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Exploring resistance to *Aphanomyces cochlioides* in sugar beet

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Department of Plant Protection Biology

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Abstract

Sugar beet (*Beta vulgaris* L.) is one of the two main sources of sucrose, second only to sugar cane, accounting for nearly 30% of sugar production worldwide. The viability of the crop is threatened by the attack of pathogens that cause various diseases, resulting in severe yield losses. The oomycete *Aphanomyces cochlioides* is one of the most important root pathogens in sugar beet due to its worldwide distribution and the ability to induce infection at any stage of the sugar beet lifecycle, causing both seedling damping-off and chronic root rot on mature roots. During the early phase of sugar beet cultivation, the infection can be controlled by chemical seed treatments. However, no major control strategies are available for the disease management in later stages during the growing season. An increased knowledge of the sugar beet responses to pathogen infection is required to find effective solutions to control the disease. The focus of this study was to enhance our understanding of the host-pathogen interactions. We explored *A. cochlioides* infection rates in different sugar beet genotypes by using molecular tools and confocal microscopy and we identified significant differences in the pathogen biomass between partially resistant and susceptible lines. Potential differences in sugar beet responses to different *A. cochlioides* isolates were also investigated through a transcriptomics study. Furthermore, the transcriptome analysis revealed a potential significant role for oxygen peroxide (H_2O_2) and cell wall modification in the defense mechanisms during *A. cochlioides* invasion. Candidate defense-genes were identified in the set of up-regulated transcripts of partially resistant plants. Lastly, we studied differences in the genetic resistance to damping-off and chronic root rot and we identified the genomic position of regions (quantitative trait loci) associated with damping-off resistance. Overall, the results of our research represent a valuable source for future studies to improve resistance breeding against *A. cochlioides*.

Keywords: sugar beet, *Aphanomyces cochlioides*, *Beta vulgaris*, damping-off, chronic root rot, immune responses, transcriptomics, QTLs, resistance breeding

Dedication

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This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Rossi, V.**, Bengtsson, J., Kiselev, A., Gaulin, E., Holmquist, L., Grenville-Briggs, L. (2023). Rapid detection and quantification of *Aphanomyces cochlioides* in sugar beet (accepted in *Journal of Plant Pathology*)

II. **Rossi, V.**, Holmquist, L., Alexandersson, E., Grenville-Briggs, L. Transcriptome analysis of sugar beet in response to the pathogenic oomycete *Aphanomyces cochlioides* (manuscript)

III. **Rossi, V.**, Kraft, T., Thorell, E., Holmquist, L., Grenville-Briggs, L. QTL mapping for *Aphanomyces cochlioides* resistance in a mapping population of sugar beet (manuscript)

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The contribution of Valentina Rossi to the papers included in this thesis was as follows:

I. Designed the study together with co-authors, planned and performed experiments with co-authors, analysed the data, wrote the manuscript with the input of co-authors.

II. Participated in the experimental design with co-authors, planned and performed all the experiments, conducted the bioinformatics analysis, wrote the manuscript with the input of co-authors.

III. Participated in the design of the project, performed self-crosses to produce the S_2 mapping population with co-authors, collected phenotypic data, performed the analysis of the data with co-authors, wrote the manuscript with the input of co-authors.

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Abbreviations

AFLP	Amplified Fragment Length Polymorphism
BChV	Beet Chlorosis Virus
BMYV	Beet Mild Yellowing Virus
BNYVV	Beet Necrotic Yellow Vein Virus
BWYV	Beet Western Yellows Virus
CAZy	Carbohydrate-Active Enzymes
CE	Carbohydrate Esterases
CLS	Cercospora Leaf Spot
CMA	Corn Meal Agar
CMS	Cytoplasmatic Male Sterility
CRN	Crinkler
DI	Disease Index
dpi	Days post inoculation
ETI	Effector-Triggered Immunity
FAA	Formaldehyde Alcohol Acetic Acid
FDR	False Discovery Rate
GH	Glycosyl Hydrolases
GlcNAc	N-acetyl-D-glucosamine
GO	Gene Ontology
GT	Glycosyl Transferases
GWAS	Genome-Wide Association Studies
HEGS	High-Efficiency Genome Scanning
hpi	Hours post inoculation
HR	Hypersensitive Response
HSD	Honestly Significant Difference
HT-NGS	High-Throughput-Next-Generation-Sequencing

ITS	Internal Transcribed Spacer
LAI	LTR Assembly Index
LOD	Logarithm of the Odds
LRR	Leucine Rich Repeat
MAMPs	Microbe-Associated Molecular Patterns
MAS	Marker-Assisted Selection
NBS	Nucleotide-Binding Site
NGS	Next-Generation-Sequencing
NMS	Nuclear Male Sterility
NO	Nitric Oxide
PAMPs	Pathogen-Associated Molecular Patterns
PL	Polysaccharide Lyases
PR	Pathogenesis-Related
PRRs	Pattern Recognition Receptors
PTI	PAMPs-Triggered Immunity
qPCR	Quantitative Polymerase Chain Reaction
QTL	Quantitative Trait Locus
R proteins	Resistance proteins
RFLPs	Restriction Fragment Length Polymorphisms
RH	Relative Humidity
ROS	Reactive Oxygen Species
RNAi	RNA Interference
SNPs	Single Nucleotide Polymorphisms
SSPs	Small Secreted Proteins
SSR	Single Sequence Repeats
TEs	Transposable Elements
WGA	Wheat Germ Agglutinin

1. Introduction

Sucrose, better known as sugar, is the pure, natural, organic chemical compound with the world´s highest production and is one of the most traded products in the international market (Parker et al., 1977; Duraisam et al., 2017). An average annual growth of 2% has been registered between 2001 and 2018 and after 3 years of decline, world sugar consumption rose again in 2021 accounting for about 168 million tons (International Sugar Organization, 2022).

All green plants produce sugar through photosynthesis, however, sugar cane and sugar beet are the crops with the greatest quantity of sugar and are the only two plants utilized for industrial sugar production. One of the earliest written reports of sugar comes from 327 BC, where Nearchus, one of Alexander the Great's commanders, described sugar cane as "a reed in India that brings forth honey without the help of bees, from which an intoxicating drink is made, though the plant bears no fruit." (Cooper, 2006).

Sugar was introduced in the Mediterranean area by the Greeks and the Romans, when small quantities of sugar were imported and traded to physicians for medical purposes (Smith, 2015). For hundreds of years, sugar was considered a necessary ingredient for the health of mankind in ancient Greece, until it began to be viewed no longer as a medicine but as a food delicacy (Smith, 2015).

In 1493, Columbus introduced sugar cane to the New World, which rapidly spread in many countries of South America (Gudoshnikov et al., 2004). Today, Brazil, India, China, Pakistan, Thailand, Mexico, the United States and Australia are the major sugar cane producers, accounting for nearly 70% of the global sugar output (International Sugar Organization, 2022).

The production of sugar from sugar beet has a much more recent history. Sugar beet production expanded between the 19th and 20th centuries up to present day and today it provides nearly 30% of sugar worldwide (Zicari et al., 2019) with Russia, France, Germany, the United States, Turkey, Poland and China being the top producing countries (FAO, 2022).

1.1 Sugar beet

1.1.1 The sugar beet crop

Sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) belongs to the Amaranthaceae family and the Caryophylalles order and is characterized by a C3 photosynthetic system (Zicari et al., 2019). It is a biennial crop, characterized by a first vegetative year in which the plant develops a fleshy taproot where sugar is accumulated and stored for the second year of growth (Fig. 1A). In the second year, after a period of vernalization and exposure to long photoperiods, the plant enters the reproductive stage and develops a primary stem in a process known as bolting, with secondary and tertiary branches that terminate in an inflorescence (Figure 1B) (Biancardi, 2005). Reproduction occurs after flower opening, when the two sperm cells of the pollen grain fuse respectively with the egg, to produce the embryo, and with the central cell of the embryo sac to produce the endosperm. Sugar beet is a cross-pollinated species, with a degree of self-incompatibility controlled by four gametophytic S-loci with complementary interactions, meaning that all the S-alleles in the pollen have to be matched in the pistil to result in an incompatible mating (Larsen, 1977). After fertilization, a true seed is developed, surrounded by a brown, lignified fruit to form the hard seed ball, typically used for propagation in sugar beet culture.

Sugar beet seeds are sown in March-April and roots are harvested at the end of the first growing season, typically 170-200 days after sowing. However, plants used for seed production are kept until the end of the second year to allow seed development and propagation of the seeds.

Due to its high adaptability to different environments, sugar beet is cultivated in diverse climates across Europe, North America, Asia, South America and more recently in North Africa (Hossain et al., 2017).

Sugar beet is primarily grown as a source of sugar for human consumption. However, it is also used for the production of bioethanol, the

extraction of pectin from the sugar beet pulp, livestock feed, and the production of molasses which is used as an antioxidant, sweetener, and colorant (Yu et al., 2020).

Figure 1. Vegetative and reproductive stages of sugar beet. A) Sugar beet plant during the vegetative stage; B) Sugar beet bolting plant at the flowering stage. Photos: Simon Jeppson

1.1.2 The history of sugar beet

In ancient times, sugar beet varieties were cultivated along the shores of the Mediterranean and their leaves were consumed as a spinach-like vegetable (Draycott, 2008). Sugar beet cultivation in fields started in the seventeenth century, when leaves and roots were mainly used as fodder for cattle in France and Germany (Cook and Scott, 2012). It was not until the second half of the eighteenth century that sugar beet became an industrial crop, cultivated for its high sucrose content. The turning point occurred in 1747, when the German chemist Andreas Sigismund Marggraf first discovered that the crystals extracted from the beet juice were identical to the sugar from sugar cane (Pathak et al., 2022). Marggraf´s student Franz

Karl Achard then revived this discovery and developed a process to extract sugar on a large scale, becoming the pioneer of the sugar beet industry (Cook and Scott, 2012). Observing the wide variability in sugar content between different beet cultivars, Achard initiated a mass selection of beets with higher sugar yield and obtained the variety "White Silesian", with a sugar content of 5-7% of the total root weight, which was then used as base population for further sugar yield improvement (Bosemark, 1979; Coons, 1936).

The industrial production of sugar in Europe was also encouraged under the Napoleonic Wars. At that time, Britain had a monopoly over the sugar cane trade, and when France was cut off from the trade routes, the country was left without a sugar supply (Draycott, 2008). In 1811, Napoleon became interested in developing the manufacturing of sugar and established more than 40 sugar beet factories throughout France, which became the world´s greatest sugar beet grower of that time (Pathak et al., 2022).

After 1830, sugar beet was introduced into North America and Chile (Peter et al., 2001). The United States, which was completely dependent on imported sugar beet from Germany in the 1800s, rapidly caught up to the levels of sugar beet production in Europe (Pathak et al., 2022).

The first sugar factory in Sweden was built in 1854 in Landskrona and 8 new factories were established in the 1880s in the southern regions of Sweden (Bosemark, 1997). Breeding activities in Sweden started in Landskrona in 1907, with the company Hilleshög becoming the only sugar beet seed supplier in Sweden in 1928. This company is still the leading sugar beet seed supplier in Sweden today, although it has been bought-out and is now incorporated into DLF Beed Seed.

Today, sugar beet is cultivated in around 50 countries, with the United States and the European Union being global leaders in the production of beet sugar-derived sugar (Rana et al., 2022).

The most important events in the sugar beet crop development are represented in Figure 2.

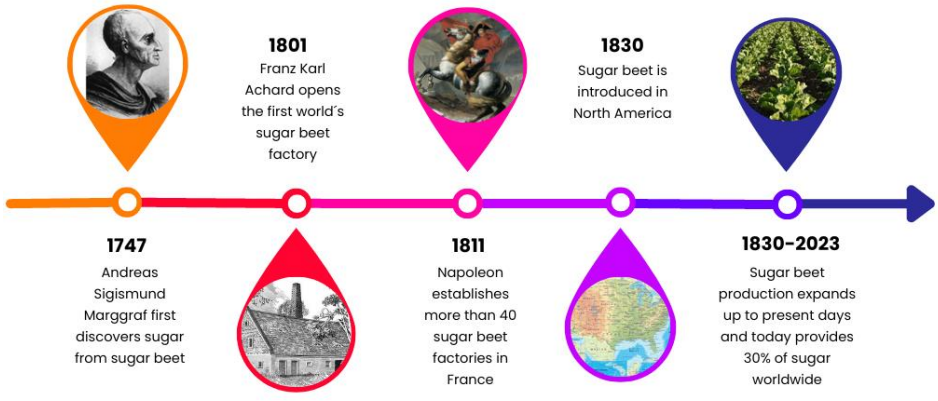

Figure 2. Important events in the history of sugar beet cultivation. Image created on Canva.com

1.1.3 Sugar beet breeding

Sugar beet is considered the first crop whose development is built exclusively on a modern understanding of genetics and a science-based selection system (Biancardi, 2005).

Cultivated beets originate from a common wild ancestor, the wild sea beet (*B. vulgaris* ssp. *maritima*). The selective pressure imposed by growers to tailor the expression of traits of interest has resulted in the loss of survival traits such as competitiveness against weeds and the annual cycle, necessary to increase seed production and the livelihood in the wild (Biancardi et al., 2010). Furthermore, the selection process has resulted in a narrow gene pool in domesticated sugar beet (Biancardi et al., 2012). Today, the wild sea beet is used in breeding not only to increase the genetic diversity of cultivated sugar beets, but also as a source of important traits such as resistance to pests and pathogens and tolerance to drought, heat, and salinity (Biancardi et al., 2012).

The discovery of monogerm plants (Savitsky, 1950) was an important achievement in sugar beet breeding. Prior to this, all sugar beet seeds were multigerm, meaning that each seed generated multiple seedlings. This required time-consuming work consisting of manual thinning in the field (Richardson, 2010). The farmers´ workload was drastically reduced with the adoption of monogerm seeds, which is nowadays the only type of sugar beet seed planted in developed countries (Biancardi, 2005).

Commercial sugar beet varieties are 3-way hybrids. The discovery of male sterility in sugar beet plants represented an important step for the development of the hybrids. Male sterility implies the inability of plants to produce or release viable pollen. Two types of male sterility are used in sugar beet breeding: the nuclear (or genetic) male sterility (NMS), governed by one or more nuclear genes, and the cytoplasmatic male sterility (CMS), maternally transmitted and regulated by the combined effect of nuclear and cytoplasmatic factors (Lasa and Bosemark, 1993). Nuclear male sterility is exploited by breeders to facilitate cross-pollination between breeding lines, while the cytoplasmatic male sterility system is used for the 3-way-hybrid production. Hence, the first hybrid is obtained by crossing a diploid, monogerm CMS plant, which is sterile, with a genetically divergent genotype, called O-type. The O-type carries the same nuclear sterility genes as the CMS line but in normal cytoplasm and it is, therefore, fertile (Draycott, 2006). CMS and O-type plants are crossed to ensure high seed yield during hybrid seed production. The resulting hybrid, referred to as F_1MS, is also male sterile and is used as a mother plant in the second cross with a second pollinator. The offspring of this cross is the final hybrid, used as commercial variety in the market, characterized by heterosis in terms of sugar yield, meaning a higher yield performance compared to the homozygous parental lines (Fig.3). The crosses are made when the plants have entered the reproductive stage, after a period of vernalization that can be induced by artificial exposure to cold temperatures.

The primary goal in sugar beet breeding is to exploit the genetic potential of the crop to develop varieties with the maximum sugar yield possible. In the past decades, sugar beet yield has continuously grown, showing an annual increase of 1.5% (Märländer et al., 2003; Jaggard et al., 2010). In addition to the high yield trait, it is important to produce uniform seeds with high germination potential and seedling emergence.

The sugar yield is also influenced by abiotic and biotic stresses. In particular, pests and diseases represent a major constraint for the development of an economically viable crop.

Remarkable progress has been made over the years in sugar beet breeding, however there is still much more to be accomplished. In particular, genetic resistance to pathogens is often incomplete or accompanied by reduced yield. Drought also significantly affects sugar beet productivity. It is, therefore, important to develop sugar beet drought-tolerant hybrids and varieties with

long-lasting resistance against pathogens to meet the market demands in a rapidly-changing environment.

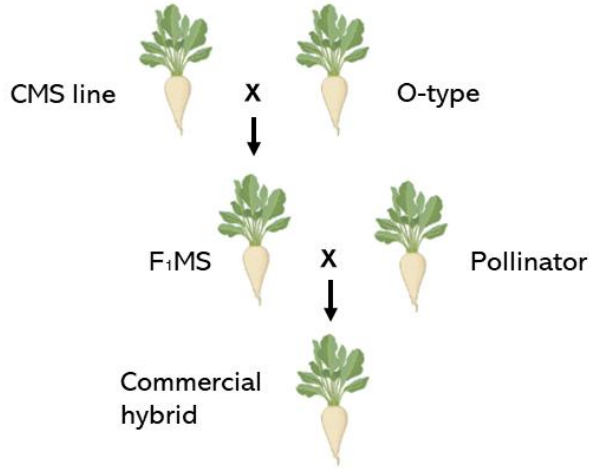

Figure 3. Schematic representation of sugar beet commercial hybrid production. Image created in BioRender.com

1.2 Plant diseases and immunity

As sessile organisms, plants must constantly adjust to the environment to cope with the myriad of abiotic and biotic stresses to which they are continuously exposed. A plant becomes diseased when the normal physiological processes are deviated from their function by some causal agent. Based on the nature of the primary causal agent, plant diseases can be infectious, if they are caused by pathogenic organisms, or non-infectious, when induced by abiotic stresses such as drought, salinity and extreme temperatures. In this thesis, the attention is focused on infectious diseases with a closer look at host-pathogen interactions.

1.2.1 Plant-pathogen interactions

In both natural and agricultural ecosystems, plants are continuously exposed to beneficial and pathogenic microorganisms, forming complex and dynamic interactions. Resistance of plants to pathogenic microorganisms is

the rule, while susceptibility is the exception (Yarwood, 1967). Resistance exhibited by an entire plant species to a specific pathogen is known as non-host resistance and involves a multiplicity of defense factors. By contrast, host resistance is expressed by a certain plant genotype of a species susceptible to a pathogen (Heath, 2000).

Plants can prevent the entry of pathogens and disease induction by developing physical barriers such as wax layers, trichomes, rigid cell walls and cuticular lipids or by producing degrading enzymes and secondary metabolites (Muthamilarasan and Prasad, 2013). When pathogens succeed in overcoming this first defensive barrier, they have to cope with the plant defense responses that are elicited upon recognition of carbohydrate- and protein-based signaling molecules classified as Microbe- or Pathogen - Associated Molecular Patterns (MAMPs or PAMPs). These molecules, which are essential for the pathogen viability, include bacterial flagellins, elongation factors, fungal cell wall components such as polysaccharides and chitin and oomycetes β-glucans (Selin et al., 2016; Judelson and Ah-Fong, 2019). The recognition of PAMPs is mediated by Pattern Recognition Receptors (PRRs), localized in the plant cell plasma membrane. PAMP-triggered immunity (PTI) is the first layer of plant immunity that triggers defense responses leading to basal or non-host resistance. PAMP-induced responses include the production of reactive oxygen species (ROS) and reactive nitrogen species such as nitric oxide (NO), alteration of the cell wall, induction of antimicrobial compounds and the synthesis of pathogenesis-related (PR) proteins (Newman et al., 2013). To counteract PTI, pathogens have evolved effector proteins, which are molecules that are key for a successful infection. They function by inhibiting the host immune system, and/or adapting the environment inside the host to allow the pathogen to grow and reproduce. In response, plants have evolved resistance (R) proteins which are intracellular receptors characterized by a nucleotide-binding site (NBS) and a leucine rich repeat (LRR) motif, which are able to recognize effector proteins. The recognition of the pathogen effectors by the corresponding R protein leads to the activation of the so-called effector-triggered immunity (ETI) (Muthamilarasan and Prasad, 2013). ETI gives rise to immune responses that partially overlap with PTI (Figure 4). However, ETI is stronger and faster and additionally induces a form of programmed cell death known as the hypersensitive response (HR) (Dodds and Rathjen, 2010). The interaction of these two immune pathways has remained unclear

for a long time, but it has now been shown that cell-surface and intracellular receptors are co-dependent in activating the defense responses to pathogens. The abundance of protein kinases and NAPDH oxidases activated by PTI is increased by intracellular receptors and the activation of surface receptors potentiates the hypersensitive response induced by ETI (Ngou et al., 2021).

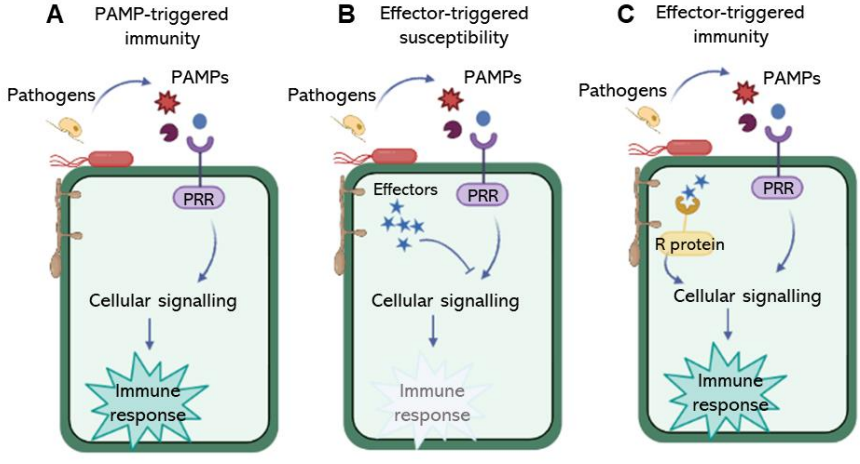

Figure 4. Schematic overview of the plant immunity response to pathogen attack. A) Recognition of Pathogen-Associated Molecular Patterns (PAMPs) by Pattern Recognition Receptors (PRRs) induces plant immune responses; B) Effector proteins secreted by the pathogen suppress the plant immune system and C) Recognition of the effectors by the host R proteins induces the effector-triggered immunity. Illustration created in BioRender.com based on figure from Pieterse et al. 2009

1.2.2 Sugar beet pests and diseases

Sugar beet cultivation is threatened by a multitude of pests and pathogens, which can attack both the root and the leaf. Sugar beet is susceptible to several viruses that can be transmitted by insects, fungi, nematodes and via seeds. Viral infections may result in reduced yield potential and affect the sugar extractability. Some of the most damaging viruses are the Beet necrotic yellow vein virus (BNYVV), the causal agent of Rhizomania, vectored by the soil-borne protist *Polymyxa betae*, as well as Beet mild yellowing virus (BMYV), Beet western yellows virus (BWYV) and Beet chlorosis virus (BChV), which are responsible for virus yellows disease and are transmitted by aphids (Draycott, 2006).

Sugar beet is also attacked by several insects and mites that can damage the crop at different stages of its growth either directly or by introducing viruses into the plant (Baitha et al., 2022). Thrips, e.g., *Hercinothrips femoralis*, beet fly (*Pegomya betae*), pygmy mangold beetle (*Atomaria linearis*) and centipedes are some of the most important pests causing feeding damage on sugar beets.

Diseases caused by oomycetes and fungi have drastic effects on sugar beet production. Soil-borne seedling diseases caused by the oomycetes *Aphanomyces cochlioides* and *Pythium* spp. have been efficiently controlled by chemical treatments applied to seeds. However, chemical pesticides are not effective in protecting adult plants from the infection caused by *A. cochlioides*. Moreover, the increasing demand in reducing the chemical load on the environment will necessarily lead to the adoption of sustainable control approaches including biological control strategies, habitat manipulation, modification of cultural practices and genetic resistance as primary means of disease management. Commercial varieties with high resistance levels to the soil-borne fungus *Rhizoctonia solani* are available, but at the cost of yield and the lack of resistance to other important diseases (Jacobsen et al., 2004). Fungicides with different active ingredients are also available to control *R. solani* damping-off and crown and root rot (Arabiat and Khan, 2016). Large amounts of fungicides are also employed to control Cercospora leaf spot (CLS), the predominant foliar disease in sugar beet, caused by the fungus *Cercospora beticola*. Without chemical treatment, the pathogen can cause up to 50% of sugar yield reduction (Rangel et al., 2020). The widespread use of synthetic fungicides has caused the development of *C. beticola* populations with a reduced sensitivity to the most common fungicides used to manage the disease (Kumar et al., 2021). A better understanding of the fungicide resistance mechanisms is needed to maintain fungicide effectiveness and to contain the rapid development of resistant isolates. Nonetheless, agronomical practices and breeding for resistance are also required to preserve sugar beet profitability.

1.3 Aphanomyces cochlioides

One of the most damaging taproot diseases in sugar beet is the root rot disease caused by *Aphanomyces cochlioides*. *A. cochlioides* is a soil-borne pathogen belonging to the class of oomycetes, a group of filamentous eukaryotic microorganisms widespread in freshwater, marine and terrestrial ecosystems (Beakes et al., 2012). More than 50 years ago, due to their morphological and ecological similarities with fungi, oomycetes were classified at the same levels as ascomycetes and basidiomycetes within the Fungal kingdom (Lévesque, 2011). However, phylogenomic and genetic studies have revealed that they are more closely related to brown algae (Becking et al., 2021) and thus they are now placed in the kingdom Stramenopila.

A. cochlioides was first described in 1929 as the causal agent of root rot disease in sugar beet in Michigan, U.S. (Deschler, 1929). The pathogen is specialized to infecting the roots of sugar beet, spinach (*Spinacia oleracea* L.), cockscomb (*Celosia argentea* L.) and other related species belonging to the Amaranthaceae (Scott, 1961). *A. cochlioides* has a worldwide distribution and it has a great economic impact in many sugar beet cultivating areas of North America, such as the Red River Valley of Minnesota and North Dakota, much of Europe and in the Hokkaido Prefecture of Japan (Harveson, 2000; Beale et al., 2002). In USA, annual losses due to *A. cochlioides* amount to 53.9 million U.S. dollars when management practices such as chemical treatment on seeds are applied, and 299.5 million U.S. dollars are estimated to be lost if no chemical treatment is used (information given by the Beet Sugar Development Foundation, BSDF, 2022).

1.3.1 Pathogenicity determinants of *Aphanomyces* species

To successfully start the infection and invade the host, oomycetes secrete a vast repertoire of effector molecules to modulate the innate host immunity. Effector proteins can either be intercellular, if they target the apoplast, or intracellular when they are addressed to the cytoplasm of the host cell (Becking et al., 2021).

Cell wall degrading enzymes such as glycosyl hydrolases families (GH), Glycosyl Transferases (GT), Polysaccharide Lyases (PL) and Carbohydrate Esterases (CE) represent a vast majority of the extracellular effectors in phytoparasitic *Aphanomyces* species (Becking et al., 2022). For example, *A. euteiches* harbors over 300 carbohydrate-active enzymes (CAZy) and carbohydrate-binding modules (Gaulin et al., 2018).

Among the intracellular effectors, the main classes of oomycete effector families include the RxLR and the Crinkler (CRN) proteins, which are defined by a conserved N-terminal motif that plays a role in targeting the host cell (Bozkurt et al., 2012). While the presence of CRNs occurs in all oomycetes examined, the RxLR family is, so far, believed to be restricted to the Peronosporales clade of the oomycete class (Schornack et al., 2010).

Whole genome sequences of the animal pathogens *A. astaci* and *A. invadans*, the phytopathogenic species *A. euteiches* and *A. cochlioides* and the saprophyte *A. stellatus*, are available and the presence of these two host targeting signals has been investigated in some of these *Aphanomyces* species. Analysis of the genomes of the legume pathogen *A. euteiches* and the crayfish pathogen *A. astaci* confirmed the absence of RxLR proteins in these two species, while several CRN genes were identified (Gaulin et al., 2018). However, in the recent *de novo* genome assembly of *A. cochlioides*, 22 RxLR putative effector proteins were identified in addition to CRN effector candidates (Botkin et al., 2022), while the genome of the fish pathogen *A. inavdans* was found to lack both classes of effectors (Iberahim et al., 2018).

A new class of putative oomycetes effectors was also recently reported in the genus *Aphanomyces*. These Small Secreted Proteins (SSPs) are less than 300 amino acids in size and are characterized by a signal peptide (Gaulin et al., 2018). Functional studies on SSPs in *A. euteiches* showed that some of these proteins target the plant nucleus and alter the plant DEAD-box RNA helicase to promote the infection (Camborde et al., 2022).

Compared to other oomycetes and *Aphanomyces* species, virulence factors of *A. cochlioides* have received less attention, due to the lack of a reference genome until recently. Further analysis of the genome of *A. cochlioides* may reveal the presence of these classes of effectors and would help in elucidating the molecular factors involved in the infection process of this pathogen. The development of molecular tools such as RNA silencing, transformation and gene editing, used to study other plant and animal pathogenic oomycetes, has not been successful in *Aphanomyces* species, except for the animal pathogen *A. invadans*. This is the only *Aphanomyces* species for which both RNAi silencing and a Crispr/Cas9 system have been effectively established (Iberahim et al., 2020; Majeed et al., 2018). Attempts to transform *A. euteiches* and *A. cochlioides*, or to otherwise manipulate their genomes, have not led to any results so far, making the lack of *Aphanomyces* stable or

transient transformation systems a major barrier in the investigation of the infection mechanisms operated by this group of oomycetes.

1.3.2 *A. cochlioides* life cycle

The life cycle of *Aphanomyces* species is characterized by sexual and asexual stages. *A. cochlioides* is homothallic with female and male reproductive structures in the same thallus and, therefore, fertilization can occur in a single strain. During sexual reproduction, thick-walled diploid oospores, 18-25 µm in size, are formed from the union of oogonia and antheridia inside infected tissues, and are released into the soil when the plant dies (Heffer et al., 2002). Oospores serve as overwintering structures and can survive in the soil for many years in the absence of a suitable host (Papavizas and Ayers, 1974).

The infection is initiated under warm and moist soil conditions, when oospores germinate and develop vegetative hyphae, which can directly infect the host or differentiate into sporangia, where uninucleate primary zoospores are produced in the asexual phase (Dyer and Windels, 2003). Primary zoospores are bi-flagellated and motile and after evacuation from the sporangial tip, they encyst and give rise to secondary zoospores, which are also equipped with two flagella that allow the zoospores to swim in the soil water towards the root surface (Walker and van West, 2007). Zoospore motility is stimulated by chemotactic signals generated by the root exudates. *A. cochlioides* zoospores are attracted by a specie-specific flavonoid known as cochliophilin A, first isolated from spinach roots (Horio et al., 1992). When attracted by cochliophilin A, zoospores encyst by shedding their flagella, develop a germ tube on the infection site and ultimately penetrate the host within 30–60 minutes (Islam, 2010; Sakihama et al., 2004). Once inside the host, hyphae proliferate in the intercellular spaces of the root cortex and eventually colonize the whole tissue.

If the encystment occurs in the absence of a suitable host, secondary zoospores also have the ability to originate a second and third generation of zoospores, in an event known as repeated zoospore emergence, or polyplanetism (Cerenius and Söderhäll, 1985; Diéguez-Uribeondo et al., 2004). The duration of first, second and third generation of *A. cochlioides* zoospores was reported to be 30, 18 and 15 hours, respectively, after which they stop and become round-shaped cysts. The first generation of zoospores

is faster and is more sensitive to the chemotactic signals generated by the host, compared to the following two generations (Islam, 2010).

1.3.3 *A. cochlioides*-sugar beet interactions

A. cochlioides can attack sugar beet plants at any stage of their life cycle, resulting in two distinct diseases: seedling damping-off, occurring 2-5 weeks after emergence and chronic root rot on adult plants (Papavizas and Ayers, 1974). The infection is favored by wet and warm (20-28 °C) soil conditions and the disease is prevalent in acidic soils, with low calcium concentrations. Infected seedlings display threadlike roots and hypocotyls, characterized by dark and water-soaked lesions (Fig. 5A). Under optimal weather conditions, the infection can destroy entire sugar beet fields. If the soil dries up, seedlings can survive but develop malformed adult roots, with reduced sugar yield and high levels of impurities (Windels, 2000). The chronic disease occurs on older plants, from late June until the harvesting season in mid-September till November, under warm and rainy weather conditions (Olsson et al. 2010). Infected plants are characterized by scabby, irregular roots displaying a dark brown discoloration (Fig. 5B). In a similar manner to the acute phase, if weather conditions become unfavorable for the pathogen, the plants can recover and develop a relatively healthy crop; however, plants may still present symptoms of previous infections such as root distortion and scarring. In severe cases, the pathogen can destroy the taproot leaving only the crown but still maintaining a healthy-looking top (Harveson, 2013).

Influence of *A. cochlioides* tap root infections has been studied also on sugar beet leaves. The efficiency of the photosynthetic apparatus and the photosystem II is reduced in young leaves together with the inhibition of the rate of net photosynthesis, while chlorophyll degradation occurs mainly in older leaves when the natural senescence processes has initiated (Chołuj and Moliszewska, 2012).

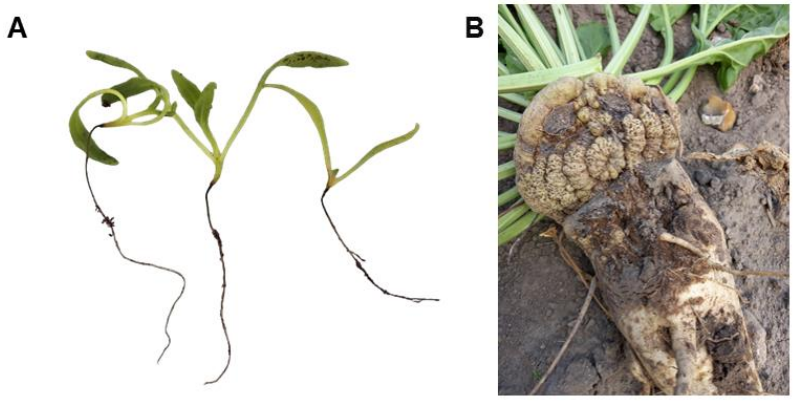

Figure 5. Sugar beet plants infected with *A. cochlioides*. A) Damping-off symptoms on sugar beet seedlings and B) *A. cochlioides* chronic root rot infection on a sugar beet root. Photos: V. Rossi

1.4 Integrated pest management (IPM)

Integrated Pest Management (IPM) is the model crop protection strategy, adopted in the late 1950s. It consists of the implementation of appropriate disease control measures, by minimizing the reliance on chemical control options, to promote the growth of healthy crops with the least possible damage to the agro-ecosystem (FAO, 2020). Undesirable side effects of an unrestrained use of chemical pesticides include the development of target pest resistance, secondary pest outbreaks and environmental pollution. The principle of IPM is, therefore, to guide growers in using a combination of different control strategies that are appropriate for their specific situations, in order to keep the chemical treatments at minimum levels. Biological control, habitat manipulation, mechanical and physical controls and resistant varieties are some of the management strategies laid down in IPM programs.

1.4.1 Management and control of *A. cochlioides* in sugar beet

Chemical control

Chemical control is the dominant strategy to protect sugar beet seedlings against damping-off caused by *A. cochlioides*. Commercial varieties are pre-treated with hymexazol, sold under the tradename Tachigaren, which is a

synthetic fungicide used to control soil-borne diseases caused by *Pythium* and *Aphanomyces* oomycete species and *Fusarium* spp. Hymexazol is the only registered product that is effective against *A. cochlioides*. In Sweden, the standard dose used in treated seeds is 14 g/unit, while in the United States 45 g/unit are applied in standard seed pellet. Hymexazol is rapidly translocated inside the plants and has locally systemic distribution properties. Once inside the plant, hymexazol is transformed into glucosides: the O-glucoside has a toxic activity interfering with the pathogen RNA and DNA syntheses, whereas the N-glucoside enhances plant growth by stimulating lateral root hair development in seedlings (Ypema, 2003).

Despite the high efficacy of hymexazol in controlling Aphanomyces damping-off, the harmful consequences of the intensive use of pesticides have been largely debated during the last decades and alternative management strategies should be pursued to limit the application of chemical fungicides. Additionally, chemical treatment is not effective in protecting adult plants against the chronic phase of the disease caused by *A. cochlioides*.

Cultural practices

Some of the cultivation methods that can help control *A. cochlioides* disease include early planting, enhanced soil drainage, crop rotation with nonhost plants, the use of partially resistant varieties and the application of calcium carbonate in the soil to increase the pH and reduce *A. cochlioides* growth (Bresnahan et al., 2001; Brantner and Chanda, 2016).

However, the success of crop rotation and soil amendment to combat *A. cochlioides* is limited due to the persistence of the oospores in the soil. Moreover, in highly infested fields and under optimal weather conditions for disease development, these measures are inadequate to secure high yields.

Host resistance

The identification of resistance sources and the development of hybrids with high levels of resistance remain the first and foremost solution to the problem to sustain agronomic practices. In these genotypes, disease incidence is limited, and the damage caused by the pathogen is less severe. Screening programs to identify resistance to *A. cochlioides* have been performed since the 1940s (Panella, 2005). However, the first quantitative trait locus (QTL) responsible for the resistance to the chronic root rot in sugar beet was described only in 2009 and it was found on chromosome III by

combining AFLP markers with high-efficiency genome scanning (HEGS) (Taguchi et al., 2009). The segregation of this QTL in a segregating population fitted the 3:1 ratio of monogenic inheritance in greenhouse conditions, suggesting that the trait is governed by a single, dominant gene (Taguchi et al., 2010). Despite explaining a major portion of the resistance to *A. cochlioides* in the field, this region did not confer full resistance. Moreover, additional information concerning the genetic background of resistance to *A. cochlioides* has not been provided since then. Thus, new resistance sources to the pathogen remain urgently needed.

Biological treatment

Fungi from the genus *Trichoderma* form intimate association with plant roots and are characterized by a direct or indirect biocontrol potential against a wide range of soilborne pathogens. In sugar beet, *T. harzianum, T. viridie,* and *T. flavus* are used for the management of root rot diseases caused by *R. solani* and *Sclerotium rolfsii*. Within the oomycetes class, *Pythium oligandrum* is also characterized by biocontrol properties. *T. afroharzianum,* and *P. oligandrum* were shown to have potential in promoting plant growth and inducing host systemic resistance in specific sugar beet genotypes (Schmidt et al., 2020; Takenaka et al., 2006). Due to their biostimulant and mycoparasitic properties, these microorganisms seem to be good candidate in controlling sugar beet plants against soilborne pathogens such as *A. cochlioides*. An alternative strategy of biological control is mycofumigation, which refers to the application of antimicrobial compounds produced by fungi. Mycofumigation by using the endophytic fungi *Muscodor albusitalic* and *M. roseus* on sugar beet, has resulted in reduced disease severity caused by *R. solani*, *P. ultimum* and *A. cochlioides* (Stinson et al., 2003).

Moreover, the microbiome of sugar beet was found to carry antagonists with the potential of preventing pathogens infection. The antagonistic potential was shown to be dependent on the location, the plant developmental stage, as well as the microenvironment and antagonists against *A. cochlioides* mostly belonged to the fungal communities (Zachow et al., 2008).

1.5 Molecular methods in plant breeding

1.5.1 Marker-assisted breeding

Many agronomically important traits such as yield and disease resistance, are defined as quantitative traits since they are controlled by many different genes with a small and cumulative effect on the phenotype. The selection of quantitative traits can be indirectly achieved by using molecular markers linked to the genes responsible for a trait, through marker-assisted selection (MAS).

QTL linkage analysis is a robust tool to detect genetic markers associated with the variation of quantitative traits and their candidate genes. It is based on the observation that genes occupying close positions on the same chromosome are linked together and are frequently inherited as a single unit (Pulst, 1999). By calculating the recombination frequencies between genes or molecular markers, it is possible to estimate their positions and relative distances. The QTL analysis allows researchers to link a trait of interest to specific regions of the chromosome and to identify number and positions of genes associated with a certain phenotype (Miles and Wayne, 2008). Single nucleotide polymorphisms (SNPs), single sequence repeats (SSR), restriction fragment length polymorphisms (RFLPs) and transposable elements (TEs) are some of the most commonly used molecular markers (Miles and Wayne, 2008). In order to perform QTL analysis, two parental lines, showing opposite phenotype for a target trait, are crossed to generate an heterozygous F_1 progeny. These individuals are then crossed following different crossing schemes and the lines of the population obtained are evaluated for their phenotypes and genotypes (Darvasi, 1998; Miles and Wayne, 2008). Markers that are genetically linked to QTLs associated to the trait of interest, will segregate more frequently with the target trait.

Linkage analysis was the predominant genetic mapping approach used in the second half of twentieth century. More recently, association analysis using genome-wide association studies (GWAS), became the tool of choice to associate genomic variants of a large population to complex traits (Ott et al., 2015). GWAS test differences in the allele frequency of genetic variants between individuals that are ancestrally related but show different phenotypes. The most commonly genetic variants studied in GWAS are the single-nucleotide polymorphisms, SNPs. This method generates significant associations to a target trait by testing thousands of SNPs (Uffelmann et al.,

2021). Association studies have the advantage to eliminate the need to perform experimental crosses and provide an increased resolution (Tibbs et al., 2021). Recent studies have used a combined QTL mapping and GWAS approach for the identification of QTLs associated to important agronomic traits such as Phytophthora blight resistance in sesame (*Sesamum indicum* L.), kernel test weight in maize (*Zea mais* L.) and soluble solids content in rapeseed (*Brassica napus* L.) shoots (Asekova et al., 2021; Zhang et al., 2020; Wu et al., 2021).

Genomic selection is another genetic approach for the selection of desirable phenotypes. In genomic selection, phenotypic and genotypic data of a training population are used to fit a prediction model. This model is then used to infer the breeding value of individuals based only on their genotypes (Crossa et al., 2017). This allows the prediction of phenotypic values of individuals without performing expensive and time-consuming field trials, which results in shorter selection cycles, accelerating the genetic improvement of crops (Kaler et al., 2022).

1.5.2 Genome sequencing

The completion of the first draft of the human genome (Yamey, 2000) was the beginning of the modern DNA sequencing era, which rapidly led to the development of high-throughput next-generation-sequencing technologies (HT-NGS). In-depth genome sequencing information provides the potential for the understanding of genetic variation of important traits.

Heavy emphasis has been put on plant genome sequencing in the last two decades. The development of NGS technologies reduced sequencing costs and led to an increased number of plant genome sequences. However, NGS technology often resulted in DNA fragments with shorter length and lower quality (Bolger et al., 2014). With the development of long-read sequencing technologies the quantity and quality of genome assemblies is improving rapidly (Belser et al., 2018). The universal reference species in plant science, *Arabidopsis thaliana*, was the first plant to be sequenced (Arabidopsis Genome Initiative, 2000) and today its genome has been nearly completely assembled (Kersey, 2019). For other major crops such as rice (*Oryza sativa* L.), maize and species of the Brassicaceae family, multiple highly contiguous assemblies are available (Zhao et al., 2018; Hufford et al., 2021; Marks et al., 2021).

Gene annotations are based on *de novo* detection and comparisons with known gene sequences from related species. Gene Ontology (GO) vocabulary is a useful tool for comparison of standardized annotations. The GO defines a universe of concepts related to gene functions, described by GO terms, and the relationship between these functions (Gene Ontology Consortium, 2017). In Arabidopsis, >94% of protein-coding genes have been annotated with a functional description from GO, with nearly 50% of the gene annotations supported by research studies. However, for less characterized species like potato (*Solanum tuberosum* L.) and sugar beet, less than 60% of the genes have been annotated from GO (Kersey, 2019).

Moreover, with the increasing availability of plant genome sequences, it is becoming clear that a single reference genome is not representative of the genomic diversity occurring within a species. The production of pangenomes, which comprise the entire set of genes from individuals of different genotypes, within the same species, provide a broader picture of gene variability and polymorphisms and is a useful tool to investigate conserved and variable genomic features as well as for the identification of novel alleles (Danilevicz et al., 2020). A combined approach using short and long reads has shown potential to overcome the drawbacks of both the short-reads (i.e., lack of spanning regions) and the long-reads (i.e., higher error rate) and resulted in the production of highly contiguous genome assemblies (Danilevicz et al., 2020).

1.5.3 The sugar beet genome

Sugar beet is a true diploid species with 2n = 18 chromosomes and is considered to have a moderate genome size. However, variability in genome size is known to occur within the species. Significant differences in genome size were observed in wild beet populations of *B. maritima* and *B. macrocarpa* in Portugal (Castro et al., 2013) and size estimates in cultivated beets ranged from 633 to 875.5 Mb (McGrath et al., 2020).

The first complete reference genome of sugar beet, namely RefBeet, has an estimated size of 731 Mb and is characterized by 252 Mb of repetitive sequences and 27.421 protein-coding genes, 63% of which are functionally annotated based on sequence homology (Dohm et al., 2014). A small number of disease resistance genes were detected in RefBeet and a single class of TIR-NB-LRR (TNL) genes, that is typical for the Amaranthaceae, was identified (Dohm et al., 2014).

Although it represents an important source for the investigation of the sugar beet genome features and evolution, RefBeet, as a fragmented genome sequence assembly, presents some limitations. Therefore, a contiguous genome assembly of the sugar beet line EL10 was recently generated, providing a broader picture of the genome organization (McGrath et al., 2020). Scaffolds of EL10 displayed high similarities to RefBeet but resulted in a higher assembly continuity (LTR Assembly Index (LAI) of 13.3 compared to 6.7 of RefBeet). More than 180.000 repetitive elements are present in the EL10 genome and a decreased gene copy number compared to annotated protein genes of other angiosperms were observed. In particular, transcription factor encoding genes are under-represented, as previously noted also in RefBeet.

The availability of a contiguous well-annotated sugar beet genome represents a valuable resource to allow us to improve our knowledge of sugar beet evolutionary history, to assess similarities between plants genes and to investigate the genetic determinants of traits of interest.

1.5.4 Transcriptomics

Molecular plant breeding has relied on molecular markers over the last two decades. More recently, modern plant breeding has taken advantage of the transcriptome analysis. While the genome provides an overview of the complete set of genetic information, the transcriptome, which is the complete set of RNA transcripts in a cell, gives information on the gene expression patterns. The study of the transcriptome can reveal useful information about the transcriptional structures of genes and changes in gene expression under specific conditions. Different methodologies have been used to quantify the transcriptome, including hybridization-based methods using microarrays and sequenced-based approaches like Sanger sequencing and tag-based methods (Wang et al., 2009). However, since the development of high-throughput sequencing technologies, RNA sequencing (RNA-seq) revolutionized the way in which transcriptomes are analyzed. This approach involves creating a library of cDNA fragments starting from RNA molecules. The fragments are then ligated to adaptors and sequenced and the obtained reads are aligned to a reference genome or assembled *de novo* (Wang et al., 2009). RNA-seq is frequently used to perform differential expression analysis to address fundamental biological questions. In plant breeding, RNA-seq is a valid tool to detect genes of interest involved in important processes such as defense

responses to biotic and abiotic stresses. The method has been widely used to gain knowledge about plant-pathogen interactions in economically important crops like tomato (*Solanum lycopersicum* L.), potato (*S. tuberosum* L.), and rice (*Oryza sativa* L.). In sugar beet, the analysis of the transcriptome through RNA-seq has been applied to investigate genes responsive to physiological processes as well as abiotic stresses. Furthermore, candidate genes involved in susceptibility and resistance to pathogens have been identified in sugar beet by RNA-seq (Table 1).

A more recent application of RNA-seq in plant breeding consists of developing SSR or SNP markers by identifying polymorphisms within the transcribed region of genes associated to a trait. The developed markers could be functional markers associated to a trait of interest, but they could be useful also as dense, genome-wide-associated markers to conduct linkage mapping and association mapping (Fu et al., 2017).

Additionally, RNA-seq is well suited for simultaneous analysis of gene expression in both the host and the pathogen and has played an important role in identifying putative virulence factors involved in the infection process. This provides a basis for understanding the molecular mechanisms underlying host-pathogen interactions.

The transcriptome sequencing has a wide range of applications and can be used to obtain useful information for plant breeding and to improve our knowledge of the molecular pathways involved in important biological processes. Other -omics technologies, including proteomics and metabolomics, could also be integrated to strengthen transcriptomic studies in analyzing biological networks associated with plant immune responses.

Table 1. Sugar beet traits for which global transcriptional responses have been analysed using RNA sequencing

Trait	Type	Reference
Vernalization and gibberellin responses	Plant Physiology	Mutasa et al., 2012
Taproot growth and sugar accumulation	Plant Physiology	Zhang et al., 2017
Neutral and alkalin salt stress responses	Abiotic stress	Geng et al., 2019; Geng et al., 2020; Zou et al., 2020

Trait	Type	Reference
Beet cyst nematode *Heterodera schachtii* resistance	Biotic stress	Ghaemi et al., 2020
Beet necrotic yellow vein virus and *Beet soil-borne mosaic virus* resistance	Biotic stress	Fernando et al., 2020
Rhizoctonia solani resistance	Biotic stress	Holmquist et al., 2021

A combination of different approaches based on breeding methods and molecular tools is a valid strategy to deepen our understanding of the complexity of plant-pathogen interactions, but also to apply the acquired knowledge on a practical level, to make sure that farmers necessities and agriculture are sustained by our research. In this thesis, we aimed to incorporate research studies on the relationship between sugar beet and *A. cochlioides* in an industry context. Therefore, the purpose of this dissertation was to answer both fundamental questions in biology, related to sugar beet responses to *A. cochlioides*, and at the same time to provide applied knowledge that could be used by breeding companies to support *A. cochlioides* disease management.

2. Aims of the study

The aim of this study was to investigate the nature of the interaction between the sugar crop *Beta vulgaris* (sugar beet), and the pathogenic oomycete *Aphanomyces cochlioides*. Severe yield losses are reported in fields infested with *A. cochlioides* and agricultural practices are inadequate to prevent the occurrence of the disease caused by this pathogen. Chemical treatments to protect plants at the seedling stage are available but are not effective on adult plants and due to their impact on the environment, their use might be banned in the future. Resistant cultivars represent an effective and sustainable way to manage the disease. Little is known about the genetic basis of the host resistance to *A. cochlioides* and the data presented in this thesis could serve as basis for future work to understand the molecular mechanisms that govern these host-pathogen interactions and to support breeding programs in developing resistant varieties with high sugar yield.

The specific objectives of the research manuscripts included in this thesis were:

- To develop a DNA-based method to measure the levels of resistance to *A. cochlioides* in sugar beet genotypes and to systematically describe the progress of the infection in partially resistant and susceptible sugar beet material (Manuscript I).

- To analyse the transcriptome profile of partially resistant and susceptible sugar beet genotypes during the early phases of infection with *A. cochlioides* isolates from major sugar beet cultivating regions, with the aim being to identify candidate genes involved in susceptibility and defence responses (Manuscript II).

- To identify quantitative trait loci (QTLs) associated with *A. cochlioides* resistance and to investigate the relationship between the genetic basis of the resistance to damping-off and to chronic root rot (Manuscript III).

3. Materials and Methods

3.1 Plant material and *A. cochlioides* isolates

In this study, several sugar beet breeding lines with varying resistance and susceptibility levels to *A. cochlioides* were used. The different breeding lines were suggested by the breeders according to their previous evaluations of the plants in the field. After phenotypic evaluation of infected plant material under controlled conditions, 10 genotypes were selected and used in different experiments (Table 2).

Table 2. Description of levels of resistance to *A. cochlioides* damping-off and chronic root rot of sugar beet lines used for the experiments in different manuscripts

Sugar beet line	Resistance to damping-off	Resistance to chronic root rot	Manuscript
G1	Low	Low	II
G2	Medium	Medium	I
G6	High	High	II, III
G8	Medium	High	I
G12	High	High	I
G17	Low	Low	I, II
G18	Low	Medium	I
G19	Low	Medium	I
G20	Low	Low	I
G21	Low	Medium	III

Three *A. cochlioides* isolates collected from different sugar beet cultivating regions were used to inoculate plants: Arhill_2012, from Sweden, Hokkaido_01, from Japan and USA_01.7.6 from U.S. All *A. cochlioides*

isolates were single-spore isolated and cultivated in Corn Meal Agar (CMA) at 21°C in the dark, to obtain single strain cultures.

3.2 Infection assays

In order to investigate the phenotypic and transcriptomic responses to *A. cochlioides* of the sugar beet genotypes evaluated in this study and to gain information on *A. cochlioides*-sugar beet interactions, plants were inoculated in climate chambers under controlled conditions. Warm temperatures (22°C) and high humidity (95% RH) were set to ensure optimal conditions for the disease development, with 16h light and 8h dark and 400 ppm CO_2.

In this research, the infection caused by *A. cochlioides* was investigated in both seedlings and older plants, depending on the biological questions we wanted to address and, therefore, different infection assays were used according to the aim of the study.

3.2.1 Phenotypic evaluation of the disease resistance

To perform pathogenicity tests for phenotyping, surface-disinfected seeds were germinated in plastic trays (21 x 35 x 6 cm) (Manuscript I) or Ø= 12 cm pots (Manuscript III) containing steamed-sterilized soil. Inoculation was performed by watering the soil with the zoospore solution produced by *A. cochlioides* mycelium. For the evaluation of damping-off resistance (Manuscript I and III), seedlings were inoculated 10 days after emergence, while 6-week-old plants were inoculated to induce chronic root rot disease (Manuscript III). Plants were inspected visually, and symptoms scored 3 weeks after inoculation, to assess damping-off resistance, and 5 weeks after inoculation for chronic root rot resistance. The scoring was performed using 5 scores in a range from 1 to 9 (1= dead plant, 3= severe infection, 5= medium infection, 7= mild infection, 9= healthy plant) (Fig. 6). Disease Indexes (DI) were expressed as the average scores of the replicates belonging to the same genotype. 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 aov function, to test for significance in DI between different genotypes x *A. cochlioides* isolates interactions.

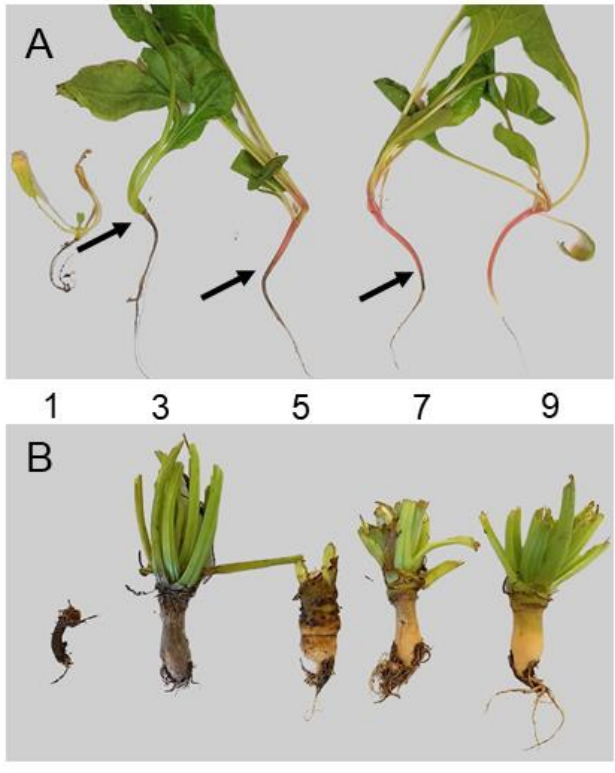

Figure 6. A) 1-9 scoring scale used to evaluate *A. cochlioides* damping-off and chronic root rot symptoms in sugar beet. A) Damping-off symptoms: 1= dead plant; 3 = 100% necrotic hypocotyl; 5= 50% necrotic hypocotyl; 7= 25% necrotic hypocotyl; 9 = no necrotic lesions and B) Chronic root rot symptoms: 1 = dead plant; 3 = severe infection, root with necrotic lesions; 5 = medium infection, root with irregular shape and brow discoloration, 7 = minor infection; root with small irregularities and brownish spots; 9 = no infection, root with no signs of infection

3.2.2 *In planta* infection assay

To enable molecular studies on the host-pathogen interaction, the *in planta* infection system previously described in pea (*Pisum sativum* L.) (Hosseini et al., 2012) was adapted to sugar beet. 8-day-old seedlings and 6-week-old plants, cultivated in steamed-sterilized soil, were harvested, washed and placed on 10 µl- and 10 ml-pipette boxes respectively, to submerged the roots in water for 2 days. After 2 days, the racks carrying the plants were transferred to clean pipette boxes, filled with zoospore

suspension to incubate the roots for 2 h, followed by incubation in water in the climate chamber.

In Manuscript I the infection assay described above was performed on seedlings to collect samples for DNA extraction, to perform qPCR and for confocal microscopy. Seedlings were collected at 0h, 4h, 24h, 2 days, 4 days and 8 days after inoculation, lyophilized and used for DNA extraction or directly fixed in Formaldehyde Alcohol Acetic Acid (FAA) fixative to prepare samples for confocal microscopy. Sugar beet and *A. cochlioides* biomass was amplified and quantified by qPCR. *A. cochlioides* DNA (target sequence: ITS region of the rRNA gene) was normalized to the sugar beet DNA (reference sequence: 11S globulin storage protein 2 gene) and *A. cochlioides* biomass was expressed as the pathogen/host DNA ratio. The variance between pathogen/host DNA ratios was calculated based on genotype-timepoint interaction and genotype-*A. cochlioides* isolates interaction in R, using the aov function.

In Manuscript II, 6-week-old plants were inoculated to perform the analysis of the transcriptome, with the aim to identify candidate genes involved in the defence responses during the initial stages of the infection in mature plants. Inoculated plants were collected at 6h and 30h after inoculation and roots were ground in liquid nitrogen and used for RNA extraction.

3.3 Confocal microscopy

In order to examine the differences in the progression of the infection inside partially resistant and susceptible sugar beet genotypes, we used confocal microscopy (Manuscript I). Confocal microscopy is a technique that uses optical imaging to create a virtual plane within the tissue observed. A small spot inside the tissue is illuminated by a beam of incoming light, and unlike conventional microscopy, only the light emitting from the desire focal spot is projected through a small pinhole, while out-of-focus signals are blocked and eliminated (Nwaneshiudu et al., 2012). This results in a less blurry image with a better contrast.

Infected sugar beet seedlings were first subjected to a fixation process, using a FAA solution to maintain intact the cellular structures, followed by dehydration in an ethanol series to remove water. Samples were embedded in agar and transversally cut into 100 μm slices. Wheat germ agglutinin

(WGA) coupled to Alexa Fluor 488 conjugate was used to stain the *A. cochlioides* mycelium inside the infected tissue. WGA is a carbohydrate-binding lectin with high affinity with sialic acid and N-acetyl-D-glucosamine (GlcNAc), which is mainly found in chitin and in the cell membrane of yeast and bacteria. Even though oomycetes lack chitin in their cell walls, GlcNAc constitutes ~10% of *Aphanomyces* cell walls, corresponding to non-crystalline chitosaccharides associated with glucans, rather than chitin (Badreddine et al., 2008).

The spatial distribution of *A. cochlioides* inside the host was examined nearby the root, in the central part of the hypocotyl and in the area below the cotyledons during the first 8 days after inoculation.

3.4 Transcriptome analysis

To gain insight into the sugar beet responses triggered by *A. cochlioides* infection, a large set of transcript data was generated by inoculating two partially resistant and two susceptible sugar beet genotypes with three different *A. cochlioides* isolates, singularly (Manuscript II). mRNA was isolated from the total RNA extracted from infected and non-infected roots and was retro-transcribed into cDNA. After fragmentation and adaptor ligation, cDNA libraries were sequenced in the Illumina platform. A standard pipeline consisting of a quality assessment of the generated reads, followed by a trimming and filtering of poor quality reads, was performed on the raw data obtained. High quality reads were then aligned onto the sugar beet reference genome EL10 and quantified. To identify the up- and down-regulated genes induced by the pathogen, a differential expression analysis was conducted between infected genotypes and their respective non-infected samples, consisting of plants inoculated with water, using the DESeq2 package (Love et al., 2014) in R. Genes with a false discovery rate (FDR) value < 0.05 and a log2 fold change > 1 were considered to be differentially expressed. To obtained functional annotations, EL10 transcripts were blasted against the *A. thaliana* genome, using BLASTp. GO enrichment analysis was conducted in ShinyGO v0.75 (Ge et al., 2020) and significance was tested by default method at $p < 0.05$, after adjustment for false discovery rate.

3.5 QTL analysis

In manuscript III, a segregating population was created by crossing sugar beet breeding lines of opposite phenotypes for *A. cochlioides* resistance, to find QTLs associated with the trait of interest. About 300 individual F_2 lines, obtained by crossing F_1 plants with each other, were self-crossed for two generations to obtain an S_2 progeny that was screened for resistance to damping-off and chronic root rot. DNA extracted from leaves was fingerprinted using a 22K Affymetrix SNP array for sugar beet, developed at DLF Beet Seed. Molecular markers with the same genetic profile in the two parental lines were removed, together with markers that segregated differently from linked markers. A genetic linkage map was created in R using the R/qtl2 package with the est_map function. A total of 230 markers were used to carry out the QTL analysis. The average DI of all evaluated lines were combined with their genetic profile, to detect number and position of QTLs linked to *A. cochlioides* resistance. QTLs with a LOD (logarithm of the odds) score > 3 were considered significant.

4. Results and Discussion

4.1 *A. cochlioides* infection in partially resistant and susceptible sugar beet plants

The assessment of resistance levels of sugar beet plants with different genetic backgrounds is the first important step in a breeding program for disease resistance. Monitoring symptoms development in infected plants is the basic procedure to detect plants with higher resistance levels. However, phenotypic evaluation based on a visual estimation of the symptoms requires expertise, time and space and does not provide an exact measure of the resistance. The host efficiency in reducing the damage caused by the pathogen can be dictated by resistance, which is the ability of the host to limit the pathogen propagation, and tolerance, that is the plant´s ability to reduce the effect of the infection regardless of the level of pathogen multiplication (Pagán and García-Arenal, 2018). In this study, we investigated the use of a DNA-based method as a potential tool to identify sugar beet genotypes with an ability to combat pathogen proliferation. A range of sugar beet lines were first evaluated for damping-off symptoms under controlled conditions. *A. cochlioides* isolates from different geographical origins were used to determine differences in their virulence against the sugar beet genotypes tested. Most of the genotypes were highly susceptible to the different *A. cochlioides* isolates, showing disease indexes < 3 and a high percentage of dead plants, during the first 2 weeks after inoculation. The sugar beet genotype G12 was the only genotype displaying a high ability to withstand the infection and develop relatively healthy plants with limited symptoms ($DI > 6$, $p < 0.001$). All *A. cochlioides* isolates used to inoculate the plants were able to cause disease and showed the same level of virulence against

the sugar beet genotypes tested, with the exception of genotype G8 that was significantly less susceptible to the isolate USA_01.7.6., compared to Arhill_2012 and Hokkaido_01 ($p < 0.001$) (Fig. 7).

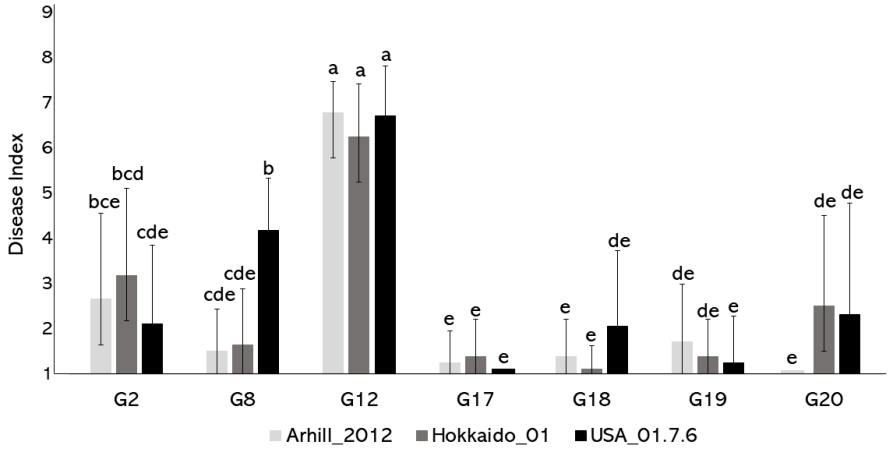

Figure 7. Disease Indexes (DIs) of sugar beet breeding lines in response to the *A. cochlioides* isolates Arhill_2012 from Sweden, Hokkaido_01 from Japan and USA_01.7.6 from U.S.A

The main objective of this study was to develop a qPCR method to identify genotypes with high resistance levels to *A. cochlioides*. The inoculation assay was designed on sugar beet seedlings to enable host-pathogen interactions to be studied in the lab, and so that we could synchronize the start of the infection and allowed a successful infection of the plants. After comparing the pathogen biomass in three sugar beet genotypes (G8, G12 and G17), with different levels of resistance to *A. cochlioides*, at 0, 2, 4 and 8 dpi, the results revealed that the pathogen DNA content was significantly lower in the partially resistant line G12 compared to the highly susceptible line G17 at 4 dpi, when inoculated with Hokkaido_01 ($p < 0.001$) and at 8 dpi, when inoculated with Arhill_2012 and USA_01.7.6 ($p < 0.001$). Furthermore, the amount of *A. cochlioides* DNA was maintained at low levels during the evaluated time in G12, indicating that the pathogen growth is inhibited in this genotype, while an increasing biomass of the pathogen was observed in G17 over time (Manuscript I, Fig. 1). Therefore, we concluded that the qPCR assay is a

valid method to separate partially resistant material from highly susceptible genotypes, between 4 and 8 dpi. Significant differences in the pathogen biomass between G12 and G8 were not observed, indicating that the qPCR assay described here was not sensitive enough to detect variation between partial resistance and intermediate susceptibility. However, these two genotypes displayed a different pattern of infection, with the pathogen/host DNA ratio showing the trend that it increased over time in genotype G8 (Manuscript I, Fig. 1). A repeated test with more replicates might be able to separate these two genotypes.

Genotype G8, that showed a degree of susceptibility to Arhill_2012 and Hokkaido_01 (DI < 2) with a plant loss > 50%, displayed a higher level of resistance against the *A. cochlioides* isolate USA_01.7.6, with a DI > 4 ($p < 0.001$) and no dead plants. Nonetheless, when comparing the pathogen biomass in G8 inoculated with the different *A. cochlioides* isolates, the analysis of variance of pathogen/host DNA ratio did not reveal any significant interaction between genotype and *A. cochlioides* isolate. However, since USA_01.7.6 had a lower zoospore concentration compared to the other two isolates in this experiment, it might be misleading to compare the actual ratios, but we can still observe the pattern of infection. For both Arhill_2012 and Hokkaido_01, G8 increases rapidly in ratio between 4 and 8 dpi, while for USA_01.7.6 only a minor increase is observed in time (Manuscript I, Fig. 1). This pattern indicates that G8 could manage the infection with USA_01.7.6 better than the Swedish or the Japanese isolates, but further investigations are needed. To date, genetic diversity in *A. cochlioides* isolates has not been investigated, but it cannot be excluded that isolates originating from different regions underwent a genetic diversification, e.g., in their effector gene complement or expression. Phylogenetic analysis revealed three separate genetic groups within European *A. euteiches* populations and different strains displayed varying levels of virulence, with the differences partly correlated with the geographic origin (Kälin et al., 2022). *A. euteiches* isolates from the United States are also highly variable in their genotypes and phenotypes (Malvick and Percich, 1998; Grünwald and Hoheisel, 2006) and 4 different virulent groups have been identified (Malvick and Percich, 1999). Other studies have been focused on the identification and characterization of different *A. euteiches* populations from France, the United States, Canada and New Zealand and the resistance of different pea genotypes was evaluated and used to develop

resistant varieties (Wicker and Rouxel, 2001, Wicker et al., 2003). Differences in virulence are common between *A. euteiches* strains, therefore genetic differentiation in *A. cochlioides* populations should also be investigated to understand the genetic structure and virulence variation in this *Aphanomyces* species.

In addition, the *A. cochlioides* infection process was monitored in the partially resistant line G12 and in the susceptible line G17, with the use of confocal microscopy. The pathogen mycelium was highlighted with the use of WGA conjugated with the green-fluorescent dye Alexa Flour 488. WGA has been largely used to observe *A. euteiches* in the model legume *Medicago truncatula*. In this study, we demonstrated that this lectin can be also used to successfully bind the cell wall of *A. cochlioides* (Fig. 8).

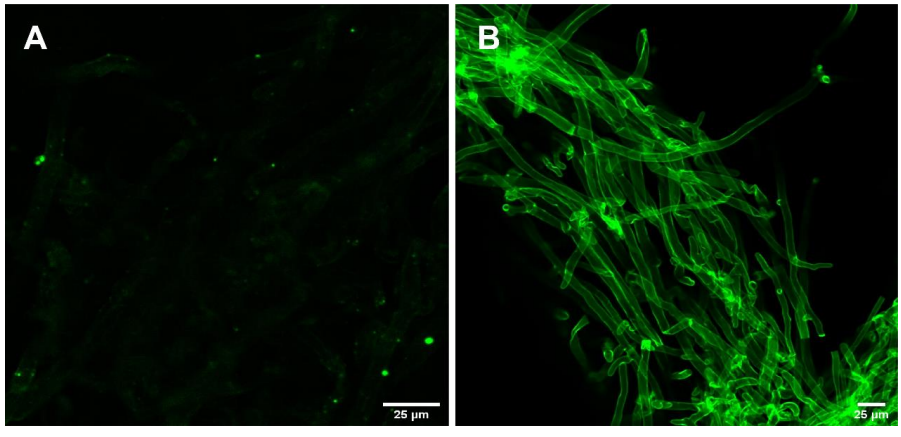

Figure 8. A) *A. cochlioides* mycelium without stain and B) *A. cochlioides* mycelium stained with Wheat Germ Agglutinin, Alexa Fluor 488 conjugate, observed using confocal scanning laser microscopy

During the first two days after exposure to *A. cochlioides* zoospores, mycelium had grown in the intercellular spaces of both the partially resistant and susceptible lines, nearby the root tip (Manuscript I, Fig. 3). The responses to *A. cochlioides* infection between the partially resistant line and the susceptible line became discernible at 4 dpi, when *A. cochlioides* hyphae had invaded the hypocotyl and colonized the apoplast in the cortex of the susceptible line, while traces of the pathogen were not detected in the same area of the partially resistant genotype. At 8 dpi, the whole tissue was invaded by *A. cochlioides* hyphae in G17, while the presence of the pathogen

was confined in the root of G12 (Fig. 9A and 9B). These results confirmed the low pathogen/host ratio detected by the qPCR experiment in G12 compared to the increasing amount of pathogen biomass in the susceptible line G17 and showed how the rapidity of the defense responses in partially resistant genotypes is determinant in preventing the disease development. *A. cochlioides* mycelium was detected also on the surface of the root of both genotypes at 8 dpi (Fig. 9C and 9D).

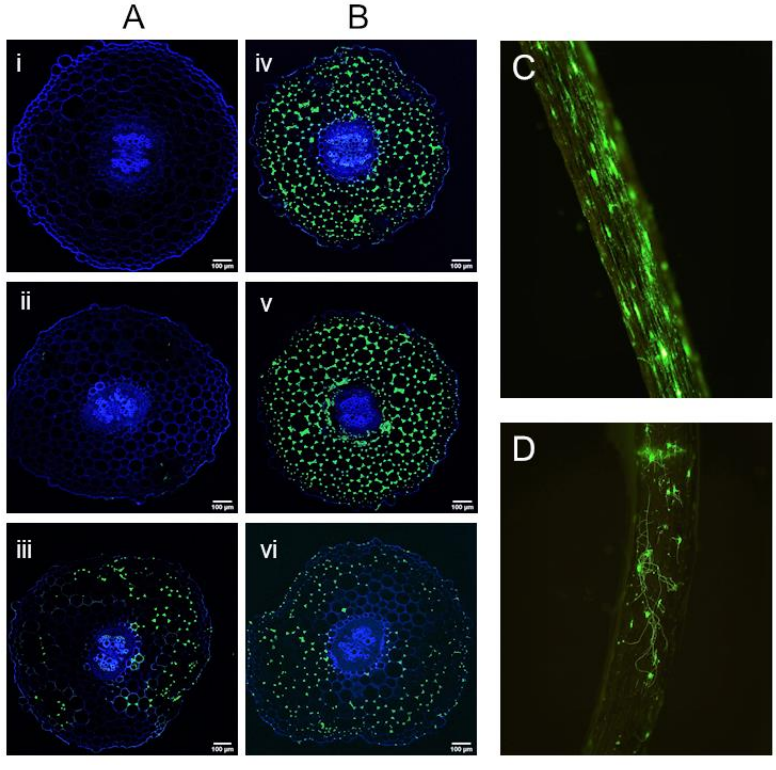

Figure 9. Infected sugar beet at 8 dpi with *A. cochlioides* zoospores, observed using confocal laser scanning microscopy. A) *A. cochlioides* hyphae were not observed in i) the upper part and in ii) the middle part of the hypocotyl but were present iii) nearby the root in the partially resistant genotype G12. *A. cochlioides* colonization was visible in iv) the upper zone, v) the middle zone and vi) nearby the root of the susceptible genotype G17. *A. cochlioides* mycelium growing on the root surface of C) the partially resistant genotype G12 and D) the susceptible genotype G17 at 8 dpi

4.2 Transcriptomic responses of partially resistant and susceptible sugar beet lines to *A. cochlioides* infection

Understanding the molecular basis of interactions between sugar beet and *A. cochlioides* would lay the foundation for improving resistance against this pathogen. In manuscript II, we analysed the transcriptomic profile of sugar beet during the initial events of the infection, with the ultimate goal being identify genes that play a major role in the defence responses. In addition, the transcriptome analysis of the host/pathogen interaction generated fundamental knowledge of the biological processes and molecular pathways that are triggered in the host upon infection with *A. cochlioides*.

Considering the variable nature of the resistance to *A. cochlioides* in the breeding lines utilized in our study, two partially resistant lines, namely G6 and G12 and two susceptible lines, called G1 and G17, genetically unrelated to each other, were selected to investigate different responses induced by this oomycete. Moreover, since effective treatments are currently available to prevent Aphanomyces damping-off, we decided to study the defence responses in older (6-week-old) plants, with the aim being to find specific genes able to control the disease in later stages, when the fungicide treatment is no longer effective on plants. Sugar beet lines were inoculated with three *A. cochlioides* isolates, individually.

In total, 1987, 2013 and 2292 genes were differentially expressed in the two susceptible genotypes G1 and G17 and in the partially resistant line G12, respectively, compared to their respective non-inoculated samples, while only 547 genes were up- and down- regulated in the partially resistant genotype G6 compared to the non-infected plants. Gene Ontology (GO) term analysis revealed common changes in the two susceptible genotypes in genes associated to the photosynthetic process, at 30 hpi. Biological processes enriched by the set of differentially expressed genes shared between these two lines but not expressed in the partially resistant genotypes included "Photosynthetic electron transport chain", "Protein-chromophore linkage", "Photosynthesis, light reaction" and glucose metabolic processes. Changes in photosynthesis and in the following glucose metabolic process have been observed in other pathosystems and might indicate an energy preservation strategy to support the induction of defense responses against pathogen invasion (Bilgin et al., 2010). Reactive oxygen species (ROS) metabolic processes were the most represented biological processes in the partially resistant line G6, at 30 hpi (Manuscript II, Fig. 4). Hydrogen peroxide

(H_2O_2), in particular, may play an important role in the response to the pathogen attack. H_2O_2 is one of the main ROS detected in plant/pathogen interactions. It can be generated in a first phase, after a compatible or incompatible interaction, directly after exposure to the pathogen or in a second, prolonged phase, in an incompatible interaction to induce the hypersensitive response. It can also directly inhibit pathogen growth and reduce pathogen viability (Kuźniak and Urbanek, 2000). In addition, biological processes such as cellular detoxification and response to toxic substance underwent significant changes in this genotype, presumably as a consequence of the high accumulation of H_2O_2. H_2O_2 metabolism was also enriched in genotype G12 at the earliest time point (6 hpi), while an enrichment in the cell wall organization process was observed at 30 hpi (Manuscript II, Fig. 4), suggesting that changes in the cell wall status could work as a trigger for defense responses.

Additionally, some candidate genes uniquely expressed in one or both partially resistant genotypes, but not in the susceptible lines, were identified as potential genes playing a role in sugar beet immune responses (Table 3). Functional studies on these genes could confirm their role in the defense mechanisms triggered by *A. cochlioides*.

Table 3. List of up-regulated genes selected as candidate defense genes in response to *Aphanomyces cochlioides*

Transcript ID	Log_2 fold change	Genotype	Time-point	Name	Description
EL10Ac7g18219	1.62, 1.83	G6, G12	30 hpi	Probable disease	CC-NBS-LRR
EL10Ac7g18203	5.70	G6	30 hpi	resistance protein At1g15890	defense response to other organism
EL10Ac5g12824	2.34, 2.19	G6, G12	6, 30 hpi	Mitogen-activated protein kinase 4 MAPK4	Signal transduction
EL10Ac4g07957	6.79	G6	30 hpi	Protein NDR1	Required for resistance conferred by multiple R genes

EL10Ac4g09732	2.23	G6	30 hpi	Peroxidase 47	Response to stresses such as wounding, pathogen attack and oxidative stress
EL10Ac5g10693	2.01	G6	30 hpi	Probable LRR receptor-like serine/threonine -protein kinase	Kinase activity
EL10Ac6g13326	3.75	G6	30 hpi	Disease resistance (R) protein RPM1	Triggers Hypersensitive Response (HR)
EL10Ac3g06517	1.41	G6	6 hpi	Receptor-like cytosolic serine/threonine -protein kinase RBK2	Kinase activity
EL10Ac4g08863	4.40	G12	6 hpi	Putative disease resistance RPP13-like protein 1	Involved in plant defense
EL10Ac2g02793	1.68	G12	30 hpi		
EL10Ac2g02511	2.13	G12	6 hpi	Disease resistance protein RGA2	Involved in plant defense
EL10Ac5g11093	2.50		6 hpi		
	2.58		30 hpi		
EL10Ac2g04100	2.03	G12	30 hpi	Putative disease resistance protein RGA3	Triggers a defense system that restricts the pathogen growth
EL10Ac2g04094	7.79				
EL10Ac2g04098	2.37	G12	30 hpi	Putative disease resistance protein At3g14460	Involved in plant defense
EL10Ac2g03197	1.07	G12	30 hpi	MLP-like protein 43	Involved in defense response
EL10Ac2g04536	1.55	G12	30 hpi	Protein NtpR	Pathogenesis-related protein

EL10Ac4g07858	3.67	G12	30 hpi	Defensin-like protein AX1	Antibiotic, antimicrobial, fungicide activity
EL10Ac6g15268	1.06	G12	30 hpi	Protein MKS1	Regulator of plant defense response

4.3 QTL analysis of resistance to *A. cochlioides* damping-off and chronic root rot

One of the main questions addressed in this project was the position of the genetic resistance to *A. cochlioides* in the sugar beet genome, in order to use the associated molecular markers in breeding programs. To detect Quantitative Trait Loci (QTLs) responsible for *A. cochlioides* resistance, a segregating mapping population, consisting of ~300 S_2 inbred lines, was assessed for damping-off and chronic root rot resistance and profiled for molecular markers. Resistance to damping-off and chronic root rot did not segregate as a single, dominant gene but displayed a quantitative nature. To dissect this quantitative trait, we calculated the linkage map of molecular markers in our segregating population and performed a likelihood ratio statistic test in the different linkage map positions, known as a LOD test. Regions on the genome showing significant values in this statistical analysis, should contain a QTL. A LOD score of 3 or higher generally means that the marker and the functional gene are physically close enough to result in a significant correlation between the marker genotype and the phenotype. The QTL analysis of the dataset from damping-off resistance tests, revealed the presence of one major QTL with a LOD score > 10 and an effect of 0.49 on the disease index (on the disease scale 1 – 9) in the homozygous resistant form RR, an effect of -0.06 in the heterozygous form RS and an effect of -0.43 in the homozygous susceptible form SS, corresponding to additive and dominance effects of 0.46 and -0.09, respectively. Additionally, two QTLs with a significant but smaller effect on the disease index were identified (LOD > 3, a difference in effect of 0.48 and 0.49, respectively, on the disease index between the RR and SS homozygous forms) (Manuscript III, Table 3). The same QTLs were not detected from the dataset from the chronic root rot test and these results were supported by a non-significant correlation between damping-off and chronic root rot disease indexes (Manuscript III, Fig. 1). Other regions associated with the resistance in older plants were not

identified. This might indicate that the resistance to chronic root rot disease is controlled by many genes, each with a small effect. In addition, a limiting factor in the analysis was the difficulty in phenotyping infected roots. While damping-off disease produces distinct symptoms, causing dark and threadlike hypocotyls, chronic root rot does not always result in necrotic lesions but can appear as malformed or undersized roots, making it difficult to accurately quantify disease severity. The quantification of the infection in seedlings using qPCR has provided promising results (Manuscript I). Therefore, further experiments to adapt and optimize this method on older roots are needed to improve the evaluation of host resistance to the pathogen.

Furthermore, it should be noted that the statistical power of the analysis performed was decreased by using a single plant from each S_2 line for the genotyping. A single S_2 genotyped individual is homozygous for only half of the markers that are segregating in the family, and since 25% of the markers are expected to segregate in each S_2 line, this will introduce an error rate of 12.5%. Therefore, genotyping the S_1 plant that generated the S_2 seeds or a bulk of the S_2 generation would have been more correct in order to increase the precision of the analysis. Nevertheless, results from this study suggest that the resistance to *A. cochlioides* is controlled by diverse genes in different stages of sugar beet life. The identification of a QTL with a marked effect on damping-off resistance is an important step to find a solution that would complement the use of chemical treatment and would result in a smaller impact to the environment as well as lower cost for seed production.

Further studies should be focused on the identification of genomic regions associated to *A. cochlioides* resistance also in the later stages of sugar beet growth. Moreover, the response to the infection caused by *A. cochlioides* should be investigated in the field, to confirm the effect of the identified QTLs in a natural environment, where other factors such as climate conditions, soil type and the presence of other abiotic and biotic stresses might affect the crop viability.

5. Conclusions

The oomycete *A. cochlioides* is one of the most damaging root pathogens in sugar beet production and it threatens sugar beet viability from the seedling stage until harvest. Fully resistant cultivars are not available on the market and management strategies are scarce and consist mainly of synthetic fungicide applications on seeds with a limited shelf-life. Identifying sugar beet germplasm with the ability to counteract the disease development and understanding the mechanisms implemented by the plants to fight off the infection was the key objective of our study.

In Manuscript I, we aimed to develop a quantitative method to measure the infection levels in young sugar beet plants. The qPCR-based experimental assay established here has the potential to separate partially resistant germplasm from susceptible material. Furthermore, we were able to observe the ability of the candidate partially resistant genotype to promptly stop the spread of the infection. These results were supported by the observation of the temporal and spatial pathogen growth inside partially resistant and susceptible sugar beet plants. By employing an *in planta* inoculation system to induce infection, *A. cochlioides* zoospores were attracted to the host surface and managed to penetrate the tissue nearby the root and start the colonization of the apoplast. However, hyphal growth inside the partially resistant genotype was inhibited after 2 dpi, indicating that host defence mechanisms are rapidly launched in response to pathogen invasion in this genotype. This suggests that pathogen recognition is likely to occur within a few hours of the onset of the infection.

To study the upstream signals triggered by the pathogen during the first stages of the infection process, we analysed the transcriptome responses in two different partially resistant genotypes and two susceptible genotypes

(Manuscript II). We concluded that H_2O_2 and changes in the cell wall organization might play a key role in the defence responses to *A. cochlioides* in the partially resistant genotypes, while photosynthetic processes underwent major changes in the two susceptible genotypes as a reaction to the pathogen invasion. Moreover, we identified 13 up-regulated genes in the partially resistant genotypes that are candidates for future studies.

The virulence of different *A. cochlioides* isolates was also tested on different sugar beet genotypes. No significant differences were observed in the ability to cause the disease. Only one genotype/isolate interaction showed a different pattern, suggesting the occurrence of a specific recognition (Manuscript I). Moreover, the differential expression analysis revealed only a small percentage of shared up- and down-regulated genes induced by different isolates, indicating that the different *A. cochlioides* strains might have specific and unique interaction with their hosts (Manuscript II). These results highlight the importance of exploring the genetic diversity in *A. cochlioides* populations as well as the genetic diversity within the host.

Lastly, we aimed to identify genomic regions linked to *A. cochlioides* resistance and to use the generated knowledge to improve sugar beet breeding programs (Manuscript III). The QTL analysis revealed the presence of three new QTLs conferring the host the capability to cope with the infection induced on sugar beet seedlings. Interestingly, the identified genomic regions did not correlate with the resistance to the chronic root rot, indicating that different genes are involved in the defence responses in mature roots. QTLs associated to the resistance to chronic root rot disease are yet to be identified and, therefore, are important targets for the future.

6. Future perspectives

A major challenge in modern agriculture is to fulfil the food demands of a rapidly increasing global population, whilst reducing the impact of food production on the environment to preserve the natural resources for future generations. Integrated pest management represents an effective and environmentally sensitive approach to control pest diseases. It consists of a combination of sustainable practices that together can successfully ensure a high productivity and limit the use of pesticides.

Effective control measures to *A. cochlioides* in sugar beet cultivation consist of predominantly chemical treatments and do not ensure an extended protection of the crop. Amalgamation of cultural practices, host resistance, chemical and biological strategies has been reported to effectively control soilborne pathogens. A suggested integrated disease management program to reduce the significant losses caused by *A. cochlioides* in sugar beet is illustrated in Fig. 10. Sugar beet can be grown in the same field every third year, in rotation with other crops. However, *A. cochlioides* oospores can survive in the soil for longer years, limiting the effectiveness of crop rotation. We, therefore, suggest longer intervals (e.g., 7 years) to reduce the pathogen levels in the soil. Crop rotation should be accompanied by soil management strategies, such as soil drainage and pH adjustments to create unfavourable conditions for the pathogen growth. Early interventions might include the application of chemical treatments complemented with the use of resistant varieties. In addition, the use of biocontrol agents and bio-stimulants represents a valuable supplement in both early and later interventions as greener alternative to chemical fungicides. A better understanding of the genetic processes related to plant-microbe interactions will open new prospects for the development of novel formulation to improve disease

management. Moreover, the identification and selection of sugar beet genotypes responding positively to biocontrol agents such as *Trichoderma* and *P. oligandrum* could be incorporated in sugar beet breeding, since the beneficial interactions with biocontrol agents seem to be genotype-dependent. Microorganisms present in the rhizosphere as well as endophytes play also an important role in disease management. Therefore, microbiome communities and root colonization of beneficial microbes are receiving increasing attention for the development of an efficient integrated disease management program.

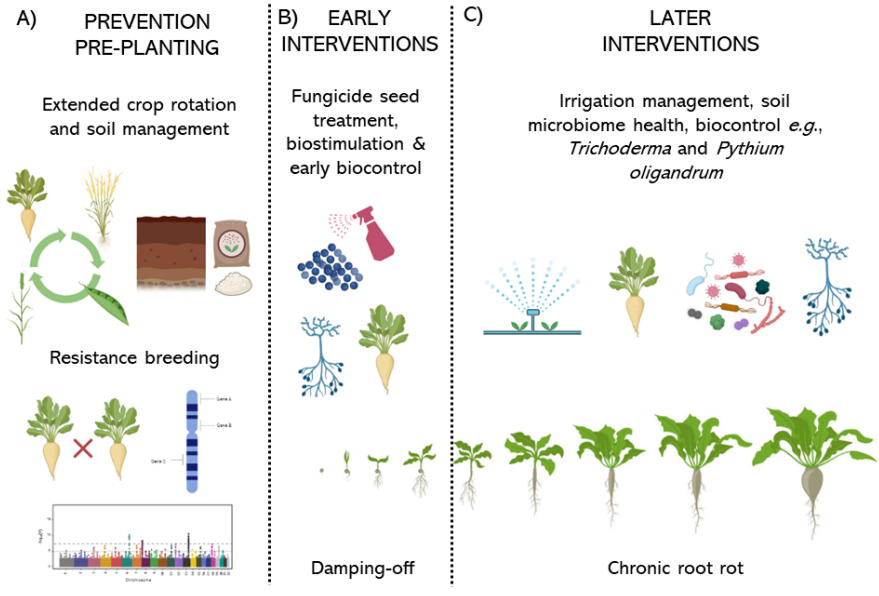

Figure 10. A suggested integrated pest management (IPM) program to control *A. cochlioides* disease in sugar beet. A) Measures to prevent the occurrence of the disease include extended crop rotation, resistance breeding and soil management, i.e., soil drainage, pH adjustment, nutrients and microbiome; B) Early interventions are fungicide seed treatment, use of biocontrol agents and bio-stimulants (e.g., *Trichoderma*) and resistant varieties and C) Late-season measures are irrigation management, resistant varieties, soil microbiome health assessment and biological control (*Trichoderma* and *P. oligandrum*). Illustration created in BioRender.com

Furthermore, to develop alternative management strategies with a reduced impact on the environment that can secure a long-lasting protection and high sugar yield, a better understanding of the host-pathogen interactions as well as the exploitation of resistant germplasm is required. The knowledge generated in this research will serve as a basis for future studies, to elucidate the mechanisms underlying sugar beet resistance to *A. cochlioides*. Genomic regions that correlate to damping-off resistance have been identified and the molecular markers linked to these regions can be used in marker assisted selection to improve the precision and efficiency of conventional plant breeding and could, hopefully, help in reducing the use of chemicals to control Aphanomyces damping-off. Nonetheless, more emphasis should be put on the characterization of the resistance to chronic root rot, since the genetic background of this trait remains unclear. QTL analysis on a new mapping population obtained using a different resistance source as a parental line could reveal loci harbouring genes that control chronic root rot resistance. Additionally, functional studies on the candidate genes identified in the transcriptome analysis will help in clarifying the role of those genes in the defence responses. The characterization of defence-related genes and their localization in QTL regions would provide a valuable resource in resistant breeding to *A. cochlioides*.

Additionally, further research should focus on studying this poorly characterized oomycete, to gain insights into the pathogenicity factors employed by the pathogen to infect its host. Functional studies by using transformation systems or genome editing technologies would help in elucidating the molecular processes involved in the host invasion. However, attempts in transforming *Aphanomyces* species, with the exception of *A. invadans*, have not been successful so far. Nevertheless, gene silencing and Crispr/Cas9 approaches have been established in other important economic oomycetes, therefore, the development of a transformation system in *A. cochlioides* should not be excluded but rather pursued. A better understanding of the effector arsenal carried by *A. cochlioides* is needed to shed light on the infection process employed by the pathogen to invade the host and perturb plant defence circuitry. Extensive studies have been conducted on other oomycete species, notably those of the genus *Phytophthora*, providing broader knowledge on their pathogenicity and evolution that could serve as base to explore *A. cochlioides* mechanisms of infection and host adaptation. With the first draft genome of *A. cochlioides*

available, comparative genomics with other closely related species, e.g., *A. euteiches*, could answer important questions about the genome content of *A. cochlioides* for the identification of genes involved in the evolution of pathogenesis and survival mechanisms of this oomycete. Moreover, the identification of effector proteins secreted by *A. cochlioides* could be used to accelerate the identification, functional profiling, and cloning of potentially broad-spectrum R genes.

There is increasing attention nowadays on ways to control *A. cochlioides* disease in sugar beet, after it has been largely ignored for many years compared to other widespread diseases. Our hope is that this research could be a useful piece of work for future studies, with the ultimate goal to find effective and sustainable management strategies to this pathogen in line with the world sugar production demand but also in harmony with the environment.

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Popular science summary

Sugar plays a dominant role in food industry, agriculture and economy. It is an important ingredient for home-made food as well as processed products such as baked goods, soft drinks, dairy products and wine. Beyond food, sugar is largely used in medicine, cosmetics and other industries, i.e., for the production of bioplastic and biofuel. Around 30% of global sugar supply derives from sugar beet, which, due to the genetic improvement of the varieties, has reached a sucrose content of 14 to 18%. However, the productivity of the crop is mined by abiotic and biotic factors. In the soil where sugar beet are grown there are a multitude of pathogens that attack the plants and cause root diseases and a reduction in sugar yield. One of the most disruptive diseases in sugar beet is caused by *Aphanomyces cochlioides*, a fungus-like soilborne pathogen that belongs to the class of Oomycetes. This pathogen is able to attack sugar beet young seedlings, causing damping-off, as well as adult plants, inducing chronic root rot. Fungicide seed treatment provides protection of seedlings and this is by far the most efficient solution to control Aphanomyces damping-off. However, chemical treatment and other effective control measures to prevent root rot in older plants are not available. The best ways of managing the disease include the use of resistant cultivars, management practices to improve soil drainage and avoidance of highly infested soils. A deeper understanding of the host-pathogen interaction is also needed to aid in the prevention of the disease and to secure a high yield production. In order to contribute to the development of sustainable control measures, the focus of this project was to investigate the nature of the relationship between sugar beet and *A. cochlioides* and to understand the plant defence mechanisms induced during infection. Molecular tools and microscopy were used to investigate how *A. cochlioides* invades plants with different levels of resistance. We observed that the speed

and the extension of host colonization by the pathogen were inversely correlated with the susceptibility of host genotypes tested. The gene expression of sugar beet plants during the early stages of the infection was also studied. Significant changes in the production of the chemical compound hydrogen peroxide (H_2O_2) and in the cell wall organization were observed in sugar beet genotypes characterized by high resistance levels, indicating their potential role in the defence responses to the pathogen. Moreover, we found regions of DNA in the sugar beet genome that make the plant more resistant to damping-off. Overall, the results from this study could help in the development of sugar beet varieties with enhanced resistance to *A. cochlioides* that could be used in agriculture to support integrated pest management strategies and to reduce the use of chemical fungicides.

Populärvetenskaplig sammanfattning

Socker spelar en viktig roll inom såväl livsmedelsindustrin som inom jordbrukssektorn och för den globala ekonomin. Socker är en viktig ingrediens i hemlagad mat såväl som i livsmedelsprodukter som bakverk, läsk, mejeriprodukter och vin. Förutom som livsmedel används socker även inom medicintillverkning, kosmetika samt i andra industrier som till exempel produktion av bioplast och biobränsle. Cirka 30% av den globala sockerproduktionen kommer från sockerbetor, som med hjälp av förädling har nått en sackaroshalt på 14 till 18 %. Avkastningen hos grödan påverkas dock av abiotiska och biotiska faktorer. I jorden där sockerbetor växer finns en mängd skadegörare som angriper växterna och orsakar rotsjukdomar och en minskning av sockeravkastningen. En av de viktigaste sjukdomarna hos sockerbetor orsakas av *Aphanomyces cochlioides*, en svampliknande jordburen patogen som tillhör klassen algsvampar. Denna patogen kan attackera unga fröplantor och orsaka rotbrand, såväl som vuxna plantor och då inducera kronisk rotröta. Fröbehandlingar med svampmedel kan skydda de unga fröplantorna från rotbrand men någon effektiv kemisk behandling för att kontrollera kronisk rotröta finns inte tillgänglig. De bästa sätten att hantera sjukdomen inkluderar användning av resistenta sorter, jordbearbetning för att förbättra jorddräneringen samt att undvika starkt infekterade jordar. En djupare förståelse för interaktionen mellan växt och patogen behövs för att förebygga sjukdomen och för att säkerställa en hög avkastning. För att bidra till utvecklingen av hållbara kontrollåtgärder var fokus för detta projekt att undersöka sambandet mellan sockerbetor och *A. cochlioides* och att förstå de växtförsvarsmekanismer som induceras under infektion. Molekylära verktyg och mikroskopi användes för att undersöka hur *A. cochlioides* angriper sockerbetor med olika nivåer av resistens. Vi observerade att hastigheten och utvidningen värdkolonisering för patogenen

var omvänt korrelerade med känsligheten hos testade värdgenotyper. Genuttrycket hos sockerbetsplantor under de tidiga stadierna av infektionen studerades också. Signifikanta förändringar i produktionen av den kemiska föreningen väteperoxid (H_2O_2) och i cellväggsorganisationen observerades i sockerbetsgenotyper med hög resistensnivå, vilket indikerar deras potentiella roll i försvarsmekanismen mot patogenen. Dessutom hittade vi regioner av DNA i sockerbetsgenomet som gör växten mer motståndskraftig mot rotbrand. Sammantaget kan resultaten från denna studie hjälpa till i utvecklingen av sockerbetssorter med ökad resistens mot *A. cochlioides* som skulle kunna användas inom jordbruket för att stödja integrerade bekämpningsstrategier och minska användningen av kemiska fungicider.

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I

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. 2010). 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 et al. 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 conditions (Windels 2000). The symptoms of the seedling 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. 2010). 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, designated 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 markers 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 selection of sugar beet lines to be used in breeding programs. Therefore, in this study we assessed a qPCR assay for detection and quantification of *A. cochlioides* in infected plants over time to investigate the association between the pathogen biomass and the levels of resistance of the host and to elucidate the progress of the infection in partially resistant and susceptible genotypes. 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 investigated by challenging the plants to isolates originated from different geographical regions. The time course and extent by *A. cochlioides* in infected seedlings was also observed and documented using confocal microscopy.

Materials and Methods

Plant material and *Aphanomyces cochlioides* strains

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 x 35 x 6 cm) in a climate chamber under controlled conditions (16h light, 22°C day/night, 95% RH). Three *A. cochlioides* field strains previously collected from Sweden (Tågarp, Skåne), USA (Marshall, Minnesota) and Japan (Hokkaido) were provided by DLF Beet Seed. The three *A. cochlioides* strains were single-spore isolated by pipetting 10 µl of a serial-diluted and filtered zoospore suspension, 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 Corn Meal Agar (CMA) medium (17 g cornmeal 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 (3 g peptone/ddH_2O, pH=7) 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_2O (2mM 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 (3-5x10^4 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 (16h 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 x 10^4 zoospores/ml) for 2 hours. 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 0h, 4h, 24h, 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

Lyophilized plant material was ground for 1 minute at 30 Hz using a TissueLyser (Qiagen, Valencia, CA, U.S.; Cat.No 85220). 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 1M, 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 minutes with occasional mixing by inversion. The plate was cooled on ice for 5 minutes 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 minutes with occasional mixing. The plate was centrifuged for 10 minutes 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 minutes at 1600 x g, 6°C. The isopropanol was discarded before the addition of 200 µl of 70% ethanol followed by 10 minutes 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 minutes with occasional vortexing.

Primer and probe design

For amplification and quantification of *A. cochlioides* target sequences, primers and a probe targeting the 5.8S ribosomal RNA gene of *A. cochlioides* (GenBank accession number: AY353911) developed by 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 11S 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.

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´

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, 0,72 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 sec 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).

Quantitative PCR

Host and pathogen biomass was quantified by qPCR performed by using an Applied Biosystem 7500 Real Time PCR System. The reaction mix consisted of 5µl Takyon™ ROX Probe 1X MasterMix dTTP blue (Eurogentec), 0,8 µl ddH_2O, 0,04 µl primers (0,2 µM), 0,02 µl probes (0,1 µM) and 4 µl DNA (1-2 µg/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 sec and 60°C for 1 min. The amount of *A. cochlioides* DNA was normalized to the sugar beet reference gene 11S 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 hours of incubation at room temperature, roots were washed for 2 minutes 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 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 minutes, 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 488nm 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

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 5.8S 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.

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 5.8S ribosomal RNA gene of *A. cochlioides*

Dilution series	11s globulin seed storage protein 2	5.8S 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

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 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.

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)

	Arhill_2012		Hokkaido_01		USA_01.7.6	
Genotype	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

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 Figure 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 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).

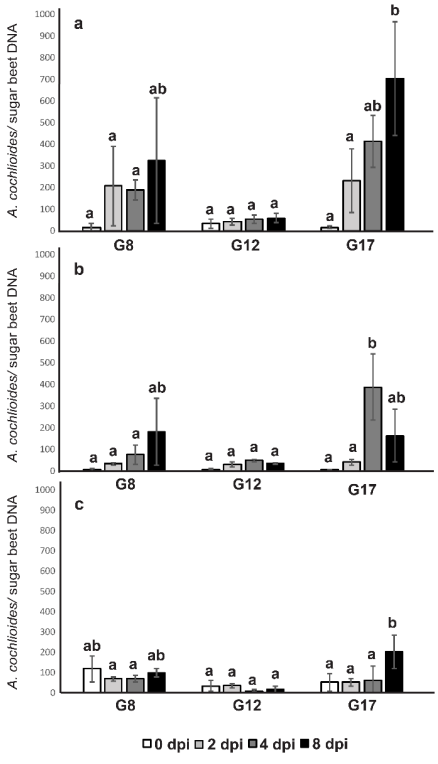

Fig 1 Ratio between *A. cochlioides* DNA and sugar beet DNA in sugar beet seedlings. Genotypes G8, G12, G17 were inoculated 12 days after sowing with a) the Swedish isolate Arhill_2012, b) the Japanese isolate Hokkaido_01 and c) the USA isolate USA_01.7.6. Seedlings were collected directly after inoculation (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

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 $5x10^4$ zoospores/ml and WGA-staining of the pathogen.

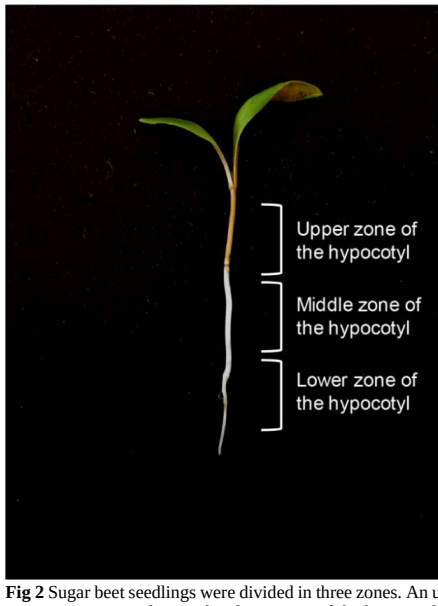

Fig 2 Sugar beet seedlings were divided in three zones. An upper zone corresponding to the closest area of the hypocotyl to the cotyledons, a middle zone corresponding to the central portion of the hypocotyl and a lower zone, including the elongation zone of the radicle, in the vicinity of the tip root

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.

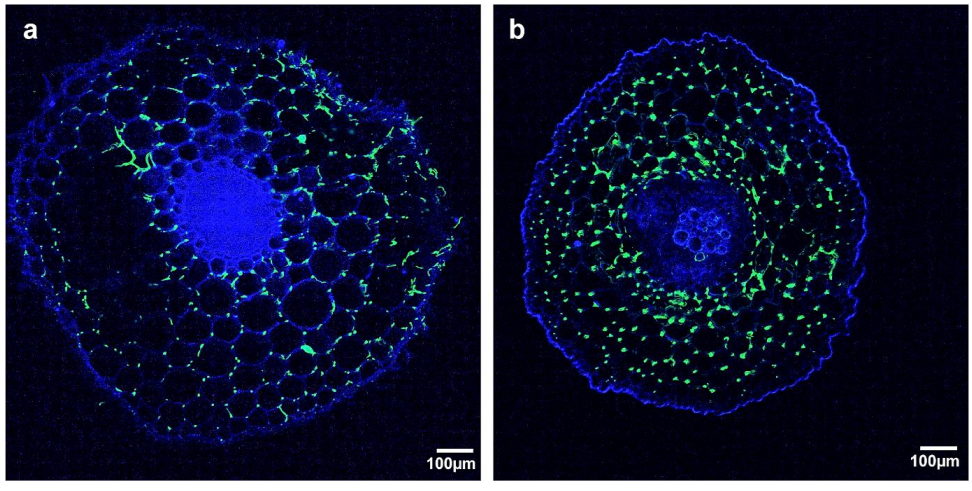

Fig 3 Cross sections of sugar beet (*B. vulgaris* L.) hypocotyl in the proximity of the radicle at 2 days after inoculation with *A. cochlioides* zoospores, observed at the confocal laser scanning microscope. *Aphanomyces cochlioides* hyphae are visible in the intercellular spaces of a) the partially resistant genotype G12 and b) the susceptible genotype 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)

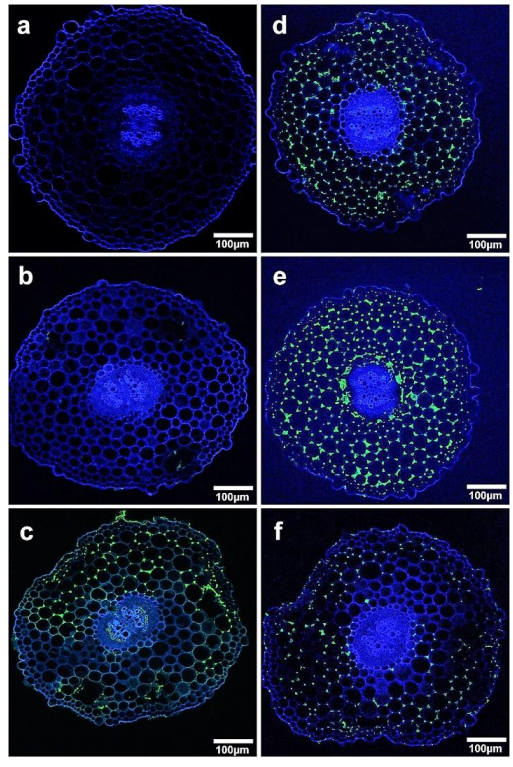

Fig 4 Cross sections of sugar beet (*B. vulgaris* L.) from different zones of the hypocotyl at 8 days after inoculation with *A. cochlioides* zoospores, observed at the confocal laser scanning microscope. In the partially resistant genotype G12 *A. cochlioides* hyphae were not observed in a) the upper 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

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, 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 minutes (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 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 pathogen 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 hypocotyl 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 genotypes 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 resistant 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. euteiches* inoculation (Djebali 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 reinforced cell walls were observed in the layers surrounding the stele, which prevent the colonization of the vascular system (Djebali 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*.

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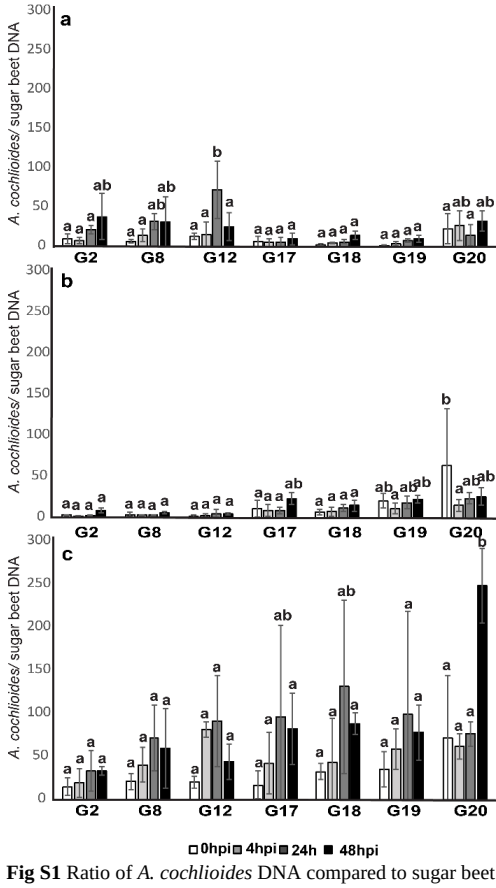

Fig S1 Ratio of *A. cochlioides* DNA compared to sugar beet DNA in sugar beet seedlings infected with a) the Swedish isolate Arhill12, b) the Japanese isolate Hokkaido01, and c) the US isolate USA01.7.6 of *A. cochlioides* two weeks after sowing of the seeds. The ratio for seven different genotypes (G2, G8, G12, G17, G18, G19 and G20) at four different time points, namely 0, 4, 24 and 48 hours post inoculation is shown. Each bar is the mean ratio of three biological replicates and the error bars show the standard deviation

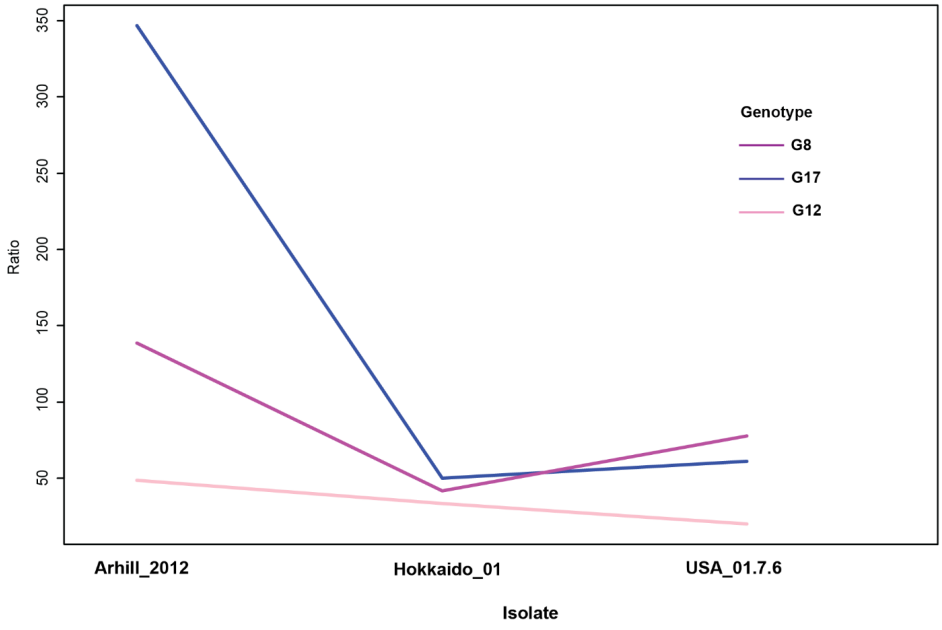

Fig S2 Genotype x isolate interaction effect on *A. cochlioides*/sugar beet ratio. The plot displays the average pathogen/host ratios on the y-axis and the *A. cochlioides* isolates on the x-axis, while the magenta, blue and pink lines represent the sugar beet genotypes G8, G17 and G12 respectively

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The pathogenic oomycete *Aphanomyces cochlioides* is the causal agent of damping-off and root rot of sugar beet, causing drastic reduction in sugar yield. The aim of this thesis was to broaden our understanding of *A. cochlioides*-sugar beet interactions and to analyse host defence responses. The host-pathogen interaction was studied at the transcriptional level and the genomic positions of genes contributing to resistance to *A. cochlioides* have been investigated, with the aim of providing new knowledge to aid future resistance breeding.

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