ORIGINAL ARTICLE

Rapid detection and quantification of *Aphanomyces cochlioides* in sugar beet

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Abstract

Sugar beet (*Beta vulgaris* L.) is an economically important crop in temperate climates providing nearly 30% of sugar production worldwide. The oomycete *Aphanomyces cochlioides* is the causative agent of seedling damping-off and root rot disease in sugar beet. The pathogen is responsible for plant degeneration and drastic yield losses in all major sugar beet producing areas. The identification of resistant germplasm is essential to reduce the use of chemical treatments as well as the costs of protective measures and to effectively limit the damage caused by the pathogen. In this study we aimed to establish a qPCR-based method to quantify the pathogen DNA in infected plants and to predict the resistance levels of different sugar beet genotypes in response to *A. cochlioides*. The difference in the response to *A. cochlioides* isolates with different geographical origins was investigated. In addition, confocal microscopy was performed in order to observe the spatial and temporal colonization pattern in infected seedlings of susceptible and partially resistant breeding lines. The research presented in this article provides a tool to understand the progress of the infection in infected tissues and to identify the genetic background of resistance to *A. cochlioides* that can be used to support breeding programs.

Keywords Damping off · Root rot · *Beta vulgaris* · Oomycete · *Aphanomyces* · qPCR

Introduction

Sugar beet (*Beta vulgaris* L.) is one of the two main sugar crops cultivated in the world. It has been bred for over a century for high sucrose yield and purity and today it provides 30% of sugar production worldwide (Zicari et al. 2019). It is mainly cultivated in temperate climates in Europe such as France and Germany and in the Northern USA (Biancardi et al. 2010). Sugar beet fields are commonly infested by soil-borne fungal and oomycete pathogens such as *Pythium* and *Fusarium* species and *Rhizoctonia solani*, which cause poor establishment, stand loss and reduced sugar yield (Amein 2006). In addition to these, *Aphanomyces cochlioides* is one of the most economically important pathogens of sugar beet. This phytopathogenic oomycete belongs to the Saprolegniales order and is specialized in parasitizing roots of sugar beet, spinach (*Spinacia oleracea* L.), cockscomb (*Celosia argentea* L) and other species of the Amaranthaceae (Scott 1961). Its presence has been reported in all sugar beet growing areas but it is a particularly problematic threat in the United States, Europe, and Japan (Beale et al. 2002). Around 50% of the sugar beet growing acreage in the Red River Valley of North Dakota, in the USA is susceptible to *A. cochlioides* outbreaks, while around 25% of the fields in Sweden are infested by *A. cochlioides* (Olsson et al. 2011). The infection occurs through direct penetration of vegetative hyphae which originate from sexually-produced oospores or via bi-flagellated motile zoospores, produced in the zoosporangia during the asexual stage (Dyer and Windels 2003). *A. cochlioides* is responsible for two diseases in sugar beet: damping-off, which affects sugar beet seedlings, and chronic root rot on mature tap roots. The development of the two diseases requires high temperatures and wet soil

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conditions (Windels 2000). The symptoms of the seed-ling damping-off can be observed after one to three weeks post-emergence and include dark lesions on the hypocotyl (Taguchi et al. 2009). Infected seedlings become threadlike and ultimately fall over and die (Windels 2000; Taguchi et al. 2009). The chronic disease occurs in late summer-early autumn when warm and rainy weather conditions prevail (Olsson et al. 2011). Attacked roots display yellowish-brown lesions which become dark-brown or black in time affecting the entire taproot, only the tip root or causing scabby lesions on the root surface (Jacobsen 2006). Severe infection results in plant death. In both stages of the sugar beet life cycle, if environmental conditions become unfavorable for disease development, plants can survive and recover, developing a relatively normal crop. Nevertheless, these plants are still characterized by root distortion, scarring and reduced sugar yield (Windels 2000).

Despite the economic impact of this oomycete on sugar beet production, in recent years attention has primarily been paid to other sugar beet pathogens, rendering Aphanomyces root rot one of the less characterized sugar beet diseases. The first and only available quantitative trait locus (QTL) mapping to *A. cochlioides* resistance dates back to 2009 when Taguchi et al. identified the presence of a QTL, desig-nated as *qAcr1*, on chromosome III, responsible for a major portion of resistance (Taguchi et al. 2009). This region has been shown to segregate in a dominant and monogenic manner and to regulate the resistant trait not only in the field but also in the greenhouse, excluding the effect of the environment on resistance (Taguchi et al. 2010). However, the genetic characterization of this region and the presence of other minor quantitative trait loci (QTLs) with additive effects remain unexplored. Furthermore, resistant sources to Aphanomyces disease are limited and molecular mark-ers linked to this trait are currently not available making it infeasible to conduct marker assisted selection (MAS). The selection of resistant genotypes that can be used to introduce resistance in commercial cultivars still relies on time-consuming phenotype-based screening. A more rapid and sensitive method for the identification of genotypes with high levels of resistance would facilitate the selec-tion of sugar beet lines to be used in breeding programs. Therefore, in this study we assessed a qPCR assay for detec-tion and quantification of *A. cochlioides* in infected plants over time to investigate the association between the patho-gen biomass and the levels of resistance of the host and to elucidate the progress of the infection in partially resistant and susceptible genotypes. Primers and a probe specific to *A. cochlioides* were developed by Almquist et al. (2016). These primers and probe were shown to be specific to *A. cochlioides* and did not amplified DNA from other fungal pathogens and oomycetes, including closely related species such as *A. euteiches* and *A. cladogamus*. Their specificity was also confirmed using BLASTN (www.ncbi.nlm.nih.gov/blast/Blast.cgi) (Almquist et al. 2016). The legume *in planta* infection system previously described by Hosseini et al. (2012) was adapted to sugar beet to enable host-pathogen interaction in sugar beet seedlings. Moreover, differences in response towards different *A. cochlioides* strains were inves-tigated by challenging the plants to isolates originated from different geographical regions. The time course and extent of *A. cochlioides* in infected seedlings was also observed and documented using confocal microscopy.

Materials and methods

Plant material

Seeds of seven sugar beet breeding lines (G2, G8, G12, G17, G18, G19, G20) were provided by DLF Beet Seed, Landskrona, Sweden. Seeds were surface disinfected by submersion in deionized water (20 °C) for 30 min followed by submersion for 5 min in a 56 °C water bath and washed in cold water before drying at room temperature. Seeds were germinated in steam-sterilized soil in plastic trays (21 × 35 × 6 cm) in a climate chamber under controlled con-ditions (16 h light, 22 °C day/night, 95% RH).

Aphanomyces cochlioides strains

Three *A. cochlioides* field strains previously collected from Sweden (Tågarp, Skåne), USA (Marshall, Minnesota) and Japan (Hokkaido) were provided by DLF Beet Seed. The identity of the *A. cochlioides* strains was verified by obser-vation of morphological features under the light microscope and by amplification of the Internal Transcribed Spacer (ITS)/5.8 S rRNA gene region (GenBank accession num-ber: AY353911), using primers and a probe developed by Almquist et al. (2016) (Table 1). These primers and probe were used to amplify pure *A. cochlioides* DNA of the three different strains, extracted from the mycelium that was collected from Corn Meal Agar (CMA) plates. Pure DNA extracted from *R. solani* mycelium, collected directly from Potato Dextrose Agar (PDA) plates, was used as a nega-tive control. To obtain a single strain from each isolate, the three *A. cochlioides* strains were single-spore isolated by pipetting 10 µl of a serial-diluted and filtered zoospore sus-pension, obtained as described in the following paragraph, onto microscope slides. The slides were observed under a light microscope at 20X magnification and drops with one zoospore were transferred onto CMA medium (17 g corn-meal agar (Sigma-Aldrich) per liter of deionized water with the addition of chloramphenicol, after autoclaving, at a final

concentration of 0.005%). CMA plates were incubated at 21 °C in the dark for two weeks. The obtained *A. cochlioides* cultures were sub-cultured every two weeks for continuous growth by cutting pieces of agar (approximately one cm^2) from the two-week-old culture and by placing them in the middle of fresh CMA plates. *Aphanomyces cochlioides* isolates were maintained at 21 °C in the dark.

Production of *Aphanomyces cochlioides* zoospores

A. cochlioides mycelial plugs were cut from 14-day old cultures grown in CMA medium and incubated in Erlenmeyer flasks containing 1500 ml of a 3% sterile peptone solution at 25 °C in the dark. After 5 days the content of the Erlenmeyer flasks was rinsed with deionized water and incubated in 3000 ml of NaCl ddH$_2$O (2 mM NaCl). The solution was aerated overnight by bubbling air through sterile glass tubes with an aquarium pump. The mycelium and agar were removed and the concentration of the zoospores contained in the solution was measured using a hemocytometer.

Evaluation of the disease symptoms

Plants were evaluated for damping-off symptoms using 12-day old seedlings that were inoculated by watering the soil with 150 ml of zoospore suspension (3-5×10^4 zoospores/ml) obtained from the three *A. cochlioides* strains. Seedling mortality was rated at 8 and 15 days after inoculation, while symptoms were scored at 20 days after inoculation. The scoring was made in 5 classes: class 1 = dead plant, class 3 = severe infection with the whole hypocotyl showing necrotic lesions, class 5 = medium infection, with half of the hypocotyl affected by necrotic lesions, class 7 = minor infection, where only the root tip presents necrosis and class 9 = healthy plant, with no visible symptoms. Dead plants scored with 1 were included in the final scoring at 20 dpi to calculate the average disease resistance index. For each genotype 15 biological replicates inoculated with *A. cochlioides* and 15 biological replicates inoculated only with water were collected from one independent experiment.

In planta infection system

Eight-day old seedlings were uprooted from soil. Roots were washed with water and plants were placed in pipette boxes filled with tap water to submerge the roots and part of the hypocotyls for 2 days in a climate chamber (16 h light, 21 °C, 95% RH). The racks carrying the plants were then moved in clean pipette boxes filled with the *A. cochlioides* zoospores suspensions (3–5×10^4 zoospores/ml) for 2 h. Plants were inoculated in three batches separately, with zoospores obtained from the three *A. cochlioides* strains, in the

same day. Roots were washed by immersion in water and incubated in new pipette boxes filled with water for 0 h, 4 h, 24 h, 2 days, 4 days and 8 days. Seedlings were collected in a 2 ml 96-well plate and lyophilized in a freeze-drier and stored at -20 °C until DNA extraction was performed or directly fixed in Formaldehyde Alcohol Acetic Acid (FAA) fixative to prepare samples for confocal microscopy. Plants inoculated with water were used as controls. Three biological replicates consisting of single plants per genotype were collected at each time point.

DNA extraction from microbial and plant material

Mycelium collected from agar plates in Eppendorf tubes was ground for 1 min at 30 Hz using a TissueLyser (Qiagen, Valencia, CA, U.S.; Cat.No 85,220) after addition of 500 µl of a lysis buffer (Tris-HCl 400 mM pH 8.0, EDTA 10 mM pH 8.0; Merck KGaA, Darmstadt, Germany). Ground tissue was incubated for 10 min at room temperature, after which 150 µl of potassium acetate (pH 4.8) were added. Tubes were vortexed and centrifuged at > 10 000 x *g* for 1 min. The supernatant was added to a new tube and centrifuged at > 10.000 x *g* for 1 min. The supernatant was then transferred to a new tube and an equal amount of isopropanol (Merck KGaA, Darmstadt, Germany) was added. Tubes were mixed by inversion and centrifuged at > 10 000 x *g* for 2 min. The supernatant was discarded and 300 µl of 70% ethanol were added. After 1 min of centrifugation at > 10 000 x *g*, the supernatant was discarded and the DNA pellet was air-dried at room temperature before resuspension in 40 µl 1X TE-buffer (10 mM Tris (pH 7.5) and 0.1 mM EDTA (Merck KGaA, Darmstadt, Germany)).

Lyophilized plant material was ground for 1 min at 30 Hz using a TissueLyser (Qiagen, Valencia, CA, U.S.; Cat. No 85,220). CTAB (1% w/v final concentration; Kebo lab, Spånga, Sweden) and β-mercaptoethanol (1% v/v final concentration; Merck KGaA, Darmstadt, Germany) were added to the extraction buffer (Tris-HCl 100 mM pH 8.0, NaCl 1 M, EDTA, 10 mM pH 8.0; Merck KGaA, Darmstadt, Germany) before use and the buffer was heated at 65 °C. 500 µl of extraction buffer was added to the ground plant tissue and incubated at 65 °C for 60–90 min with occasional mixing by inversion. The plate was cooled on ice for 5 min followed by a short centrifugation. 280 µl of cold (-20 °C) chloroform/isoamylalcohol (24:1) (Sigma-Aldrich, Saint Louis, MO, U.S.) were added followed by incubation on ice for 30–60 min with occasional mixing. The plate was centrifuged for 10 min at 1600 x *g* at 6 °C. The supernatant was transferred to a plate containing 450 µl isopropanol (Merck KGaA, Darmstadt, Germany) (stored at -20 °C) and mixed by inversion of the plate. The plate was centrifuged for 20 min at 1600 x *g*, 6 °C. The isopropanol was discarded

before the addition of 200 µl of 70% ethanol followed by 10 min of centrifugation at 1600 x *g*. The ethanol was discarded and the pellets were air-dried before resuspension in 100 µl 1X TE-buffer (10 mM Tris (pH 7.5) and 0.1 mM EDTA (Merck KGaA, Darmstadt, Germany)) at 65 °C for 10 min with occasional vortexing.

Primer and probe design

For amplification and quantification of *A. cochlioides* target sequence, primers and a probe targeting the 5.8 S ribosomal RNA gene of *A. cochlioides* (Almquist et al. 2016) were used. Primers and a probe for sugar beet DNA amplification and quantification were designed based on a gene sequence encoding the 11 S globulin storage protein 2 available in GenBank (GenBank accession number: XM_010680997) and evaluated using Primer3Plus (Untergasser et al. 2012). The 5′ terminal reporter dye used for the *A. cochlioides* specific probe was FAM (excitation at 492 nm and emission at 516 nm). The sugar beet specific probe was labeled with HEX (excitation 535 nm and emission 555 nm) at the 5′terminal and the quencher TAMRA (excitation 556 nm and emission 580 nm) at the 3′end. Primers and probes are listed in Table 1.

Validation of primer specificity and efficiency

Primer annealing temperatures were examined by performing three separate PCRs with different annealing temperatures. The PCR program was performed on GeneAmp PCR System 9700 in a total reaction volume of 10 µl consisting of 4 µl of DNA and 6 µl of PCR reaction mix (5 µl Takyon Master mix, 072 µl ddH2O, 0.12 µl Primer F (50 µM), 0.12 µl Primer R (50 µM) per reaction) and consisted of an initial step of denaturation at 94 °C for 5 min followed by 40 cycles of denaturation for 30 s at 94 °C, annealing for 1 min at either 58 °C, 60 °C or 62 °C and extension for 30 s at 72 °C. The primers targeting sugar beet were tested on pure *A. cochlioides* DNA, previously extracted from the Swedish isolate Arhill_2012 and provided by DLF Beet Seed and DNA from non-inoculated sugar beet. The primers targeting *A. cochlioides* were tested on pure *A. cochlioides* DNA and on DNA from sugar beets infected with the same isolate. Both primer pairs were also tested on a no-template control as a negative control. The resulting PCR products were examined by gel electrophoresis. Primer efficiency was tested by performing an absolute quantification by qPCR. The *A. cochlioides* primers were tested on pure *A. cochlioides* DNA (20 ng/µl), and the sugar beet primers were tested on pure sugar beet DNA (10 ng/µl). 10-fold, 5-fold and 3-fold dilution series, consisting of 6 dilutions each, were performed with each DNA sample. The primer efficiency was calculated based on the slope resulting from the average Ct value of two technical replicates and the logarithmic value of the sample quantity (dilution factor).

PCR and quantitative PCR

DNA extracted from the mycelium was amplified with an Applied Biosystem 7500 Real Time PCR System by running a presence/absence analysis to confirm the identity of the *A. cochlioides* isolates. Host and pathogen biomass was quantified by running a "Quantitation - comparative Ct (ΔΔCt)" analysis on the Applied Biosystem 7500 Real Time PCR System. The reaction mix consisted of 5 µl Takyon™ ROX Probe 1X MasterMix dTTP blue (Eurogentec), 0.8 µl ddH2O, 0.04 µl primers (0.2 µM), 0.02 µl probes (0.1 µM) and 4 µl DNA (1–2 ng/µl) for a total volume of 10 µl. The following thermal profile was used: 50 °C for 2 min, 95 °C for 3 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The amount of *A. cochlioides* DNA was normalized to the sugar beet reference gene 11 S globulin seed storage protein 2 and the pathogen biomass was expressed as the pathogen/host DNA ratio in comparison to the non-inoculated negative controls to account for false background signals using the Pfaffl method (Pfaffl 2001). The ratio was calculated from the mean of three biological replicates.

Root fixation and dehydration

Seedlings inoculated in pipette boxes were collected at 0, 2, 4 and 8 days post inoculation and the roots (including the hypocotyls) were fixed in 10 ml of FAA (10% formalin (37%), 50% ethanol (95%), 35% ddH2O and 5% glacial acetic acid) in 15 ml centrifuge tubes. After 24 h of incubation at room temperature, roots were washed for 2 min by inversion in new tubes with 50% ethanol. Roots were then dehydrated in an ethanol series of increasing concentration (50%, 70%, 80%, 95%) with 1-hour incubation at room

Table 1 qPCR primers and probes used for amplification of *A. cochlioides* and sugar beet genes. *A. cochlioides* primers and probe were from Almquist et al. (2016). Sugar beet primers and probe were designed on Primer3Plus

Primer	Sequence
Ac-F (*A. cochlioides* forward primer)	5′-TCC GGG CTA GCC GAA GGT T-3′
Ac-R (*A. cochlioides* reverse primer)	5′-ACA AGC AAT CAT TTC TGA TGC TAG ATA G-3′
Ac-P (*A. cochlioides* probe)	5′-CGA AAG GAA CCG ATG TAT-3′
Sb-F (Sugar beet forward primer)	5′-ATG CAG GTG AAG GGA TAT TGG G-3′
Sb-R (Sugar beet reverse primer)	5′-TTG TAG CAC CAG TGA ACA GC-3′
Sb-P (Sugar beet probe)	5′-AGG CGC GGC GAT ATC TTG GC-3′

temperature for each concentration. Samples were stored at +4 °C in 95% ethanol.

Embedding, sectioning and staining

Dehydrated plant material was cut in order to obtain three parts of ~2 cm each: an upper part representing the closest area of the hypocotyl to the cotyledons, a middle part in the center of the hypocotyl and a lower part corresponding to the closest section to the distal elongation zone. Each fragment was embedded in 5% agar. Embedded samples were sliced using a vibrating-blade microtome (Leica VT1000 S) to a thickness of 100 µm. To specifically stain *A. cochlioides* hyphae the stain WGA (wheat germ agglutinin) coupled to Alexa Fluor 488 conjugate was used. Specimens were stained in a 10 µg/ml staining solution for 5 min, placed in a water drop on a microscope slide and directly observed at the confocal microscope.

Confocal microscopy

Imaging was performed by using a confocal laser scanning microscope (Leica TCS SP8) and the settings were operated in the LAS X software. Specimens were observed using a 10X dry objective (HC PL FLUOTAR 10x/0.30). A 405 nm diode laser was used to detect emitted auto-fluorescence from the sugar beet tissue collected in a wavelength range of 415–465 nm. An OPSL 488 nm laser was used to detect the fluorophore Alexa Fluor 488 in a wavelength range of 500–565 nm. All images were processed in ImageJ version 1.53.

Statistical analysis

A two-way ANOVA test with interactions, followed by a Tukey HSD (Honestly Significant Difference) test was performed with the R-package tidyverse, using the function aov in R (version 4.0.5) in order to test for statistical significance. Analysis of variance between the disease resistance indexes from the phenotypic test was based on genotype-*A. cochlioides* isolates interaction. The variance between pathogen/host DNA ratios collected by qPCR was calculated based on

genotype-timepoint interaction and genotype-*A. cochlioides* isolates interaction.

Results

Identification of *Aphanomyces cochlioides* isolates

The ITS/5.8 S rRNA gene region was amplified in the DNA extracted from all the three different isolates, confirming their identity as *A. cochlioides*, while the presence of the target region was not detected in *R. solani* DNA.

Primer specificity and efficiency

In order to develop a method for the quantification of *A. cochlioides* DNA in infected sugar beets, specific primers and probes for both *A. cochlioides* and sugar beet were used. Optimal annealing temperatures for all primer pairs was tested on the pathogen and the plant DNA respectively by performing a temperature gradient test. By analysis of PCR products with gel electrophoresis, an annealing temperature of 60 °C was concluded to be optimal for further use. Primer efficiencies were examined to properly calculate the ratio of *A. cochlioides* and sugar beet DNA in sugar beet roots. The average efficiency between the different fold dilution series was 107.4 for the primers targeting the 11s globulin seed storage protein 2 gene of sugar beet and 109.3 for the primers targeting the 5.8 S ribosomal RNA gene of *A. cochlioides* (Table 2). In all experiments the *A. cochlioides* primers and probe gave a signal in the negative control consisting of non-inoculated plants, indicating either the presence of minor contamination or presumably a slightly low specificity of the primers. However, the Ct values in the negative controls were always above 29 (between 29 and 36) and all values were calibrated against the negative controls to take this error into account.

Disease resistance indexes from the phenotypic evaluation

All the non-inoculated plants were healthy when the final scoring was performed and, therefore, were not scored. The first week after inoculation, dead plants belonging to genotypes G17, G18, G19 and G20 were observed, while no dead plants were counted in genotypes G8 and G12 in response to all *A. cochlioides* isolates and in genotype G2 in response to Arhill_2012 and Hokkaido_01. The first dead plants from genotypes G2 and G8 were observed two weeks after inoculation. However, no dead plants were recorded in genotype G8 inoculated with USA_01.7.6 and from genotype G12 (Table 3). Most of the breeding lines evaluated in this study

Table 2 Primer efficiency for the different primer pairs in different dilution series. The average efficiency was 107.4 for the primers targeting the 11s globulin seed storage protein 2 of sugar beet, and 109.3 for the primers targeting the 5.8 S ribosomal RNA gene of *A. cochlioides*

Dilution series	11s globulin seed storage protein 2	5.8 S ribosomal RNA
10-fold	111.6	108.1
5-fold	100.5	110.6
3-fold	109.7	109.1
Average	107.3	109.3

Table 3 Percentage of dead plants observed during the first and second counting at 8 and 15 days post inoculation (dpi) respectively in all sugar beet genotypes in response to the three *A. cochlioides* isolates (Arhill_2012, Hokkaido_01, USA_01.7.6)

Genotype	Arhill_2012		Hokkaido_01		USA_01.7.6	
	8 dpi	15 dpi	8 dpi	15 dpi	8 dpi	15 dpi
G2	0%	53%	0%	47%	27%	40%
G8	0%	67%	0%	73%	0%	0%
G12	0%	0%	0%	0%	0%	0%
G17	33%	53%	53%	20%	40%	60%
G18	27%	47%	73%	20%	33%	40%
G19	40%	33%	33%	40%	20%	73%
G20	87%	13%	47%	7%	47%	27%

Table 4 Disease resistance indexes. Average scores of different sugar beet genotypes (G2, G8, G12, G17, G18, G19, G20) in relation to the different *A. cochlioides* isolates (Arhill_2012, Hokkaido_01, USA_01.7.6). The scoring is done in 5 classes: 1 = dead plants, 3 = 100% of the hypocotyl is necrotic, 5 = 50% of the hypocotyl is necrotic, 7 = 25% of the hypocotyl is necrotic and 9 = no visible symptoms. 15 biological replicates for each genotype were collected to calculate the average scores at three weeks after inoculation

	G2	G8	G12	G17	G18	G19	G20
Arhill_2012	2.67 bce	1.53 cde	6.80 a	1.27 e	1.40 e	1.73 de	1.00 e
Hokkaido_01	3.20 bcd	1.67 cde	6.27 a	1.40 e	1.13 e	1.40 de	2.53 de
USA_01.7.6	2.13 cde	4.20 b	6.73 a	1.00 e	2.07 de	1.27 e	2.33 de

showed a low resistance level against the seedling damping-off, with genotypes G17, G18, G19 and G20 presenting the most severe symptoms. Genotype G12 was the exception, showing significantly higher disease resistant indexes (above 6), compared to the other genotypes ($p < 0.001$). All the genotypes, with the exception of G8, performed similarly under the pressure of the different isolates. Interestingly, genotype G8 showed a significantly higher disease resistance index of 4.20 when inoculated with USA_01.7.6 compared to Arhill_2012 from Sweden, or Hokkaido_01 from Japan ($p < 0.001$) (Table 4). Therefore, genotype G12 was classified as partially resistant, while genotypes G17, G18, G19 and G20 were the most susceptible to Aphanomyces damping-off. The three *A. cochlioides* isolates appeared to have the same degree of virulence against the different sugar beet lines, with the exception of USA_01.7.6, which caused milder symptoms in genotype G8 compared to Arhill_2012 and Hokkaido_01.

Quantification of *A. cochlioides* DNA in sugar beet infected seedlings

To quantify the pathogen biomass in infected seedlings a relative qPCR was performed. The colonization of plant tissue over time was expressed as the accumulation of pathogen biomass compared to the negative controls. In all sugar beet lines tested, low levels of *A. cochlioides* DNA were detected at 0 hpi, after immersion for 2 h in the zoospore suspension. The ratio between *A. cochlioides* DNA and sugar beet DNA in seedlings infected with Arhill_2012, Hokkaido_01 and USA_01.7.6 is shown in Fig. 1. All sugar beet lines were evaluated at 0, 4, 24 and 48 hpi (Fig. S1). However, the

individual genotypes could not be significantly differentiated during this time span, therefore the three most interesting genotypes were selected and analyzed for a longer time span (0, 2, 4 and 8 dpi). Genotype G12 was selected because of the high disease resistance index shown in the phenotypic test, genotype G17 was selected as being one of the lines most susceptible to the disease and genotype G8 was included since it significantly differed in disease resistance index between the different isolates.

Quantification of the infection with the Swedish isolate, Arhill_2012

When inoculated with Arhill_2012 the *A. cochlioides*/sugar beet DNA ratio in genotypes G8 and G17 increased over time, while the amount of the pathogen DNA remained stable at low levels over time in genotype G12. In the susceptible genotype G17 the pathogen/host DNA ratio was significantly higher at 8 dpi compared to 0 and 2 dpi ($p < 0.001$ and $p < 0.05$) and compared to the ratio in genotype G12 at the same time ($p < 0.001$) (Fig. 1a).

Quantification of the infection with the Japanese isolate, Hokkaido_01

Plants inoculated with Hokkaido_01 showed a pattern of *A. cochlioides*/sugar beet ratio similar to that of plants inoculated with Arhill_2012, however the ratio in genotype G17 reached a significantly higher peak at 4 dpi compared to the other time points (0 hpi ($p < 0.001$), 2 dpi ($p < 0.001$) and 8 dpi ($p < 0.05$)) and to the other two genotypes at the same time point (G12 ($p < 0.001$), G8 ($p < 0.01$)) (Fig. 1b).

Quantification of the infection with the USA isolate, USA_01.7.6

When inoculated with USA_01.7.6, both genotypes G8 and G12 showed a stable *A. cochlioides*/sugar beet DNA ratio over time, while an increase of the pathogen/host ratio was observed in genotype G17 at 8 dpi, significantly higher than the ratio at the other timepoint (0 ($p < 0.01$), 2 ($p < 0.01$) and 4 dpi ($p < 0.05$)) and significantly larger than the ratio in genotype G12 at 8 dpi ($p < 0.001$) (Fig. 1c).

An increasing amount of *A. cochlioides* DNA was detected during the first 8 dpi in genotype G17 and a similar

Fig. 1 Ratio between *A. cochlioides* DNA and sugar beet DNA in sugar beet seedlings. Genotypes G8, G12, G17 were inoculated 12 days after sowing with **(a)** the Swedish isolate Arhill_2012, **(b)** the Japanese isolate Hokkaido_01 and **(c)** the USA isolate USA_01.7.6. Seedlings were collected directly after inoculation (0 h) and at 2, 4 and 8 days post inoculation. Each bar is the mean ratio of three biological replicates and the error bars show the standard deviation

trend was observed in genotype G8, when inoculated with Arhill_2012 and Hokkaido_01. Limited and stable *A. cochlioides*/sugar beet ratios were detected in genotype G12 during the examined period.

The analysis of variance on pathogen/host ratios also showed that both the genotype and the isolates affect the amount of *A. cochlioides* DNA in infected plants ($p < 0.001$), but the interaction between these two variables was not significant ($p = 0.063$) (Fig. S2).

To confirm that significant differences in the pathogen/host DNA ratio between the partially resistant and susceptible lines were detectable at 8 dpi, a new inoculation was performed on genotypes G12 and G17, using Arhill_2012. In this experiment, 6 biological replicates were used for each genotype and samples were collected at the latest time point (8 dpi). In agreement with the results from the first experiment, *A. cochlioides* DNA content was significantly higher in genotype G17, with an *A. cochlioides*/sugar beet DNA ratio of 585 compared to a ratio of 135 observed in G12 ($p < 0.05$).

Confocal microscopic observations of the *in planta* infection process

In order to understand differences in the infection patterns between different genotypes, three sugar beet seedling hypocotyl and root zones (Fig. 2) of the partially resistant line G12 and the susceptible line G17, inoculated with Arhill_2012, were analyzed using confocal laser scanning microscopy after inoculation with 5×10^4 zoospores/ml and WGA-staining of the pathogen.

As expected at 0 hpi the presence of mycelium corresponding to *A. cochlioides* was not observed in either of the analyzed genotypes. The first detection of *A. cochlioides* hyphae was documented at 2 dpi in both genotypes in the vicinity of the elongation zone (Fig. 3). At 4 dpi *A. cochlioides* structures were observed in genotype G17 also in the middle zone of the hypocotyl. However, the presence of the pathogen was not detected in the middle zone of genotype G12 at the same time point. At 8 dpi *A. cochlioides* was observed in all areas of the hypocotyl in the susceptible genotype G17 and the intercellular spaces of the cortex were fully colonized by the hyphae while the pathogen was confined in the elongation zone in the proximity of the tip root in genotype G12 (Fig. 4). In genotype G17 the formation of clusters of hyphae surrounding the endodermis was observed but hyphae were not detected within the vascular system.

Aphanomyces cochlioides hyphae were able to invade the whole hypocotyl in the susceptible genotype within 8 days, by proliferating in the intercellular spaces of the cortex,
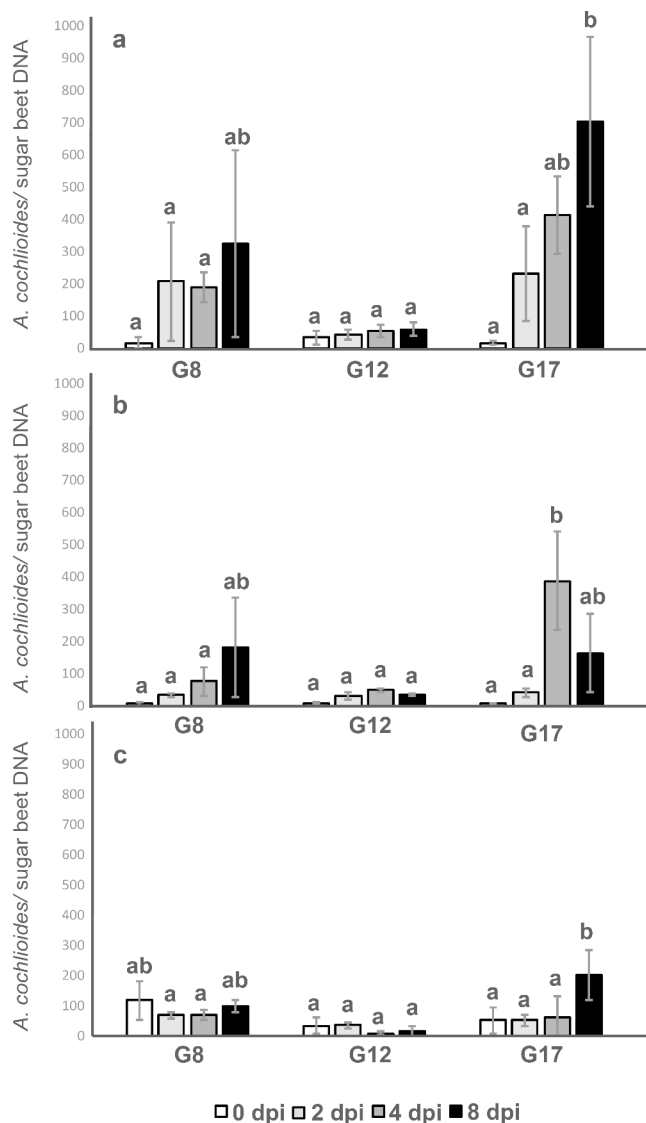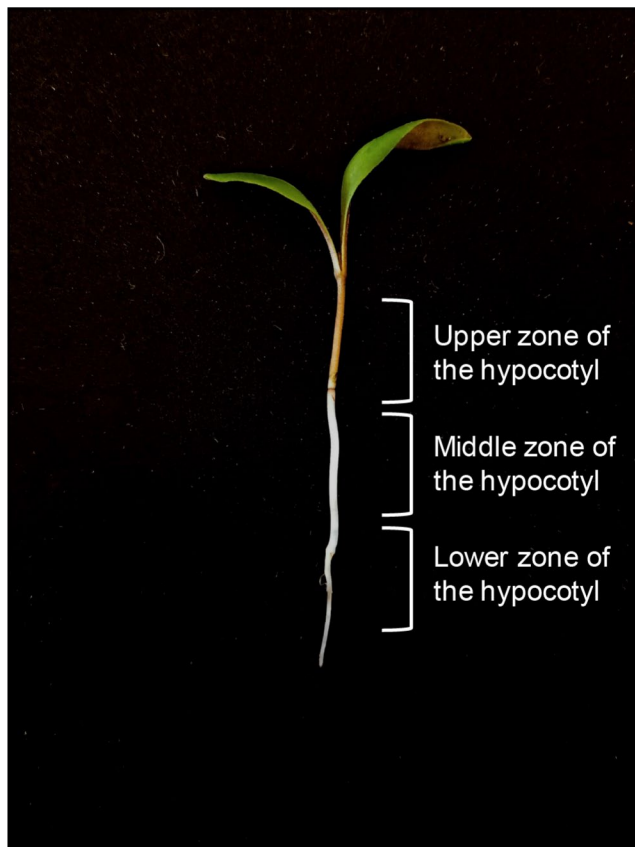
Fig. 2 Sugar beet seedlings were divided in three zones. An upper zone corresponding to the closest area of the hypocotyl to the cotyledons, a middle zone corresponding to the central portion of the hypocotyl and a lower zone, including the elongation zone of the radicle, in the vicinity of the tip root

while the spread of the infection was constrained to the root tip in genotype G12.

Discussion

The sugar yield losses and consequent economic damage caused by *A. cochlioides* represent a major problem in sugar beet cultivation. Fully resistant varieties are not available on the market due to the lack of germplasm carrying this trait. Therefore, the identification and selection of genotypes showing a high level of resistance to this pathogen are essential. In this study, a qPCR assay was applied to sugar beet seedlings infected with *A. cochlioides* to investigate whether the quantification of *A. cochlioides* biomass in infected plants could predict the phenotypic response of different genotypes to Aphanomyces damping-off. We hypothesized that partially resistant genotypes should contain a lower amount of the pathogen DNA and therefore a lower pathogen biomass and higher disease resistance index compared to the susceptible genotypes. The phenotypic

screening of the sugar beet genotypes revealed G17, G18, G19 and G20 to be the most susceptible to damping-off. Genotypes G2 and G8 displayed a certain level of resistance and G12 had a higher level of resistance towards the disease. The encystment of *A. cochlioides* zoospores occurs few minutes after exposure to the host-specific attractant cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone), exuded by spinach roots. This is followed by germination of cystospores within 30–60 min (Sakihama et al. 2004). In our qPCR test, the pathogen DNA was detected in the host directly after 2 h of submersion in the zoospore solution. In the first qPCR test performed at 0, 4, 24 and 48 hpi some trends in the progress of the infection were observed in genotypes infected with USA_01.7.6 and Hokkaido_01. In particular, two groups could be distinguished: genotypes with lower pathogen/host DNA ratios, i.e., G2, G8 and G12 and genotypes with higher pathogen/host DNA ratios, i.e. G17, G18, G19 and G20. These results were in agreement with the phenotypic data collected in the phenotypic test. However, the same pattern was not observed when the sugar beet lines were infected with Arhill_2012. Moreover, for most of the genotypes, regardless of the isolate, the ratio at different time points did not change significantly. This led to the conclusion that a longer time span was needed to considerably differentiate the pathogen biomass in different genotypes. In the extended test, just three among the most interesting genotypes were included (i.e. G8, G12 and G17) and plants were collected at 0 hpi, 2 dpi, 4 dpi and 8 dpi. The pattern of the infection was clearly different between the different genotypes. At the latest timepoints, the ratio of *A. cochlioides*/sugar beet DNA in the susceptible genotype G17 was significantly higher compared to the ratio in the resistant genotype G12. This observation was also consistent across independent replicates of the experiment, indicating that the qPCR assay can be used to effectively distinguish highly resistant genotypes from the most susceptible plant material between 4 and 8 dpi. In addition, even if it was not possible to significantly differentiate genotype G8 from G12, the infection rate in these two lines showed a diverse trend. The infection in G12 was almost constant at any time point while the ratio in G8 tended to increase between 4 and 8 dpi when inoculated with Hokkaido_01 and Arhill_2012. It was not surprising to observe a different infection pattern in G8 inoculated with USA_01.7.6 since this genotype had a higher disease resistance index in the phenotypic evaluation in response to this isolate. These results suggest that G8 might respond differently to USA_01.7.6 compared to Hokkaido_01 or Arhill_2012. Two races (race 1 and race 2) have been identified in *A. euteiches*, a close relative of *A. cochlioides*. Race 1 was reported to be highly virulent on the susceptible alfalfa (*Medicago sativa* L.) cultivar Saranac and less
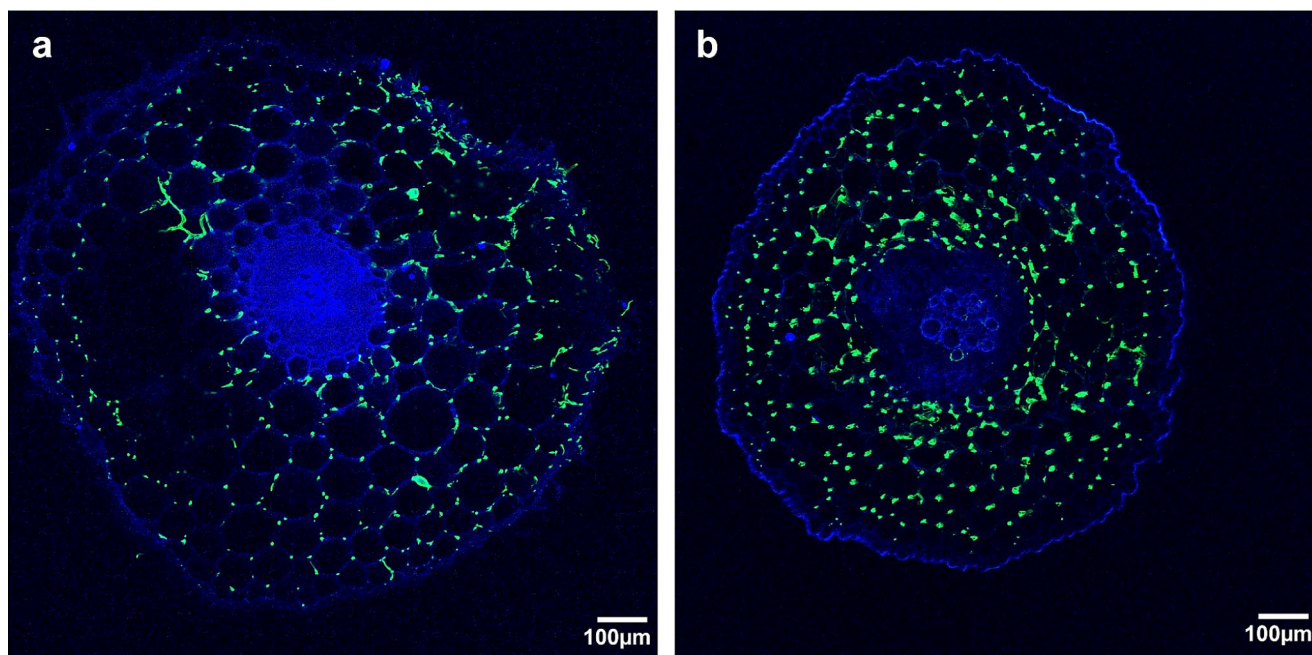
Fig. 3 Cross sections of sugar beet (*B. vulgaris* L.) hypocotyl in the proximity of the radicle at 2 days after inoculation with *A. cochlioides* zoospores, observed at the confocal laser scanning microscope. *Aphanomyces cochlioides* hyphae are visible in the intercellular spaces of (**a**) the partially resistant genotype G12 and (**b**) the susceptible geno- type G17. Sections were stained with wheat germ agglutinin (WGA) conjugated with Alexa Fluor 488 to visualize *A. cochlioides* hyphae (in green). Autofluorescence from sugar beet tissue was detected using UV excitation (in blue)

virulent on the alfalfa population WAPH-1, while race 2 is able to infect both genotypes (Grau et al. 1991; Malvick and Grau 2001). It is therefore possible that the isolates of *A. cochlioides* tested in this study correspond to two different races of the pathogen. However, population studies are needed to confirm this hypothesis. The difference in the responses to *A. cochlioides* infection between the partially resistant and the susceptible genotypes was further investigated by confocal microscopy. During the first two days after the inoculation period, zoospores had germinated and developed hyphae which colonized the intercellular spaces of the cortex and reached the endodermis in both genotypes. However, the pathogen was restricted in the elongation zone in proximity to the radicle. Previous studies have shown that *A. euteiches* initiates the infection in the elongation zone of pea (*Pisum sativum* L.) roots while the root cap and border cells were free of colonization (Cannesan et al. 2011). The absence of the pathogen in the root cap was explained as a consequence of the synthesis and secretion of defense- related proteins such as pisatin by the root border cells, while very low amount of this isoflavonoid were produced in the elongation zone, making it a suitable infection site for pathogen attack (Cannesan et al. 2011). On the basis of our study it is not possible to exclude the presence of the patho- gen in the root cap, however the presence of *A. cochlioides* hyphae in the lower part of the hypocotyl but not in the middle and upper zones during the early stages of the

infection (2 dpi) strongly suggests that the infection initiates in the vicinity of the radicle before spreading up to the hypo- cotyl in later stages, in a similar manner to that described for *A. euteiches*. At 2 dpi, no considerable differences were observed between the two genotypes. The presence of hyphae inside the infected tissues indicates that both geno- types are prone to the pathogen attack and initial ingress. From 4 to 8 dpi *A. cochlioides* had invaded the elongation zone, the maturation zone and hypocotyl of the susceptible genotype, while the spread of the pathogen was still limited to the apical part of the elongation zone in the partially resis- tant genotype. These results suggest that within four days after exposure of the plants to *A. cochlioides* zoospores, the pathogen is able to induce the disease by overcoming the host defense mechanisms in the susceptible genotype. On the other hand, the pathogen growth seems to be suppressed or delayed in partially resistant plants. Cytological analysis of a *Medicago truncatula* susceptible line, named F83005.5 and the partially resistant line A17-Jemalong revealed major differences in plant defense responses following *A. eutei- ches* inoculation (Djébali et al. 2009). All root cortical cells of the susceptible line F83005.5 were colonized at 6 dpi, hyphae had invaded the stele at 15 dpi, and most root cells appeared to be dead at 21 dpi. Conversely, mycelium was restricted in the cortical cells in A17-Jemalong and rein- forced cell walls were observed in the layers surrounding the stele, which prevent the colonization of the vascular
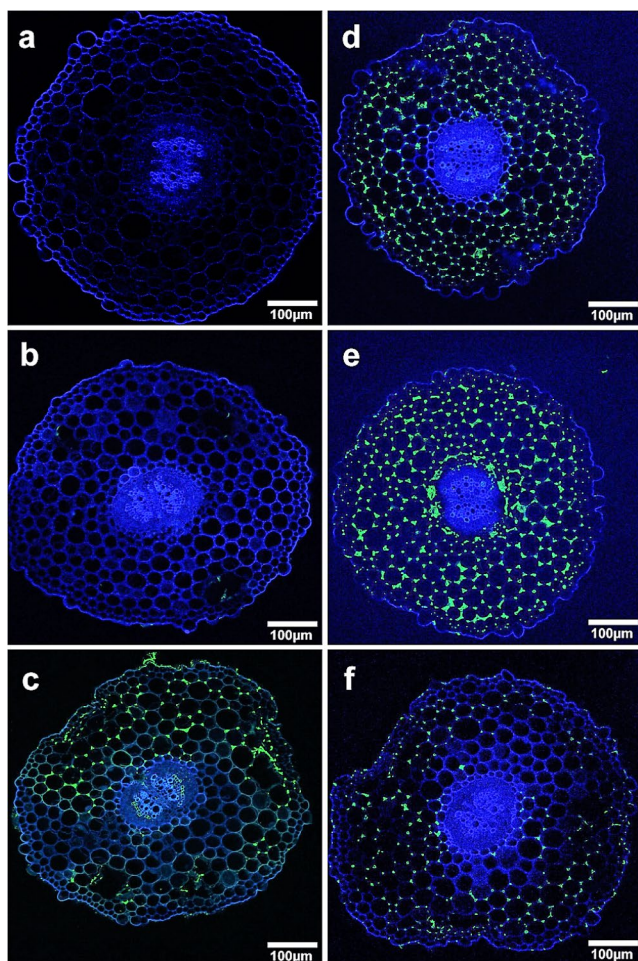
Fig. 4 Cross sections of sugar beet (*B. vulgaris* L.) from different zones of the hypocotyl at 8 days after inoculation with *A. cochlioides* zoospores, observed at the confocal laser scanning microscope. In the partially resistant genotype G12 *A. cochlioides* hyphae were not observed in **(a)** the upper part and in **(b)** the middle part of the hypocotyl but were present in **(c)** the elongation zone. *Aphanomyces cochlioides* hyphae colonization was visible in **(d)** the upper zone, **(e)** the middle zone and **(f)** the elongation zone of the hypocotyl of the susceptible genotype G17

system (Djébali et al. 2009). Furthermore, a strong autofluorescence which correlated with the accumulation of soluble phenolic compounds was observed in the cortex of the partially resistant line. Changes in the autofluorescence in the *A. cochlioides*-sugar beet pathosystem were not detected in our study, possibly due to the use of ethanol during the fixation and dehydration of the examined samples resulting in the dissolution of the phenolic compounds. However, the production and accumulation of such molecules in the partially resistant line could potentially play a role in stopping the pathogen spread within the hypocotyl, similarly to the partial resistance strategy described in *M. truncatula*. It is also tempting to infer that the recognition of molecules secreted by the pathogen triggers immune responses in the partially resistant genotype, resulting in a localized response

that prevents the pathogen from spreading. However, more insights into pathogenicity genes that play a role in the infection process as well as defense-related genes activated in the host are needed, in order to elucidate the molecular mechanisms underlying these host-pathogen interactions. Nevertheless, noticeable differences in coping with *A. cochlioides* invasion between partially resistant and susceptible sugar beet breeding lines emerged from this study, highlighting the importance of selecting suitable material able to control the disease.

Conclusion

More knowledge about the infection biology of *A. cochlioides* is needed, in order to successfully manage the disease. This study is the first attempt to elucidate the progress of the infection inside infected plants. The qPCR-based approach used in this study provided data which were in agreement with the variations observed in the responses to damping-off of plants of different genotypes and thus could represent a fast and reliable system for the selection of resistant germplasm that can be used in breeding programs. The low pathogen levels detected by qPCR in the partially resistant genotype was confirmed by confocal microscopy and suggested that the rapidity of host responses could prevent the progression of the disease. Future studies on transcript and metabolic levels would provide a deeper understanding of *A. cochlioides*/sugar beet interaction and could reveal specific immune mechanisms in response to *A. cochlioides*.

Statements and Declarations

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