar industry (Draycott, 2006). Sucrose extraction is performed on sugar beet taproots providing approximately 30% of the world's sugar (Dohm et al., 2014). In 2021, 36.7 million tons of sugar beets were harvested in the United States with a market value of 1.8 billion dollars (USDA NASS, 2022). The Red River Valley (RRV) of Minnesota and North Dakota produces 51% of the total U.S. sugar beet crop. In the RRV, American Crystal Sugar Company (ACSC), a major sugar beet production cooperative, produces 3 billion pounds of sugar from 425,000 acres of sugar beet annually (ACSC, n.d.). Sugar beets are utilized as a fuel feedstock for ethanol production, and the pulp byproduct of sucrose extraction is used to produce non-starch livestock feed (Alexiades et al., 2018). Overall, sugar beets are agriculturally and economically valuable in the USA, as well as many other countries, including France, Canada, Russia, Ukraine, Turkey, Chile, Germany, Spain, Hungary, Sweden, the Netherlands, Australia, and Japan (Draycott, 2006; Harveson et al., 2007; Olsson et al., 2019).

The soil-dwelling oomycete, *Aphanomyces cochlioides*, is the causal agent of damping-off and root rot of sugar beet, causing yield losses in all major sugar beet growing regions. In the RRV, it was estimated that Aphanomyces root rot (ARR) occurs in half of all sugar beet fields, and in southern Minnesota it occurs in nearly all sugar beet fields (Beale et al., 2002; Harveson et al., 2007). The disease risk is higher in water-saturated regions of a field and affected area can range from a square meter to an entire field (Windels, 2000). Growing seasons with above average precipitation typically have a higher prevalence of ARR (Windels & Nabben-Schindler, 1996; Harveson, 2007). Severe ARR can reduce sucrose yields up to 27% and increase impurities in the roots which reduce its value for sucrose extraction (Olsson et al., 2011; Windels, 2000). ACSC

70 estimates that its monetary losses are approximately 10 million dollars annually due to this disease
71 (Harveson et al., 2007).

72 Aphanomyces seedling damping-off occurs after emergence, causing the hypocotyl to
73 become necrotic and thread-like with the absence of foliar wilting. When ARR occurs on mature
74 sugar beets the symptoms include chlorosis of older leaves, wilting during hot days, and necrotic
75 water-soaked lesions that spread across the surface of the taproot, which result in stunting or plant
76 death (Windels, 2000). Commercial sugar beet seeds are often treated with Tachigaren™
77 (hymexazol), which effectively protects from Aphanomyces and Pythium damping-off for a few
78 weeks after planting. As sugar beets mature throughout the growing season, the chronic phase of
79 ARR cannot be prevented with Tachigaren™. To reduce ARR severity, growers practice early
80 planting of resistant sugar beet varieties, improve drainage by tiling and tillage practices, apply
81 waste lime (precipitated calcium carbonate) to fields, implement a 3 to 5 year crop rotation with
82 non-host crops, and control of alternative host weeds (Draycott, 2006; Harveson, 2007).

83 The hosts of *A. cochlioides* includes some Caryophyllales and dicots in the Amaranthaceae
84 such as sugar beet (*Beta vulgaris* L.), table beet (*Beta vulgaris subsp. vulgaris* L.), spinach
85 (*Spinacia oleracea)*, New Zealand spinach (*Tetragonia tetragonioides*), chard (*Beta vulgaris ssp*.
86 *vulgaris*), cockscomb (*Celosia argentea* and *Celosia cristata* L.), and bouncing bet (*Saponaria*
87 *officinalis*) (Wen et al., 2006; Windels, 2000). Additionally, wild weed and perennial flower
88 species such as pigweed, lambsquarters (*Chenopodium album*), carpetweed (*Mollugo verticillata*),
89 and fireweed can serve as inoculum reservoirs (Papavizas and Ayers, 1974; Grünwald et al., 2003;
90 Harveson, 2007). ARR is an intractable disease due to its thick-walled oospores, which can persist
91 in a dormant state for 10 to 20 years of adverse conditions without a host (Windels & Brantner,
92 2000; Harveson, 2007). Overwintering oospores serve as the primary inoculum for the following

sugar beet crop (Harveson, 2007). When a host is present, under warm and saturated soil conditions, *A. cochlioides* oospores germinate by producing hyphae and form zoosporangia, which release large quantities of zoospores that migrate through soil water by sensing host root exudates and initiate infection (Islam, 2010). Overall, the ability to mass produce asexual zoospores that are capable of re-infecting host plants makes *A. cochlioides* a devastating sugar beet pathogen in warm and water-saturated soils.

Largely, traditional and molecular assays have been used to diagnose ARR, however these assays have limitations. A growth-chamber bioassay has been traditionally used to evaluate the ARR disease severity index (DSI) of infested field soil over a 4-week period (Fink and Buchholtz, 1954). However, this bioassay is limited by sensitivity and speed, and lacks the ability to quantify initial amounts of *A. cochlioides* inoculum in soil (Windels and Nabben-Schindler, 1996). Second, an ELISA targeting *Aphanomyces cochlioides* was developed to assess ARR of sugar beet. This assay is quantitative, but not specific to *A. cochlioides* and lacks sensitivity in samples with low inoculum amounts (Weiland & Shelver, 2004). A PCR assay was designed to amplify the *A. cochlioides* actin gene and the ITS region of the rRNA gene, but lacks specificity, and was only able to identify *A. cochlioides* with a restriction enzyme analysis of the amplicons (Weiland & Sundsbak, 2000). Another PCR assay specific to *A. cochlioides* was developed that multiplexed a primer pair specific to *A. cochlioides* with another primer pair specific to *A. euteiches* (Vandemark et al., 2000). This allowed the detection of both pathogens to be performed in one PCR run, but lacked the ability to quantify the pathogens. Recently, a specific qPCR assay was designed to detect and quantify *A. cochlioides* from infested soil using the internal transcribed spacer (ITS) region of the rRNA gene (Almquist et al., 2016). This qPCR assay had a reported limit of detection in the range of 1-50 oospores per gram of soil under artificial infestation depending on soil clay

content. However, this qPCR assay did not detect *A. cochlioides* in 68.9% of naturally infested soil samples that were positive for *A. cochlioides* with the bioassay with a DSI below 80. Largely, traditional and molecular assays have been used to evaluate ARR, however these assays have apparent drawbacks.

The limitations of the available assays for ARR highlight the importance of designing an effective assay to detect and quantify *A. cochlioides* in soil and *in planta*. This study developed a qPCR assay that is sensitive, specific, and able to accurately quantify *A. cochlioides* in naturally infested field soil and infected plant samples. This qPCR assay will be useful for growers to accurately assess the Aphanomyces root rot risk level of a field prior to planting sugar beets, without having to wait 4 to 5 weeks for the soil bioassay results. This time is valuable because management decisions are determined by the infestation risk level in the field. Additionally, this qPCR assay could be useful for sugar beet diagnosticians to assess sugar beets infected with *A. cochlioides*. Overall, sugar beet growers have a need for a more sensitive, accurate and rapid assay that can consistently quantify initial infestation levels of naturally infested field soils. Having this information will help growers make informed decisions, such as planting date, selecting a resistant cultivar, use of appropriate dose of Tachigaren seed treatment, applying sugar beet factory waste lime, managing soil moisture, length of crop rotation, and other sanitation methods.

Materials and Methods

qPCR primer and probe development. To design the primers and probe, the mitochondrial genomes of 14 *Aphanomyces* isolates representing 8 species, including *A. cladogamus, A, cochlioides* and *A. euteiches*, were assembled and compared (F. Martin, unpublished). These included the mitochondrial genome of 5 *A. cochlioides* isolates, 13-35-5 (MN), 14-SMAN-M-1

(MN), 61ss (TX), 64ss (TX), C10 (ND), that were assembled from Illumina sequencing data. In addition, 250 available mitochondrial genomes from *Pythium, Phytophthora*, downy mildews and other oomycetes were used to identify differences in conserved gene order of *Aphanomyces* spp.. Within the mitochondrial genomes, 4 loci were specific to Saprolegniales, and 3 loci were specific to *Aphanomyces*. The forward primer (Aphcoc-F) was designed to anneal to a conserved region just downstream from the mitochondrial *rps4* gene for any *Aphanomyces* spp.. (Supplementary Figure S1). The reverse primer (Aphcoc-R) was designed to anneal to the 5' end of the mitochondrial *nad2* gene for the plant pathogens *A. cladogamus*, *A. cochlioides*, and *A. euteiches*. The probe (Aphcoc-Pr) was designed to anneal to a region just before the 5' end of the mitochondrial *nad2* gene of *A. cochlioides* alone. Using these primers for amplification of *A. cochlioides* resulted in a 234 bp amplicon.

Development of qPCR detection assay. Quantitative PCR (qPCR) was executed using a LightCycler 480 Instrument II (Roche Life Science, Pleasanton, CA, USA) with a reaction volume of 25 µL per sample. The standard *A. cochlioides* assay reaction contents consisted of 1x PerfeCTa Multiplex qPCR ToughMix (Quantabio, Beverly, MA, USA), 0.6 µL (10 µM) forward primer (Aphcoc-F, 5'-GACCCTATTTAAAAATAGGTAT-3'), 0.6 µL (10 µM) reverse primer (Aphcoc-R, 5'-AAAAATTCAGGAATTAGAAATAAA-3'), 0.2 µL (10 µM) probe (Aphcoc-Pr, 5' 6-FAM ATATAAATAATAATAATACATACATGATT-3' BHQ), 1 µL template DNA, and the remaining volume made up to 25 µL with molecular grade water. The Roche LightCycler instrument measured the intensity of FAM (465-510 nm) for the *A. cochlioides* probe (Aphcoc-Pr). The thermal cycler conditions were as follows; a reaction initialization at 95°C for 3 minutes, followed by 40 cycles of denaturing at 95°C for 15 seconds and annealing at 60°C for 45 seconds,

with a final elongation at 37°C for 30 minutes. Each qPCR plate included a no template negative control and a positive control consisting of 1 ng *A. cochlioides* DNA, which was also used in the standard curve. The standard curve was applied externally to each run using a fit point analysis.

A standard curve was made using eight 10-fold dilutions of *A. cochlioides* isolate 13-69-4 DNA, from 1 ng/µL to 0.1 fg/µL. The 1 ng/µL DNA dilution level was initially quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The 0.1 ng concentration was prepared by adding 100 µL of the 1 ng/µL DNA dilution level to 900 µL molecular-grade water, then vortexing for 3 s, and repeating the process for all eight dilutions. Aliquots of 10 µL and 100 µL were prepared for each DNA dilution, and all DNA standards were stored at -80°C until use. One microliter of each DNA standard was tested with the qPCR assay to attain Ct values for the 1 ng to 1 fg dilutions. The standard curve Ct values were saved as an external standard curve, which was imported for each run using the fit point analysis.

The specificity and inclusivity of the qPCR assay was evaluated using the standard primer and probe set (Aphcoc-F, Aphcoc-R, Aphcoc-Pr). First, the specificity was evaluated *in silico* using NCBI's nucleotide BLAST. Next, the qPCR assay was tested against DNA from *Beta vulgaris*, *A. euteiches*, *A. cladogamus*, Ascomycota fungi, non-Aphanomyces species of Oomycota, and Protozoa isolates (Table 1). Additionally, DNA from 14 *A. cochlioides* isolates obtained in the Red River Valley of Minnesota and North Dakota, southern Minnesota, and Texas were included to test the inclusivity of the qPCR assay (Table 1). *A. cochlioides* isolates were grown on 0.5X PDA and DNA was extracted from mycelial tissue using the FastDNA Spin Kit for Fungi (MP Biomedicals, Irvine, CA, USA) following the manufacturer's specifications. All DNA samples were quantified using the Nanodrop Spectrometer (Thermo Fisher Scientific,

184 Waltham, MA, USA) and 10 ng of DNA from each sample was used to test the specificity and
185 inclusivity of the qPCR assay.
186
187 **Naturally infested soil samples**. Twelve naturally infested soil samples were collected from fields
188 in the RRV of Minnesota and North Dakota, and Southern Minnesota from 2017 to 2020 (Table
189 2). Soils were used directly to conduct the bioassay and subsampled for DNA extraction. To
190 prepare soil for subsampling, each soil was mixed thoroughly by hand in a 5-gallon bucket, 1 kg
191 of soil was removed and homogenized with a mortar and pestle then passed through a 3 mm sieve.
192 Homogenized soil samples were poured into a large tray and thoroughly mixed by hand. Three
193 500 mg and 5 g soil subsamples were removed and placed in 2 mL micro-centrifuge tubes and 50
194 mL centrifuge tubes, respectively, and stored at 4°C until DNA extraction.
195
196 **Aphanomyces root rot bioassay for infested soils**. The ARR DSI of infested soils was assessed
197 using the ARR growth chamber bioassay. For each soil sample, 25 seeds of Crystal 257 RR were
198 planted in pots (10 x 10 x 10 cm) filled with naturally infested field soil. Each pot was considered
199 a replicate and six replicates were used for each soil sample. Seed was commercially treated with
200 a metalaxyl (0.15 g a.i. per kg seed) and thiram (2.49 g a.i. per kg seed) to protect against *Pythium*
201 spp. Pots were arranged in a randomized block design and incubated in a growth chamber for 1
202 week at 20°C, followed by 3 weeks at 25°C, with a 14-hour photoperiod. To create favorable
203 infection conditions for *A. cochlioides* pots were watered once per day. Seedlings were counted
204 every day during emergence and three times weekly thereafter. Dying seedlings were removed,
205 surface sterilized in a 0.5% sodium hypochlorite solution, rinsed with sterile deionized water,
206 plated in sterile deionized ultra-filtered water (Fisher Chemical, Waltham, MA, USA) and

207 microscopically evaluated to verify presence of *A. cochlioides* zoosporangia or other pathogens
208 after 24 hrs. Four weeks after planting, remaining seedlings were removed from soil, washed, and
209 rated using a 0 to 3 disease severity scale (Figure 1). The number of seedlings that died during the
210 4-week bioassay along with the disease severity ratings were used to calculate the DSI (Equation
211 1). The DSI values range from 0 to 100, with 0 representing no disease, and 100 representing the
212 most severe ARR (Windels and Nabben-Schindler, 1996; Beale et al., 2002).
213

214 $$\text{DSI} = \frac{\sum(\text{Rating x No.plants with rating}) \text{ x } 100}{\text{Total No.plants emerged x } 3} \qquad (1)$$

215 Equation 1. The Aphanomyces root rot disease severity index (DSI) is calculated based on the
216 number of seedlings with a 0 to 3 rating (0 = healthy hypocotyl, 1 = light brown hypocotyl
217 discoloration, 2 = moderate hypocotyl discoloration, 3 = severe hypocotyl discoloration or dead
218 plant) (Windels & Nabben-Schindler, 1996).
219

220 **Naturally infected plant samples.** Plant samples of diseased adult sugar beet roots were
221 submitted to the sugar beet pathology lab at the University of Minnesota, Northwest Research and
222 Outreach Center, Crookston for diagnosis during the summer of 2020. A total of 60 adult sugar
223 beet roots were submitted from 11 fields in the RRV of Minnesota and North Dakota, and Southern
224 Minnesota (Table 3). The soil was removed from roots with a brush under running water, an image
225 was taken, and observations of root rot symptoms were recorded for each individual root. Of these
226 samples, 40 roots with ARR symptoms such as irregular water-soaked or necrotic lesions, root
227 constriction, scarring and malformation were selected to be tested for *A. cochlioides* using the
228 culture-based and qPCR assay. Tissue subsamples, approximately 1 x 1 x 0.5 cm (150 mg), were
229 excised from the margin of necrotic lesions on the root with a sterile scalpel. Two subsamples were

taken right next to each other (Figure 2). One subsample was used for a culture-based assay and the other subsample was flash frozen in liquid nitrogen for DNA extraction and the qPCR detection assay.

Culture-based assay for infected plants samples. To conduct the culture-based assay, excised tissue subsamples were surface sterilized as previously noted, rinsed with sterile deionized water, plated in 10 mL of sterile deionized ultra-filtered water, and incubated at room temperature. Water cultures of plant subsamples were examined with a stereo microscope one to two times a day over a three-day period. The presence of nonseptate hyphae, zoosporangia, zoospores or oospores was treated as confirmation that the infection was caused by *A. cochlioides*. Attempts were made to isolate *A. cochlioides* from diseased root tissue by culturing on PDA-RP [potato dextrose agar amended with rifampicin (18.8 mg/L) and penicillin G (50 mg/L)], or PDA-MBR [potato dextrose agar amended with metalaxyl (30 mg/L), benomyl (5 mg/L), and rifampicin (18.8 mg/L)] media.

DNA extraction from soil and plant tissue. The FastDNA Spin Kit for Soil and Plants (MP Biomedicals, Irvine, CA, USA) was used for soil DNA extractions based on previous qPCR assays for soilborne plant pathogens (Almquist et al., 2016; Bilodeau et al., 2012; Wallenhammar et al., 2012). The standard protocol for the FastDNA Spin Kit for Soil and Plants was optimized for extracting *A. cochlioides* DNA by increasing the duration and speed of mechanical lysis. The optimized procedures for soil and plant tissue are described below.

For naturally infested soil samples, each sample was 500 mg (dry wt.), which was stored at 4°C until DNA extraction. An extra ceramic bead was added to each sample tube with Lysing Matrix E. Samples were subjected to mechanical cell lysis for 80 seconds using the FastPrep-24™

253 5G (MP Biomedicals, Irvine, CA, USA) instrument at speed 7.5 m/s, or for 160 seconds with the
254 Bead Beater instrument (BioSpec Products, Bartlesville, OK, USA) at the highest speed. The rest
255 of the protocol was conducted according to the manufacturer's recommendations. Final eluted soil
256 DNA samples were stored at -20°C.

257 For naturally infected plant samples, tissue was flash frozen in liquid nitrogen and stored
258 at -80°C. Each 150 mg (fresh wt.) tissue sample was placed in Lysing Matrix A, and an extra
259 ceramic bead was added to each sample tube. Mechanical cell lysis was done for 80 seconds using
260 the FastPrep instrument set at speed 7.5 m/s, or for 160 seconds with the Bead Beater instrument
261 at the highest speed. The rest of the protocol was followed according to the manufacturer's
262 recommendations, and final eluted DNA samples were stored at -20°C.

263

264 **Detection of *A. cochlioides* in sugar beet seedlings**. Four pots (10 cm x 10 cm x 10 cm) were
265 filled with naturally infested field soil (Perley, MN, Table 2) and four pots (10 cm x 10 cm x 10
266 cm) were filled with the same soil, which had been recently used to conduct the bioassay (referred
267 to as used bioassay soil from here on). Ten untreated sugar beet seeds (cultivar Crystal 257 RR)
268 susceptible to *A. cochlioides,* were planted in each pot, and pots were placed in the growth chamber
269 at 25°C with a 14-hour photoperiod. Seedlings were carefully uprooted at 3, 5, 7, 9, and 11 days
270 after planting (DAP) to keep roots intact. Root tissue was washed under running water for 2
271 minutes on a screen, and a paintbrush was used to remove excess soil. Root infection was
272 confirmed by examination with a stereo microscope. For each pot, all ten seedlings were removed,
273 5 seedlings were placed in a microcentrifuge tube, representing 2 replicates per collection time
274 point. Sample tubes containing 5 seedlings were immediately frozen in liquid nitrogen and stored
275 at -80°C until use. DNA extractions were performed using the FastDNA Spin Kit for Plants (MP

Biomedicals, Irvine, CA, USA) using the optimized procedure described previously, and DNA was stored at -20°C until use. The qPCR assay was conducted for all seedling DNA samples using the procedure described previously, and Ct values were obtained using the fit point analysis.

Detection of *A. cochlioides* in naturally infested soils. A total of 12 field soil samples naturally infested with *A. cochlioides* were used to validate the qPCR assay. All samples were subjected to the growth chamber bioassay for *A. cochlioides* described earlier. DNA was extracted from two samples received in 2017 using the DNeasy PowerMax Soil Kit starting with a 5 g soil sample and following the manufacturer's recommendations. DNA was extracted from ten naturally infested field soil samples received in 2019 and 2020 using the FastDNA Spin Kit for Soil optimized protocol. All 12 samples were tested using the qPCR assay following the procedure described above. Ct values were obtained using the fit point analysis.

Detection of *A. cochlioides* in naturally infected plant samples. Three *A. cochlioides* detection methods were used in parallel on diseased adult sugar beet roots collected from 11 fields known to have ARR in past growing seasons (Table 3). The detection methods included a visual assessment for ARR symptoms, the culture-based assay, and the qPCR assay. Adjacent subsamples, excised from each diseased adult sugar beet root, were either plated in sterile water cultures to recover the pathogen or subjected to DNA extraction using the FastDNA Spin kit for plants optimized protocol to obtain DNA, and the qPCR assay was used to confirm the presence of *A. cochlioides* DNA. Ct values were obtained using a fit point analysis and the results of the three detection methods were compared.

Detection of *A. cochlioides* in artificially infested potting soil. The growth chamber soil bioassay and qPCR assay were performed on potting soil infested with different amounts of *A. cochlioides* isolate 103-1 oospores. Oospores were produced on excised sugar beet hypocotyls following the procedure described earlier (Dyer & Windels, 2003). Oospores were quantified with a Speirs-Levy counting chamber, added to autoclaved fine vermiculite, and air dried at 25°C for 2 days. Potting soil (Sun Gro Professional Growing Mix, Sun Gro Horticulture, Agawam, MA, USA) was amended with 60% sand (Difco Laboratories Inc, Franklin Lakes, NJ, USA) by mass and autoclaved. The oospore-vermiculite inoculum was weighed and mixed into the soil at 0, 1, 5, 10, 20, 50, and 100 oospores/g soil (dry wt.) in trays by hand. The infested soil was watered with reverse osmosis water, and air dried at 25°C for 10 days. Infested soil was plated on PDA-RP medium to confirm *A. cochlioides* mycelium was inactive. Five seeds from the susceptible sugar beet cultivar Crystal 093 RR were planted in infested soil following established methods, with eight technical replicates for each soil-oospore dilution (Windels & Brantner, 2001). After mixing oospores into soil, two 500 mg samples for each infestation level were removed and used for DNA extraction following the FastDNA Spin Kit for Soil optimized procedure. Finally, the qPCR assay and the ARR bioassay were conducted, as described above, to assess the Ct value and ARR DSI for each oospore infestation level.

Results

Specificity of the qPCR assay. NCBI's nucleotide BLAST was used to computationally evaluate the specificity of the *A. cochlioides* forward (Aphcoc-F) and reverse (Aphcoc-R) primers, which were designed to be specific to the genus *Aphanomyces* and *Aphanomyces* spp. that are plant pathogens, respectively, as well as the probe (Aphcoc-Pr) that is specific to *A. cochlioides*. The

322 results showed both the forward and reverse primers and probe have 100% identity with the *A.*
323 *cochlioides* isolate 103-1 (accession JAHQRK010000050.1) and did not have high similarity with
324 plant pathogens outside of the genus *Aphanomyces.* Additionally, the forward primer has 100%
325 identity to a region in the mitochondrial genomes of *A. astaci* isolate NJM9701 (accession
326 KX405005.1), *A. invadans* isolate AP03 (accession KX405005.1), and other *Aphanomyces* spp.
327 Finally, the reverse primer has 100% identity to *Aphanomyces euteiches* strain ATCC 201684
328 (accession VJMJ01000379.1).

329 To evaluate the specificity of the qPCR assay, it was tested against 10 ng of DNA from a
330 variety of other taxa, and multiple isolates of *A. cochlioides*. These included 9 isolates of *A.*
331 *euteiches*, 2 isolates of *A. cladogamus*, 15 species of Ascomycota fungi, 3 non-Aphanomyces
332 species of Oomycota, and a species of Protozoa (Table 1). DNA from 14 isolates of *A. cochlioides*
333 originated from North Dakota, Minnesota, and Texas was used to test the inclusivity of the qPCR
334 assay. The results of this analysis showed that the qPCR assay is specific to *A. cochlioides*, as
335 DNA amplification did not occur for any species tested other than *A. cochlioides*. Also, the qPCR
336 assay was shown to be inclusive, with the DNA from each *A. cochlioides* isolate being detected
337 consistently (Table 1).
338
339 **Sensitivity of the qPCR assay.** A standard curve of 10-fold serial dilutions of pure *A. cochlioides*
340 genomic DNA was tested with the qPCR assay in order to assess the linearity of amplification and
341 limit of detection (LOD). The LOD of the qPCR assay was 1 fg, which had a mean Ct value of
342 34.66 (Table 4). Each standard from 1 ng to 1 fg was detected in every technical replicate. For
343 DNA standards that were detected consistently, the difference between mean Ct values of
344 concentrations ranged from 3.41-4.15, giving an amplification efficiency of 83.3% (Figure

345 3). However, since the standards were not tested in the background of soil or sugar beet DNA, the
346 qPCR amplification efficiency and the LOD may differ in DNA samples of naturally infested soils
347 or infected plants that may contain different levels of PCR inhibitors.
348
349 **Detection of *A. cochlioides* in sugar beet seedlings.** Seedlings were collected over five time
350 points (Table 5) for DNA extraction and qPCR to validate that *A. cochlioides* DNA could be
351 detected in the seedling tissue, and to determine if *A. cochlioides* could be detected prior to
352 development of visible symptoms. For naturally infested field soil, *A. cochlioides* DNA was first
353 detected 5 DAP with a mean Ct value of 29.62, and consistently detected in all samples 7 DAP
354 with a mean Ct of 25.03. For the used bioassay soil, *A. cochlioides* DNA was first consistently
355 detected at 5 DAP, with a mean Ct value of 24.21. *A. cochlioides* DNA was consistently detected
356 2 days prior to visible ARR symptoms in both soil samples. These results confirmed that the qPCR
357 assay could detect *A. cochlioides* in infected sugar beet seedling tissues ahead any visible
358 symptoms.
359
360 **Detection of *A. cochlioides* in naturally infested soils.** The qPCR assay was validated with DNA
361 extracted from twelve soil samples that were naturally infested with *A. cochlioides*. The ARR
362 bioassay confirmed each soil sample was infested with *A. cochlioides*, and determined the ARR
363 DSI value. The ARR DSI values ranged from 48-100, with a mean DSI value of 87 (Table 6).
364 Eleven samples tested positive for *A. cochlioides* DNA in every technical replicate, while one soil
365 sample (Perley) was positive in 3 of 4 technical replicates. For 12 naturally infested soil samples
366 the mean Ct values ranged from 26.72 - 34.64, indicating the assays capability in detecting *A.*
367 *cochlioides* (Table 6).

Detection of *A. cochlioides* in naturally infected plants. Out of 40 roots with ARR symptoms, culture-based diagnostics identified *A. cochlioides* in 23% of samples and *A. cochlioides* DNA was detected in 95% of samples. *A. cochlioides* DNA was detected in diseased sugar beet roots from every field whereas culture-based diagnostics identified *A. cochlioides* in 3 of 11 fields (Table 7). Finally, *A. cochlioides* DNA was detected in each of the 9 sugar beet roots that *A. cochlioides* was detected with using the culture-based assay, and in an additional 29 sugar beet roots where *A. cochlioides* was not recovered. These results validate that the qPCR assay can detect *A. cochlioides* in naturally infected mature sugar beet roots with high accuracy.

Detection of *A. cochlioides* in artificially infested potting soil. The qPCR assay and ARR bioassay were performed on infested potting soil samples with various densities of *A. cochlioides* oospores to determine their mean Ct values and ARR DSI value and compare the two assays. In this experiment, the LOD for the qPCR assay was 10 oospores/g soil (dry wt.), which was consistently detected in all technical replicates, and had a mean Ct value of 33.26 (Table 8). A strong negative linear correlation (R^2= 0.96, p = 0.00054) was observed between oospore density and mean Ct value. *A. cochlioides* DNA was not detected in the negative control, or in the lowest oospore density of 1 oospore/g soil (dry wt.). After completing the ARR bioassay for the soils, the mean ARR DSI value was calculated for each oospore density. The mean ARR DSI values ranged from 10.83 for 1 oospores/g soil (dry wt.) to 75 for 100 oospores/g soil (dry wt.) (Table 8). A strong positive linear correlation (R^2= 0.968, p = 0.000032) was observed between the oospore density of the soil and the mean ARR DSI value. These results showed that the qPCR assay can

390 detect *A. cochlioides* DNA in oospore infested potting soil, which had a range of DSI values from
391 23.33 to 75.00.
392
393 **Discussion**
394 The ultimate goal of developing qPCR assays for soilborne pathogens is qualitative
395 detection and quantification of pathogen inoculum in field soils in order to assess the risk level of
396 disease prior to planting. Having this information enables growers to make informed management
397 decisions concerning which crop to plant, choosing varieties with appropriate level of disease
398 resistance, use and rates of seed treatment, and other cultural management strategies. We report
399 development of a specific, sensitive, and rapid mitochondrial DNA-based qPCR-based detection
400 assay for *A. cochlioides* from various soil and plant samples. The assay was species-specific when
401 tested against other *Aphanomyces* spp. and common soilborne fungi and oomycetes and detected
402 all 14 isolates of *A. cochlioides* collected from MN, ND and TX. The gene order unique to
403 *Aphanomyces* that was targeted improves specificity and repeatability across different real time
404 PCR thermal cyclers. The forward primer (Aphcoc-F) was designed to anneal to multiple
405 *Aphanomyces* spp., including pathogens of both plants and aquatic fish. The reverse primer
406 (Aphcoc-R) was designed to anneal to *Aphanomyces* spp. that are plant pathogens; including *A.*
407 *cladogamus, A. cochlioides*, and *A. euteiches*. The probe (Aphcoc-Pr) was designed to anneal to
408 *A. cochlioides*. Based on alignments of this locus (Figure S1) modifying bases in the probe
409 sequence would enable the development of a qPCR assay that can targets the mitochondrial
410 genome of *A. cladogamus*, or *A. euteiches* while using the same forward and reverse primers
411 developed in this study. The mitochondrial genome was chosen as the amplification target because
412 of its high copy number, which may improve sensitivity. Also, mitochondrial genomes have been

found to evolve more rapidly compared to nuclear genomes, making it easier to identify variation among closely related species for primer and probe design (Makkonen et al., 2016). The standard curve of *A. cochlioides* DNA isolated from pure cultures indicated that as little as 1 fg of pathogen DNA could be accurately detected with this assay. The LOD for oospore infested potting mix was 10 oospores per gram (dry wt.) of soil, which highlights the sensitivity of this assay. Although, the DNA standards for the qPCR assay were not run in the background of soil or plant DNA, therefore, the LOD of 1 fg does not necessarily represent the sensitivity that would be observed for some real-world samples, such as soil DNA that contains PCR inhibitors. For soil DNA samples from the field there is an expected reduction in template DNA amplification, compared to the PCR amplification of the same quantity of *A. cochlioides* DNA obtained from pure culture. For example, when 0.1 pg of *A. cochlioides* DNA was added to various soil DNA samples the Ct values were 0.69-2.14 higher than that from testing 0.1 pg of *A. cochlioides* DNA without soil DNA in the background (details of the qPCR inhibition are summarized in the Supplementary Text and Supplementary Table S1). In order to determine the actual sensitivity of this assay for naturally infested soil or infected plant samples from a specific field, DNA standards need to be tested with the appropriate background sugar beet or soil DNA to account for PCR inhibitors.

Different modifications have been made to DNA extraction procedures in order to improve the sensitivity of this qPCR assay. During the development of a qPCR assay for *V. dahliae*, improved detection was achieved by pre-treating samples with three cycles of freezing in liquid nitrogen for 2 min followed by heating to 70 °C (Bilodeau et al., 2012). In Almquist et al., (2016), they tested heating the soil samples and lysis buffer for 10 min at 65 °C, and the addition of skim milk powder prior to DNA extraction, but neither technique improved the LOD for *A. cochlioides* DNA. In this study, freezing and heating cycles, as in Bilodeau et al., (2012), were applied to soil

436 samples naturally infested with *A. cochlioides*, and detection improved for some samples, but
437 lacked consistency (data not shown).

438 During periods of dormancy, the oospores of *A. cochlioides* can persist in field soil for a
439 decade (Papavizas and Ayers, 1974). These durable thick-walled oospores within soil samples
440 present a challenge for DNA extractions, and therefore detection of *A. cochlioides* in naturally
441 infested soil samples. Various methods and DNA extraction kits have been employed in attempts
442 to determine reliable techniques to acquire DNA from these samples for qPCR diagnostic assays.
443 We utilized bead beating, which has been shown to be a dependable method of mechanical cell
444 lysis for extracting DNA from soil samples infested with *A. cochlioides* and *A. euteiches*
445 oospores (Almquist et al., 2016; Sauvage et al., 2007).

446 Previous research showed that the FastDNA Spin Kit for Soil with a sample size of 350
447 mg was preferred over the DNeasy PowerMax Soil Kit with a sample size of 10 g because the
448 larger sample size did not improve the LOD, was more time consuming, and more costly for
449 detecting *A. cochlioides* (Almquist et al., 2016). We determined the FastDNA Spin Kit for Soil
450 with a sample size of 500 mg was preferable to the DNeasy PowerMax Soil Kit with a sample size
451 of 5 g. We found the FastDNA Spin Kit for Soil had a higher DNA extraction efficiency, produced
452 more overall positive samples, and resulted in lower Ct values for soils with active *A. cochlioides*
453 (data not shown). Therefore, the FastDNA Spin Kit for Soil was chosen for conducting the soil
454 DNA extractions in this study.

455 When DNA is extracted from soil samples, PCR inhibitors like humic acid can reduce or
456 inhibit DNA amplification during qPCR (Hebda & Foran, 2015). Field soil, which was confirmed
457 to be positive for *A. cochlioides* using the bioassay, tested negative for *A. cochlioides* DNA when
458 the standard protocol of the FastDNA Spin Kit for Soil was used. Before modifications were made

459 to the DNA extraction protocol, this field soil DNA was tested for qPCR inhibition using the spiked
460 positive technique, where 0.1 pg *A. cochlioides* DNA was added to each soil DNA sample. The
461 results showed that each soil DNA sample had slightly reduced amplification, compared to the
462 amplification of 0.1 pg DNA without the background soil DNA, but no reaction was completely
463 inhibited (Table S1). Some qPCR assays for field soil DNA samples have successfully utilized
464 internal controls to determine when a sample has potential inhibition, and provide more accurate
465 quantification results (Bilodeau et al., 2012; Burkhardt et al. 2018). In the future, the addition of
466 an internal control multiplexed with the *A. cochlioides* assay would reduce the number of assays
467 that need to be run.

468 Several existing qPCR assays for soilborne plant pathogens have successfully utilized the
469 FastDNA Spin Kit for Soil and FastPrep instrument for DNA extractions, but had varied durations
470 and speed for mechanical cell lysis (Almquist et al., 2016; Bilodeau et al., 2012; Wallenhammar
471 et al., 2012). In qPCR assays for *A. cochlioides* and *P. brassicae*, the cell lysis duration was 30 s
472 at a speed of 5.5 m/s (Almquist et al., 2016; Wallenhammar et al., 2012). In Bilodeau et al., (2012),
473 the cell lysis duration was 45 s at a speed of 6.5 m/s, which successfully extracted *V. dahliae* DNA
474 from microsclerotia in the soil. In our study, a cell lysis duration of 40 s at speed 6.0 m/s
475 consistently extracted *A. cochlioides* DNA from infected sugar beet tissue and bioassay soil where
476 *A. cochlioides* was active. However, this lysis duration and speed was unsuccessful at extracting
477 *A. cochlioides* DNA from naturally infested field soil, where *A. cochlioides* was likely in a dormant
478 oospore state. Increasing the cell lysis time and speed to 80 s and 7.5 m/s, respectively, with the
479 addition of an extra ceramic bead allowed for the detection of *A. cochlioides* DNA from naturally
480 infested soil samples with this qPCR assay. The optimization of the DNA extraction resulted in

more positive samples and lower Ct values for each sample type we tested, therefore improving the detection of *A. cochlioides*.

Under field conditions, symptoms of ARR can be confounding, especially if fields dry out after initial infections which results in only superficial scarring on the surface of roots without any active rotting. We have observed that roots with scarring have poor rates of *A. cochlioides* recovery in the culture-based assay. It is also common for diseased roots to have mixed infections with other root rot fungi such as *Rhizoctonia solani* or *Fusarium* spp., which can outgrow *A. cochlioides* in culture and compromise its recovery. This qPCR assay reliably detected *A. cochlioides* in 95% of adult roots with ARR symptoms, compared to culture-based recovery where only 23% of the same roots were positive. Each of the roots that were positive with the culture-based assay were also positive with the qPCR assay, and the qPCR assay detected *A. cochlioides* DNA in an additional 29 other sugar beet roots where the pathogen was not cultured. This suggests that *A. cochlioides* DNA can be detected in adult diseased sugar beets after the pathogen is no longer active. Having this information expands our ability to detect this pathogen, and provides valuable information to growers concerning the presence of ARR in their fields.

A. cochlioides causes post-emergence damping-off of seedlings, and it can be challenging to distinguish damping-off caused by other pathogens such as *R. solani* and *Pythium* spp. under field conditions. Infected seedlings tend to have higher rates of *A. cochlioides* recovery in the culture-based assay, but the presence of *R. solani* or *Pythium* spp. can compromise the recovery of *A. cochlioides*. This qPCR assay detected *A. cochlioides* in seedlings as early as 5 DAP and before the appearance of visible damping-off symptoms at 7 to 9 DAP. The ease of *A. cochlioides* detection in infected plant material highlights the value of using a culture-independent assay.

The qPCR assay developed in this study was validated using naturally infested field soil and oospore infested potting soil, naturally infected adult sugar beet roots, and sugar beet seedlings. Recently, a qPCR assay was developed to quantify *A. cochlioides* DNA from soil samples with primers that target a rRNA gene in the nuclear genome (Almquist et al., 2016). This qPCR assay was specific to *A. cochlioides*, and had an LOD in the range of 1-50 oospores/g soil (dry wt.) depending on soil characteristics, which was similar to qPCR assays for other oomycetes (Almquist et al., 2016). They found their assay was more sensitive for soils with higher clay content. For artificially oospore infested soil, they observed a consistent LOD of 10 oospores/g soil (dry wt.), which was also observed in our study. The Almquist et al., (2016) qPCR assay was only tested on soil samples, and consistently detected *A. cochlioides* in naturally infested soil samples with a DSI above 75, detected *A. cochlioides* in 50% of the samples when the DSI was 64-74, and did not detect *A. cochlioides* when DSI was below 50. In comparison, for naturally infested soil samples, our qPCR assay detected *A. cochlioides* when DSI values were as low as 48 (mean Ct = 32.89), although the majority of these samples (10 out of 12) had DSI values above 86. Furthermore, for oospore infested potting soil our qPCR assay detected *A. cochlioides* when mean DSI values ranged from 23.33-75. This assay had a high correlation (R^2= 0.96) with oospore inoculum density ranging from 5 to 100 oospores per gram of soil using an artificially infested potting mix. However, for the bioassay of the artificially infested potting mix there was significant variability between the technical replicates, so means were used to compare the oospore density and soil DSI value (Table 8). Quantification of *A. cochlioides* at these levels is very useful for sugar beet growers to devise a disease management plan. In the future, validating these results using field soil with a broader range of DSI values would support the reliability of this assay for accurate quantification. Overall, this qPCR assay may be a useful tool to provide accurate

526 information to sugar beet growers concerning the quantity of *A. cochlioides* inoculum in their
527 fields.

528 Quantitative PCR assays for soilborne pathogens aim to provide an accurate estimate of
529 disease potential in the soil. While this assay has been demonstrated to be effective for pathogen
530 detection with asymptomatic seedlings, adult plants exhibiting root rot symptoms, and from
531 infested soil samples, before it can be effectively used for quantification of the pathogen in field
532 soil samples additional work is needed. This would include 1) multiplexing with an internal control
533 to improve evaluation of PCR inhibition while reducing the number of assays that need to be run;
534 2) rerunning the standard curve with background DNA extracted from pathogen-free soil or plant
535 samples to evaluate the effectiveness of DNA extraction techniques and provide an LOD that
536 accurately reflects conditions under which the assay will be used; 3) evaluating field sampling
537 strategies to determine the number of samples needed to accurately evaluate risk of ARR for the
538 grower. The importance of this was highlighted by the variable qPCR results observed for two soil
539 subsamples from the same infested field soil (data not shown). While it could be due to presence
540 of oospore inoculum in disintegrating organic matter, additional experimentation is needed to
541 reduce this level of variation; and 4) determine how the ARR DSI of a field soil correlates with
542 the *A. cochlioides* Ct value for naturally infested soils. It will be vital to apply an optimal sampling
543 strategy to provide an accurate representation of risk for a field, and test a large collection of soil
544 samples with a wide range of DSI values. It would also be useful to evaluate the effect of soil type
545 and microflora on the predicted disease potential compared to the realized diseased in the field.
546

547 **Acknowledgements**

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661 Table 1. The isolates of fungi, oomycetes, protista and sugar beet used to test the specificity of

662 the qPCR assay

Isolate	Species	Kingdom / Phylum	[a]qPCR Amplification
20-23	*Beta vulgaris*	Plantae / Spermatophyta	-
C5	*Alternaria alternata*	Fungi / Ascomycota	-
H4	*Diaporthe eres*	Fungi / Ascomycota	-
CT3	*Cladosporium cladosporioides*	Fungi / Ascomycota	-
EP17	*Botryosphaeria dothidea*	Fungi / Ascomycota	-
NM54	*Penicillium flavigenum*	Fungi / Ascomycota	-
NM55	*Penicillium spinulosum*	Fungi / Ascomycota	-
NM56	*Penicillium dierckxii*	Fungi / Ascomycota	-
NM99	*Aspergillus europaeus*	Fungi / Ascomycota	-
NM156	*Aspergillus ustus*	Fungi / Ascomycota	-
LS-17	*Verticillium dahliae*	Fungi / Ascomycota	-
14-20	*Macrophomina phaseolina*	Fungi / Ascomycota	-
[€]NA	*Verticillium albo-atrum*	Fungi / Ascomycota	-
[€]NA	*Fusarium oxysporum f.sp medicaginis*	Fungi / Ascomycota	-
[€]NA	*Fusarium virguliforme*	Fungi / Ascomycota	-
F8	*Fusarium solani*	Fungi / Ascomycota	-
TC-1	*Polymyxa betae*	Protista / Protozoa	-
Br-2	*Polymyxa betae*	Protista / Protozoa	-
110-02	*Pythium ultimum*	Chromista / Oomycota	-
43-5	*Pythium aphanidermatum*	Chromista / Oomycota	-
96-4	*Pythium aphanidermatum*	Chromista / Oomycota	-
[€]NA	*Phytophthora sojae*	Chromista / Oomycota	-
WI98	*Aphanomyces euteiches*	Chromista / Oomycota	-
617-24	*Aphanomyces euteiches*	Chromista / Oomycota	-
[¥]Ae 1	*Aphanomyces euteiches*	Chromista / Oomycota	-
[¥]Ae 206	*Aphanomyces euteiches*	Chromista / Oomycota	-
[¥]Ae 315	*Aphanomyces euteiches*	Chromista / Oomycota	-
[¥]Ae 1309	*Aphanomyces euteiches*	Chromista / Oomycota	-
[¥]Ae 1352	*Aphanomyces euteiches*	Chromista / Oomycota	-
[¥]Ae Morden	*Aphanomyces euteiches*	Chromista / Oomycota	-
[¥]Ae BridgeRd	*Aphanomyces euteiches*	Chromista / Oomycota	-
[¥]Br 693	*Aphanomyces cladogamus*	Chromista / Oomycota	-
[¥]NA	*Aphanomyces cladogamus*	Chromista / Oomycota	-
16-35-2	*Aphanomyces cochlioides*	Chromista / Oomycota	+
16-16-3	*Aphanomyces cochlioides*	Chromista / Oomycota	+

35ss	*Aphanomyces cochlioides*	Chromista / Oomycota	+
24ss	*Aphanomyces cochlioides*	Chromista / Oomycota	+
11-169-4	*Aphanomyces cochlioides*	Chromista / Oomycota	+
18-56-4	*Aphanomyces cochlioides*	Chromista / Oomycota	+
WL405	*Aphanomyces cochlioides*	Chromista / Oomycota	+
C-23	*Aphanomyces cochlioides*	Chromista / Oomycota	+
15-81	*Aphanomyces cochlioides*	Chromista / Oomycota	+
15-48	*Aphanomyces cochlioides*	Chromista / Oomycota	+
15-50	*Aphanomyces cochlioides*	Chromista / Oomycota	+
103-1	*Aphanomyces cochlioides*	Chromista / Oomycota	+
13-69-4	*Aphanomyces cochlioides*	Chromista / Oomycota	+
13-66-1	*Aphanomyces cochlioides*	Chromista / Oomycota	+

[a] + = amplification; - = no amplification

[¥] DNA was kindly provided by Dr. Syama Chetterton, Agriculture and Agri-Food Canada

[€] Unknown

681 Table 2. Location and year of collection for 12 soils naturally infested with *Aphanomyces*

682 *cochlioides* in Minnesota and North Dakota.

Sample Name	County	State	Year
AC10 S3	Marshall	Minnesota	2017
AC10 S4	Marshall	Minnesota	2017
19-38	Redwood	Minnesota	2019
19-43	McLeod	Minnesota	2019
19-47	Renville	Minnesota	2019
19-59	Renville	Minnesota	2019
19-60	McLeod	Minnesota	2019
SM-AN	Renville	Minnesota	2019
Climax South	Polk	Minnesota	2020
Grandin	Traill	North Dakota	2020
Perley	Norman	Minnesota	2020
Climax North	Polk	Minnesota	2020

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693 Table 3. Location of sugar beet fields from which sugar beet roots naturally infected with

694 *Aphanomyces cochlioides* were received during the summer of 2020.

Sample Name[a]	State	County
20-51	Minnesota	Polk
20-52	Minnesota	Polk
20-53	Minnesota	Chippewa
20-54	Minnesota	Renville
20-55	Minnesota	Chippewa
20-56	Minnesota	Chippewa
20-57	Minnesota	Kandiyohi
20-58	Minnesota	Kandiyohi
20-59	Minnesota	Kandiyohi
20-60	North Dakota	Grand Forks
20-61	Minnesota	Washington

695 [a] Each sample consisted of 5-6 roots collected from a field

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705 Table 4. Standard curve of 10-fold serial dilutions of pure *Aphanomyces cochlioides* genomic

706 DNA.

Quantity of DNA (ng)	**Log_{10} (ng DNA)**	**Mean Ct value ± SE**	**No. of positive technical replicates**	**Technical replicates positive (%)**
1	0	11.62 ± 0.11	6	100
0.1	- 1	15.77 ± 0.15	6	100
0.01	- 2	19.66 ± 0.20	6	100
0.001	- 3	23.25 ± 0.22	6	100
0.0001	- 4	26.66 ± 0.27	6	100
0.00001	- 5	30.67 ± 0.22	6	100
0.000001	- 6	34.66 ± 0.85	6	100

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Table 5. The qPCR-based detection of *Aphanomyces cochlioides* DNA in susceptible sugar beet seedlings planted in soil naturally infested with *A. cochlioides* and used bioassay soil. Ct values were derived from the fit point analysis..

	Field soil naturally infested with *A. cochlioides*				**Used bioassay soil**			
DAP[a]	**Mean Ct value**	**No. of positive technical replicates**	**Technical replicates positive (%)**	**ARR symptoms present**	**Mean Ct value**	**No. of positive technical replicates**	**Technical replicates positive (%)**	**ARR symptoms present**
3	NA[b]	0	0	No	NA	0	0	No
5	29.62	3	75	No	24.21	4	100	No
7	25.03	4	100	No	20.97	4	100	Yes
9	23.87	4	100	Yes	21.87	4	100	Yes
11	17.27	4	100	Yes	17.56	4	100	Yes

[a] DAP = Days after planting

[b] NA = No amplification

730 Table 6. Ct values and Disease severity index (DSI) values for 12 field soil samples naturally
731 infested with *Aphanomyces cochlioides*. Ct values were obtained using fit point analysis.
732 Technical replicates with no qPCR amplification were not included in the calculation of mean Ct
733 values.

Sample Name	Mean Ct value	No. of positive technical replicates	% of positive technical replicates	Mean DSI[a] value
19-38	26.72	6	100	95
Perley	31.58	4	75	98
19-59	31.87	4	100	99
Climax North	32.41	2	100	100
AC10 S3	32.89	5	100	48
19-60	33.02	4	100	88
19-47	33.86	4	100	99
AC10 S4	33.98	5	100	53
19-43	34.10	4	100	95
SM-AN	34.04	4	100	90
Climax South	34.63	4	100	99
Grandin	34.64	4	100	86

734 [a] DSI = Disease severity index
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740 Table 7. Number of roots that *Aphanomyces cochlioides* was detected in using the qPCR and

741 culture-based assay.

Sample Name	DNA detection	Culture-based detection
20-51	4	0
20-52	4	0
20-53	7	3
20-54	2	0
20-55	2	0
20-56	1	0
20-57	4	0
20-58	3	1
20-59	2	0
20-60	7	5
20-61	2	0
Total	38	9

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751 Table 8. Mean Ct values and Aphanomyces root rot disease severity index (DSI) values for

752 *Aphanomyces cochlioides* oospore infested potting soil samples.

	qPCR assay			Bioassay		
Oospore(s)/g soil (dry wt.)	**Mean Ct value ± SE**	**No. of positive technical replicates**	**Technical replicates positive (%)**	**Mean DSI value ± SE**	**No. of positive technical replicates**	**Technical replicates positive (%)**
1	Negative	0	0	10.83 ± 4.86	5	62.5
5	34.58 ± 0.46	14	87.5	23.33 ± 12.54	5	62.5
10	33.26 ± 0.73	16	100	35.83 ± 10.76	8	100
20	32.18 ± 0.71	16	100	39.16 ± 13.36	8	100
50	31.96 ± 0.91	15	100	64.17 ± 9.99	8	100
100	30.53 ± 0.64	15	100	75.00 ± 12.01	8	100

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762 **Figure Legends**

763 Figure 1. Disease severity rating scale used for the Aphanomyces root rot bioassay.

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765 Figure 2. Locations of two subsamples taken from an adult sugar beet root for culture-based

766 assay (blue square) and qPCR assay (red square).

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768 Figure 3. Standard curve of 10-fold dilutions of pure *Aphanomyces cochlioides* isolate 13-69-4

769 genomic DNA standards. Standard error bars are displayed with vertical black lines.

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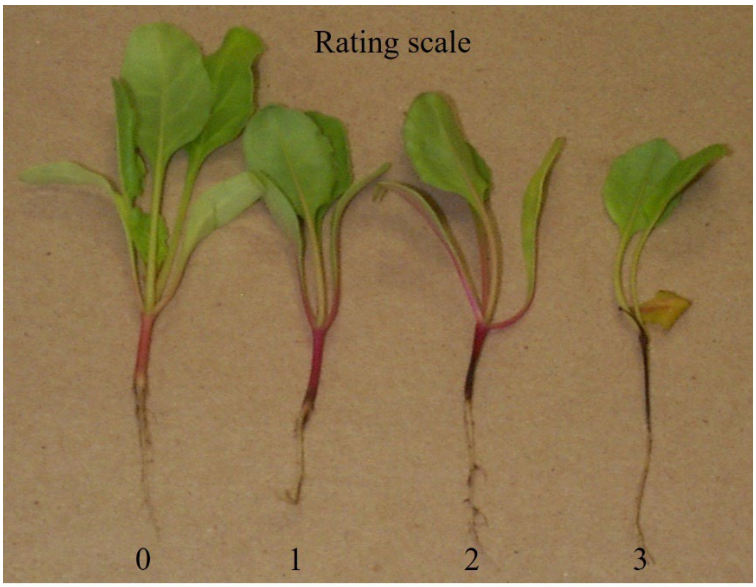

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785 Figure 1. Disease severity rating scale used for the Aphanomyces root rot bioassay.

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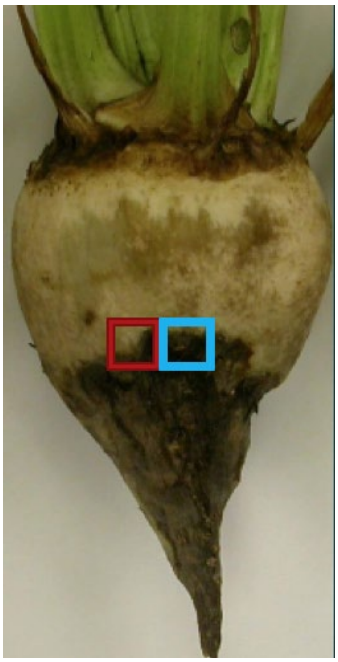
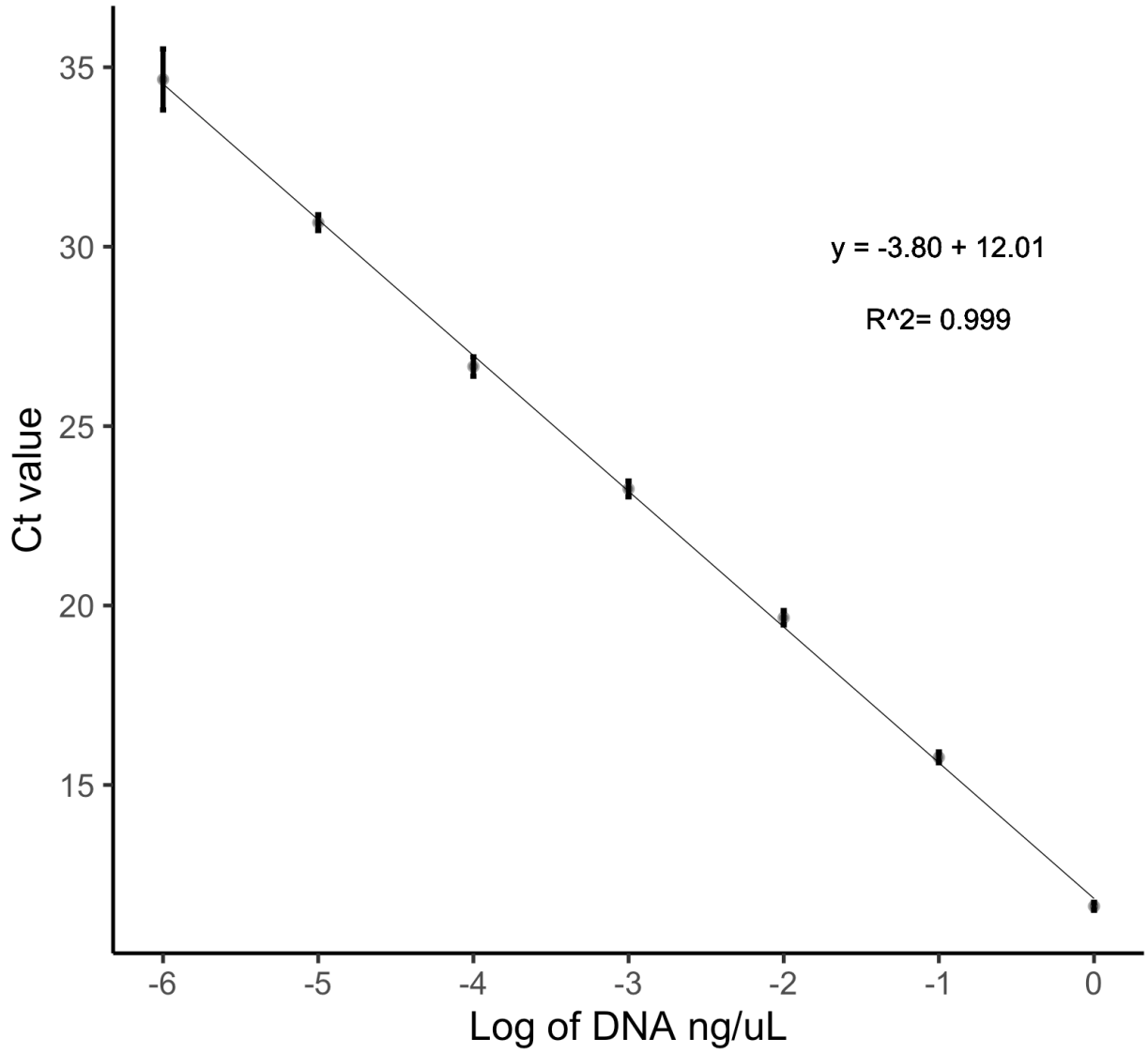


Figure 2. Locations of two subsamples taken from an adult sugar beet root for culture-based assay (blue square) and qPCR assay (red square).



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801 Figure 3. Standard curve of 10-fold dilutions of pure *Aphanomyces cochlioides* isolate 13-69-4

802 genomic DNA standards. Standard error bars are displayed with vertical black lines.

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