Occurrence and genetic diversity of *Verticillium longisporum* lineages in Europe and their interaction with oilseed rape, *Brassica napus* L.

Dissertation

to obtain the Ph.D. degree

in the international Ph.D. Program for Agricultural Sciences in Göttingen (IPAG)

at the Faculty of Agricultural Sciences,

Georg-August University Göttingen, Germany

by

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Göttingen, December 2020

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Date of disputation: 02.03.2021

Aos meus devanceiros labregos e á terra que labraban

(To my peasant ancestors and the land they farmed)

You cannot know what you will discover on the journey, what you will do with what you find, or what you find will do to you.

James Baldwin

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Chapter I: General Introduction

1. *Brassica* rapeseed crops

The Brassicaceae family comprises about 338 genera and over 3,700 species distributed worldwide. It putatively originated around the Mediterranean Sea and further extended into Southwest and Central Asia (Prakash et al. 2011). Brassicas are widely distributed throughout the world and cultivated as vegetables, condiments, and fodder crops, as well as for vegetable oil production (Downey and Rimmer 1993). The earliest written record of the cultivation of brassicas was found in China and dates to 3,000 BC. References to brassicas have also been found in ancient records from India, Greece, and Rome, as well as from the Assyrian Empire (Prakash et al. 2011). Their cultivation spread to Europe during the Middle Ages, where they were mainly cultivated for lamp oil and cooking fat (Gunstone 2009; Raymer 2002).

The demand for oilseed has grown exponentially in the last century, expanding the production of *Brassica* rapeseed. Rapeseed is the second most important oilseed crop, accounting for 14% of the global vegetable oil production after oil palm (35%) and soybean (28%) (US Department of Agriculture & USDA Foreign Agricultural Service 2020) (Figure 1, A). Canada and the European Union are the leading producers of rapeseed oil, followed by China and India (FAOSTAT 2018) (Figure 1, B). In the European Union, the biggest players are Germany, France, Great Britain, and Poland (Carré and Pouzet 2014). Since the 2000s, rapeseed production has increased by more than two-fold, an increase mainly driven by the demand for biofuels. In Europe, 80% of the rapeseed oil is used in the food sector, and the remaining 20% in the biodiesel industry (Ismail et al. 2017). The cultivation of rapeseed crops has also increased due to the benefits it provides when included in the rotation of cereal-based agricultural systems, as it improves soil structure and fertility (Gunstone 2009). Rapeseed crops contribute to the prevention of soil erosion by providing more than 80% ground cover during the winter (Robson et al. 2002). Additionally, rapeseed residues reduce nitrogen leaching in the winter, contribute to nitrogen immobilization, and reduce weed emergence (Haramoto and Gallandt 2004 and 2005; Sattell et al. 1998).

The different species and varieties of rapeseed are between 90 and 150 cm high. The flowers are bright yellow and four-petaled. The root is fibrous near the surface, and it has a deep taproot system. The round seeds are small (40,500–67,500 seeds/kg) and have a brown-black color (Sattell et al. 1998). There are several major *Brassica* rapeseed species, but the most important are *B. napus* and *B. rapa.* Both species have winter and spring cultivars. Spring cultivars do not require winter chilling (vernalization) to reach flowering. They are not winter-hardy and are therefore sown in the spring. Winter cultivars, on the contrary, survive the winter in a rosette stage (Friedt and Snowdon 2010). *B. rapa* is predominantly grown in Canada, India,

Europe, and China (Gunstone 2009; Raymer 2002). Winter *B. napus* is mostly cultivated in Europa, China, and Canada (Gupta 2016). In Australia and the Southeastern United States, spring *B. napus* is cultivated as a winter crop due to the mild winters (Raymer 2002). Other minor rapeseeds include *B. juncea*, which is grown in Northern India and China, and *B. carinata*, restricted to East Africa (Gunstone 2009).

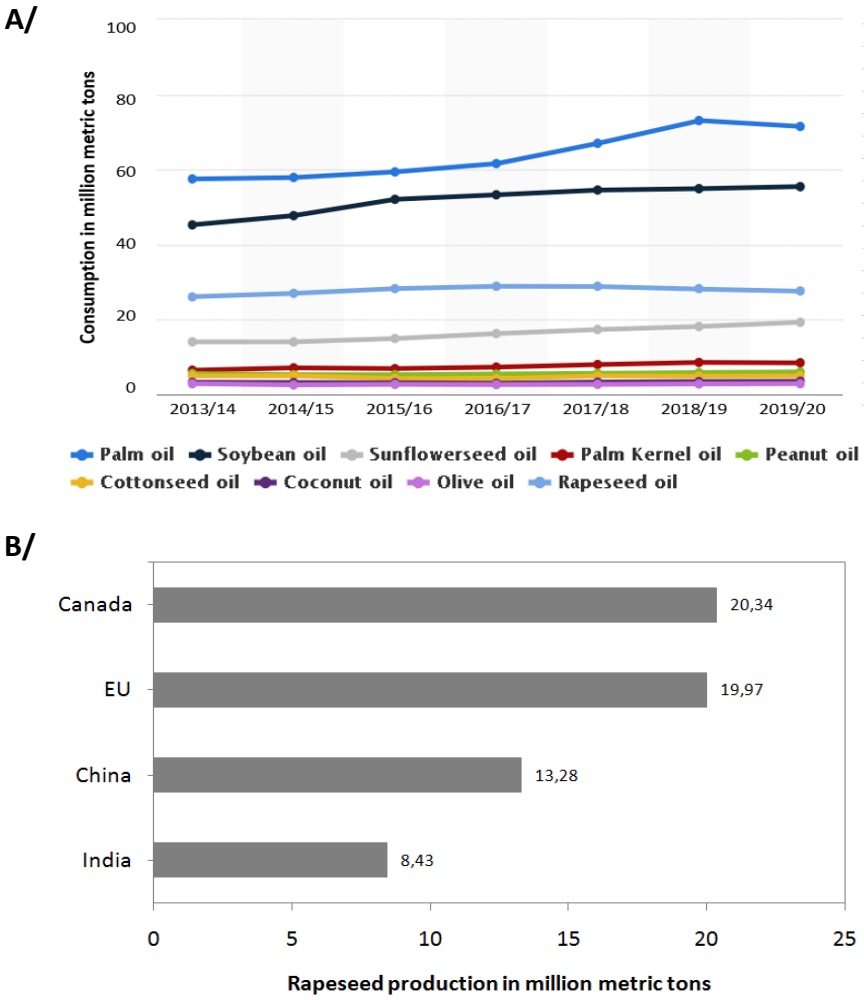

Figure 1. A) Global vegetable oil production of the ten most important oil crops from 2013/2014 to 2019/2020 (adapted from US Department of Agriculture & USDA Foreign Agricultural Service 2020) and B) leading countries of rapeseed production in 2018 (adapted from FAOSTAT 2018).

The genetic relationship between the four main rapeseed crops and other brassicas is well known. In the 1930s, Morinaga and coworkers revealed through karyological studies that *B. nigra* (*n* = 8; B), *B. oleracea* (*n* = 9; C) and *B. rapa* (*n* = 10; A) are the primary *Brassica* species. On the other hand, *B. carinata* (*n* = 17; BC), *B. juncea* (*n* = 18; AB) and *B. napus* (*n* = 19; AC) are amphidiploids which originated from different combinations of crosses from the primary species (Downey and Rimmer 1993; Raymer 2002).

Besides the production of rapeseed crops for oil, the oilseed product, rapeseed cake, is an excellent protein fodder (Office of the gene technology regulator 2008; Robson et al. 2002). The cultivation expansion of *B. napus* and *B. rapa* in the 1970s was possible due to the introduction of breeding programs to reduce the

amount of erucic acid and glucosinolates. Erucic acid has a bitter taste, and there were concerns about its potential link to heart diseases. In monogastric animals, the by-products produced from the ingestion of glucosinolates cause liver and kidney damage, as well as lymphatic dysfunction (Friedt and Snowdon 2010). These rapeseed cultivars with low seed concentrations of glucosinolates and erucic acid are referred to as 'double-low' cultivars. The term 'canola' is used for rapeseed varieties that contain less than 30 mmol of aliphatic glucosinolates per gram of air-dried oil-free solid, and whose oil has less than 2% erucic acid (Haramoto and Gallandt 2004). The term canola was registered in Canada in the 1970s, and it is used for varieties of *B. napus*, *B. rapa* and *B. juncea* (Office of the gene technology regulator 2008). Brassica rapeseed varieties with 50-55% erucic acid in the final oil are produced for the chemistry industry. Additionally, varieties with high oleic acid have been developed for frying oil due to their higher oxidative stability (Friedt and Snowdon 2010).

2. *Brassica napus*

Brassica napus, commonly known as oilseed rape, is considered the most ancient *Brassica* amphidiploid, followed by *B. juncea* and *B. carinata* (Dixon 2006) In field conditions, fertilization predominantly occurs through self-pollination, although 5 to 30 % outcrossing rates have been reported (OECD 1997). *B. napus* putatively originated through a spontaneous interspecific hybridization, where the maternal donor was related to *B. oleracea* and *B. rapa* (Friedt and Snowdon 2010; Office of the gene technology regulator 2008). The lack of wild forms of *B. napus* suggests that it emerged when the parents started being cultivated in geographical proximity. The geographical constraints and selection bottlenecks during the origin of the species have caused low genetic diversity in this species. The low genetic diversity was further intensified with the breeding carried out to develop double-low cultivars because those traits came from single sources (Friedt and Snowdon 2010).

Radiation, temperature, and drought can impact the yield of winter oilseed rape if they occur during the onset of pods and seeds (Zając et al. 2016). The increasing oilseed rape production is subjected to different biotic and abiotic stresses. To prevent the build-up of diseases, insects, and weeds, oilseed rape should not be grown on the same field more often than every three to four years (Hegewald et al. 2018). However, the worldwide demand for biodiesel is driving the oilseed rape production and shortening crop rotations (Ismail et al. 2017). The most important oilseed rape pests in Europe are the cabbage stem flea beetle, pollen beetle, cabbage stem weevil, rape stem weevil, and brassica pod midge (Zheng et al. 2020). With such a high number of pests, prophylactic pesticide applications are very common. Pyrethroids pesticides are typically used in sequence within one season to control major oilseed rape pests. The intensive use of pyrethroids has caused a widespread resistance, which has worsened since 2013 due to the European Commission ban of neonicotinoid seed dressings in bee-attractive crops (Heimbach and Müller 2013; Scott and Bilsborrow 2019;

Zheng et al. 2020). Weeds can cause severe yield losses and are an important limiting factor in this crop (Robson et al. 2002). Regarding oilseed rape diseases, the most significant ones in Europe are Sclerotinia stem rot *(Sclerotinia sclerotiorum*), stem canker —also known as blackleg disease— (*Leptosphaeria maculans*), clubroot (*Plasmodiophorabrassicae*), light leaf spot (*Pyrenopezia brassicae*), and Verticillium stem stripping (*Verticillium longisporum*) (Zheng et al. 2020).

3. *Verticillium* spp.

Verticillium spp. are widely distributed soil-borne vascular pathogens, responsible for important economic losses in many high-value crops, trees, and flowers all over the world, particularly in temperate zones (Agrios 2005; Inderbitzin and Subbarao 2014; Pegg and Brady 2002). *Verticillium* spp. are monocyclic pathogens that survive in the soil as microsclerotia, resting mycelium, or chlamydospores. They form conidia on phialides, which grow in whorls from conidiophores with transverse septae. The genus comprises ten species, from which *V. dahliae* is responsible for most economic losses. The species are divided into two clades, Flavexudans or Flavenonexudans, based on their ability to produce yellow hyphal pigmentation (Depotter et al. 2016a; Inderbitzin et al. 2011a). Whereas *V. dahliae* has more than 200 hosts, none of the other nine species have been found in more than ten hosts (Agrios 2005; Inderbitzin et al. 2011a). Most species are not host-specific and have a relatively wide geographic distribution. However, some have only been found in one host in a specific region, such as *V. klebahnii* on lettuce in the USA or *V. nubilum* in potato fields in the UK (Inderbitzin et al. 2011a; Inderbitzin and Subbarao 2014). *Verticillium longisporum* typically infects *Brassica* crops, of which cabbage, cauliflower, and oilseed rape are the most important economically (Depotter et al. 2016a; Inderbitzin et al. 2011b). Pathogenicity assays have revealed that host range and aggressiveness can vary among isolates of a *Verticillium* species. Isolates are typically more aggressive in their original host, which indicates host adaption (Berbegal et al. 2010; Bhat et al. 2003; Bhat and Subbarao 1999; Douhan and Johnson 2001; Iglesias-Garcia et al. 2013; Korolev et al. 2008; Novakazi et al. 2015). In addition, it has been reported that isolates obtained from asymptomatic hosts can induce symptoms when inoculated to other hosts (Gurung et al. 2015).

4. *Verticillium longisporum*

4.1. *V. longisporum* lineages

Verticillium longisporum is the only diploid species of the genus *Verticillium* (Inderbitzin et al. 2011a). It was first described in the 1960s as a subspecies of *V. dahliae* that produced wilting symptoms on horseradish and had conidia twice as long as *V. dahliae* (Stark 1961). Based on randomly amplified polymorphic DNA (RAPD) band profiles, pathogenicity studies, microscopic observations, and biochemical analysis, Karapapa et al.

(1997) proposed to assign it to a distinct species. *V. longisporum* is amphidiploid, and the amount of DNA, which may vary between isolates, is about 1.8-fold of haploid *Verticillium* species (Collins et al. 2003; Steventon et al. 2002). With phylogenetic analysis based on five protein-coding genes and the nuclear ribosomal internal transcribed spacer region (ITS), Inderbitzin et al. (2011b) concluded that *V. longisporum* consists of three lineages (A1/D1, A1/D2, and A1/D3) that originated from three independent hybridization events involving four haploid Verticillium parents. Although the lineages cannot be macroscopically distinguished, differences between the lineage A1/D1 and A1/D3 on microsclerotia shape and spore size have been reported (Banno et al. 2015; van Tran et al. 2013). Two *V. longisporum* parents, D2 and D3, correspond to *V. dahliae*, whereas A1 and D1 are unknown *Verticillium* species. Phylogenetic studies indicate that A1 was genetically more similar to *V. albo-atrum* than D1, which was more closely related to *V. dahlia* (Inderbitzin et al. 2011b). It is assumed that the hybridization events resulted in an increase in fitness, which allowed the newly formed species to out-compete the two unknown haploid parents. Those parents were presumably Brassicaceae specialists, whereas the other two parental lineages have a broader host range (Depotter et al. 2016b; Inderbitzin et al. 2011b).

Verticillium longisporum has so far been reported in different *Brassica* crops in Europe, Japan, China, Russia, USA, and Canada (Banno et al. 2015; CFIA 2015; Depotter et al. 2016a; Depotter et al. 2017; Gladders et al. 2011; Inderbitzin et al. 2011a; Yu et al. 2016; Yu et al. 2015). The A1/D2 lineage is the one with the narrowest host and geographic range, as it has only been reported on horseradish in Illinois and Ontario (Inderbitzin et al. 2011b; Yu et al. 2016). Both A1/D3 and A1/D1 have been isolated from cabbage, oilseed rape, radish, and cauliflower (Depotter et al. 2016a; Inderbitzin et al. 2011b). *V. longisporum* is present in most oilseed rape growing regions; including Canada, the Czech Republic, France, Germany, Sweden, Slovakia, Poland, Russia, and the UK (Bokor et al. 2014; CFIA 2015; Gladders et al. 2011; Inderbitzin et al. 2011b; Steventon et al. 2002; Spitzer and Matušinsky 2017; Yu et al. 2016). In the UK and Canada, the first reports describing *V. longisporum* in oilseed rape are quite recent, from 2011 and 2014 respectively (CFIA 2015; Gladders et al. 2011). In China and the USA, *V. longisporum* has never been reported in oilseed rape, despite infecting cabbage and cauliflower in those regions (Inderbitzin et al. 2011b; Yu et al. 2015). In Australia, another important oilseed rape producer, *V. longisporum* has never been described (Murray 2012). Previous greenhouse pathogenicity tests with different *Brassica* and non-*Brassica* hosts have revealed that A1/D1 is the most aggressive lineage on oilseed rape; while A1/D2 is the least aggressive one on all the tested crops and A1/D3 has a more diverse pathogenicity (Novakazi et al. 2015).

A1/D1 is putatively the most prevalent lineage in oilseed rape (Depotter et al. 2017; Inderbitzin et al. 2011b; Zou et al. 2020). However, an extensive lineage characterization in oilseed rape growing regions is still missing. A recent genetic analysis of the lineage A1/D1 based on microsatellites could separate isolates from different *Brassica* crops into two groups: one consisting of isolates from the UK, the Netherlands, Japan, and

the USA; and another one of North-Central European isolates (Depotter et al. 2017). Before the characterization of *V. longisporum* lineages, other studies had also shown genetic subgrouping of European *V. longisporum* populations through of amplified fragment length polymorphisms (AFLP) and restriction fragment length polymorphisms (RFLP) (Fahleson 2003; Steventon et al. 2002). Nevertheless, an extensive analysis of the *V. longisporum* population structure in the main oilseed rape growing regions is still missing. In addition, there is still a lack of knowledge of the potential relation between genetic subgroups, geographic origin, and pathogenicity.

4.2. *V. longisporum* in oilseed rape

It is believed that microsclerotia germination is stimulated by root exudates of the host plant (Pegg and Brady 2002). Germinated hyphae grow towards the roots and penetrate the epidermis with a hyphal swelling. After penetration, hyphae grow inter- and intracellularly towards the xylem (Eynck et al. 2007). *V. longisporum* typically induces wilting, which includes plant stunting, wilting, chlorosis, defoliation of lower leaves, and vascular necrosis (KemmochiI and Sakai 2004). In oilseed rape, however, it causes a dark unilateral striping on the stem during ripening of the crop. The striping symptoms originate from necrosis produced after the fungus leaves the xylem and colonizes the parenchyma, which leads to premature ripening of the crop. At plant senescence, microsclerotia start to develop in the stem and in the roots, and they are released into the soil after plant decomposition (Dunker et al. 2008). Yield losses caused by *V. longisporum* have been recorded by Dunker et al. (2008) in individual plants under controlled conditions. In the field, yield losses depend on the cultivar, disease incidence, and weather conditions (Depotter et al. 2019; Zheng et al. 2019b).

The symptoms caused in oilseed rape by *V. longisporum* in the greenhouse differ from those in field conditions. After root-dip inoculation with spore suspension at the seedling stage in the greenhouse, plants show chlorosis, vascular discoloration, and stunting (Eynck et al. 2007). The absence of such symptoms in the field is due to the delay in fungal colonization that plants experience at the beginning of the growing season due to the low soil temperature (Zheng et al. 2019b). Despite the symptoms being distinctly different, Knüfer et al. (2017) have shown a significant correlation between the severity of greenhouse symptoms and the level of fungal colonization in the field at the beginning of ripening. The lack of wilting symptoms in oilseed rape after infection by *V. longisporum* both in the greenhouse and in the field is due to the presence of sufficient xylem vessels that are not blocked by occlusions, which are formed infrequently and in late stages of infection (Eynck et al. 2009a).

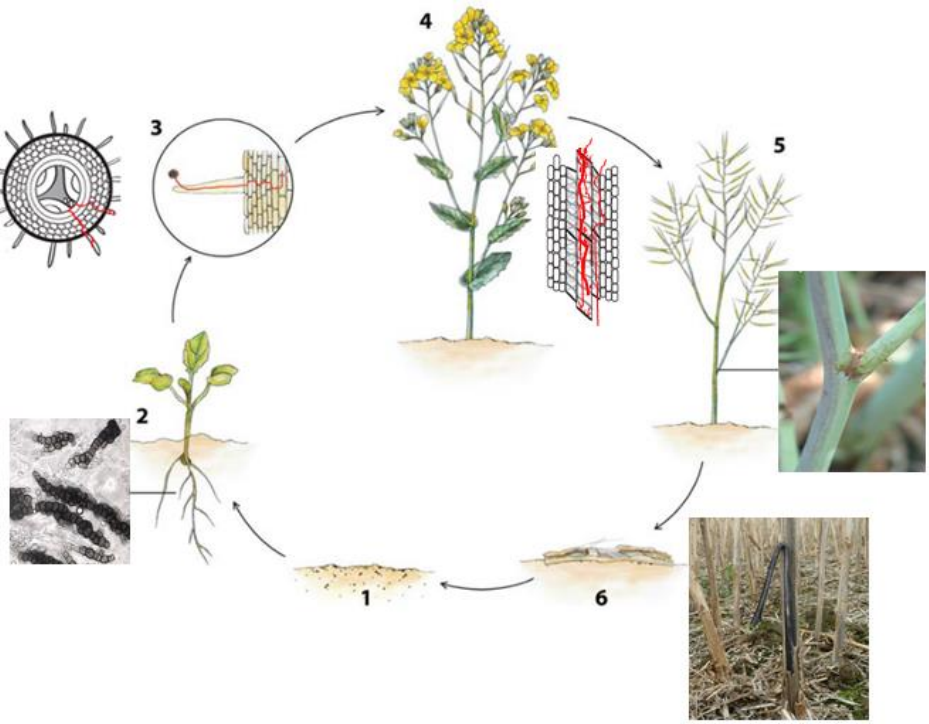

Figure 2. Life cycle *of Verticillium longisporum* in winter oilseed rape (*Brassica napus*) (adapted from Depotter et al. 2016a). 1) Latent microsclerotia in the soil. 2) Microsclerotia germination. 3) Root penetration of the hyphae followed by inter- and intracellular growth towards the xylem. 4) Xylem colonization. 5) Colonization of parenchyma and stem stripping symptoms. 6) Microsclerotia development on the stubbles.

4.3. *V. longisporum* control strategies

The long survival of the fungal resting structures in the soil and the lack of effective curative measures make vascular pathogens very difficult to control. Due to environmental and health concerns, chemical soil treatment is restricted (Deketelaere et al. 2017; Yadeta and Thomma 2013). Resistance breeding is the most sustainable and effective strategy against vascular pathogens (Yadeta and Thomma 2013). However, there is limited resistance against *V. longisporum* in the current oilseed rape varieties, due to the past intensive breeding for quality traits and the subsequent reduction of the genetic basis of the species (Friedt and Snowdon 2010). In 2003, resistant *B. oleracea* accessions were identified (Happstadius et al. 2003): Thereafter, resynthesized *B. napus* lines with enhanced *V. longisporum* resistance were developed by interspecific hybridization of *B. oleracea* with *B. rapa* (Eynck et al. 2009b; Happstadius et al. 2003; Rygulla et al. 2007). The identification of quantitative trait loci (QTL) for resistance is the starting point for marker-assisted breeding of resistant cultivars. Rygulla et al. (2008) and Obermeier et al. (2013) have identified two and three resistance QTLs in the C genome, respectively, by using segregating double haploid (DH) populations generated from resynthesized *B. napus* lines combined with *B. napus* cultivars.

Obermeier et al. (2013) have reported that the three resistance QTLs they identified co-localized with loci associated with soluble phenylpropanoids. Microscopic assays revealed that occlusions and cell-wall bound phenolics and lignin are formed faster in vascular tissues of less susceptible cultivars (Eynck et al. 2009a). In

addition, it has been shown that salicylic acid is involved in the induction of basal and cultivar-related resistance (Zheng et al. 2019a). Despite the progress in unveiling the resistance mechanisms, the genetic basis of oilseed rape quantitative resistance against *V. longisporum* is not fully understood (Friedt and Snowdon 2010). Additional efforts need to be made to map additional QTLs that can provide a broad and stable resistance, which can be used to develop genetic markers to localize important resistance genes (Obermeier et al. 2013). The identification of QTLs for disease resistance requires extensive and laborious phenotypic evaluations (Sehgal et al. 2016). Resistance screening in the greenhouse is a high throughput method that allows the assessment of a high number of genotypes in a short period. However, the generated data needs to be validated in the field (Eynck et al. 2009b).

Due to the limited resistance of oilseed rape against *V. longisporum*, integrated management with a combination of different cultural control methods remains the only available control strategy against *V. longisporum* (Deketelaere et al. 2017; Depotter et al. 2016a; Yadeta and Thomma 2013). These methods include crop rotation, soil solarization, management of potential weed reservoirs, or organic soil amendments (Depotter et al. 2016a). The limited effectivity of the currently available control measures against vascular pathogens calls for investigating new alternatives, such as the use of microorganisms as biocontrol agents (Deketelaere et al. 2017; Yadeta and Thomma 2013). The potential use of several biocontrol agents against *V. longisporum* has already been reported. However, research on *V. longisporum* biocontrol in oilseed rape has so far been limited (Depotter et al. 2016a). One biocontrol approach is using non-aggressive strains from the same genus as the plant pathogen. This biocontrol strategy, called cross-protection, provides the advantage that protective isolates share the same ecological niche as the pathogen (Deketelaere et al. 2017). Cross-protection typically involves several modes of action, such as space competition in the rhizosphere (Pantelides et al. 2009), direct induction of plant defense (Angelopoulou et al. 2014; García et al. 2011), or plant growth promotion (Shittu et al. 2009). Another biocontrol mechanism is priming, which consists in inducing rapid activation of defense responses upon pathogen infection (Conrath et al. 2002). The mode of application of the biocontrol agent is critical for effective control. Moreover, the application must be practically feasible and economically justified, which is particularly critical in large field crops. In arable crops, the best application method of a biocontrol agent against soil-borne vascular pathogens is as a seed coat (Alabouvette et al. 2009; Rocha et al. 2019).

5. Goals of the thesis

Based on knowledge gaps and relevant areas of study, the four goals of this work were as follows:

a) Genetic and phenotypic characterization of pathogen populations are essential to establish effective disease management programs (Dung et al. 2019). Consequently, the first objective of this study

was the assessment of the genetic structure and pathogenicity of *V. longisporum* populations in oilseed rape production areas. For that, an extensive lineage monitoring with a broad geographical scope was performed. Afterwards, genotyping by sequence (GBS) was used to finely identify genetic subgroups. Finally, potential pathogenicity differences between genetic subgroups and geographic regions were assessed in the greenhouse.

b) The so far scarce success in research on cross-protection against *V. longisporum* in oilseed rape calls for the screening of new biocontrol candidates. Thus, the biocontrol potential of the non-aggressive A1/D2 lineage in oilseed rape against aggressive isolates of *V. longisporum* was investigated. Thus, different *in vitro* and *in vivo* assays were carried out to assess the biocontrol potential of A1/D2. Because salicylic acid is involved in *V. longisporum* resistance (Zheng et al. 2019a), its induction by A1/D2 was also investigated, to determine whether A1/D2 induces priming of the plant. Additionally, the biocontrol efficacy of A1/D2 was also evaluated mimicking field conditions by applying it as a seed coat.

c) The mechanisms that shape the pathogenicity diversity and host preferences within *V. longisporum* are not well understood. To shed light on those differences, the interaction of the aggressive and non-aggressive lineages A1/D1 and A1/D2 with oilseed rape was evaluated. For that, microscopic, phenotypic, and qPCR assessments were carried out to assess systemic colonization, colonization patterns, and ultrastructural changes they induce in the plant.

d) The fourth and final objective of this thesis was to evaluate the level of resistance in two double haploid populations in the field and greenhouse for future QTL mapping. Furthermore, a comparative assessment of the parameters used for resistance screening was performed. Finally, the influence of climatic conditions on disease level and reproducibility of the assessments was evaluated.

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Chapter II: Genotype diversity related to pathogenicity in *Verticillium longisporum* populations recovered from European and Canadian oilseed rape fields

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Abstract

Verticillium longisporum is a vascular fungal pathogen of *Brassica* crops that segregates in at least three hybrid forms originating from independent fusions of four different haploid *Verticillium* genomes, leading to the amphidiploid hybrid lineages A1/D1, A1/D2, and A1/D3. Until today, the genetic diversity and population structure within and across lineages from different geographic origins and their relevance for pathogenicity has not been well studied. To assess whether there are genetic subgroups within *V. longisporum*, a phylogenetic analysis of 273 single spore isolates from 62 locations in eight countries based on genotyping by sequencing was conducted. In addition, the potential agronomic importance of different subgroups was assessed with fourteen representative isolates that were tested for their pathogenicity on oilseed rape under greenhouse conditions. This study confirmed that A1/D1 is the prevalent *V. longisporum* lineage in European and Canadian oilseed rape fields. The phylogenetic analysis revealed a closer genetic relationship of lineages A1/D2 and A1/D3 to *V. dahliae* than to lineage A1/D1. Genetic clusters within the A1/D1 lineage did not illustrate a clear-cut geographic separation, except between English isolates and isolates from Canada, Latvia, and Denmark. This indicates a different origin of the recent introductions of *V. longisporum* in England and Canada. French and German isolates were the most distributed among all A1/D1 clusters, which indicates a higher genetic diversity in those regions. In addition, the phylogenetic analysis showed no genetic clustering of isolates based on field of origin. Finally, the pathogenicity test revealed pathogenicity diversity within *V. longisporum* lineage A1/D1, as well as among isolates within the same field. The reported genetic and pathogenicity diversity may further complicate management strategies against this disease.

1. Introduction

Verticillium longisporum is an amphidiploid vascular pathogen of Brassicaceous plants (Depotter et al. 2016a; Inderbitzin et al. 2011b), which was first described in the 1960s as a subspecies of *V. dahliae* that produced wilting symptoms on horseradish and had conidia twice as long as other strains of *V. dahliae* (Stark 1961). In 1997, Karapapa et al. proposed to assign this *V. dahliae* variety to a distinct species, based on randomly amplified polymorphic DNA (RAPD) band profiles, pathogenicity studies, microscopic observations, and biochemical analyses. The amount of *V. longisporum* DNA, which may vary between different isolates, is about 1.8-fold of haploid *Verticillium* species (Collins et al. 2003; Steventon et al. 2002). With phylogenetic analysis based on five protein coding genes and the ribosomal internal transcribed spacer region (ITS) region, Inderbitzin et al. (2011b) concluded that *V. longisporum* consists of three genetically distinct lineages that originated from three independent hybridization events involving four haploid *Verticillium* parents. Two of these parents, D2 and D3, correspond to *V. dahliae*, whereas A1 and D1 are unknown *Verticillium* species. Phylogenetic studies based on five protein coding genes indicate that A1 was genetically more similar to *V. albo-atrum* than D1, which was more closely related to *V. dahliae* (Inderbitzin et al. 2011b). It is assumed that the hybridization events may have increased fitness, which made the newly formed species of *V. longisporum* outcompete two of the haploid parents. These two parents were presumably host specialized on Brassicaceae, whereas the other two parental lineages D2 and D3 have a broader host range, and therefore were not outcompeted (Depotter et al. 2016b; Inderbitzin et al. 2011b).

Both A1/D3 and A1/D1 have been isolated from cabbage, oilseed rape, radish, and cauliflower in Europe, China, Japan and North America (Banno et al. 2015; Depotter et al. 2016a; Inderbitzin et al. 2011b; Yu et al. 2016; Yu et al. 2015). In contrast, A1/D2 has so far only been found on horseradish in Illinois and Ontario (Inderbitzin et al. 2011b; Yu et al. 2016). Previous greenhouse pathogenicity tests on different *Brassica* and non-*Brassica* crops revealed that A1/D1 was the most aggressive lineage on oilseed rape, while A1/D2 was the least aggressive on all tested crops (Novakazi et al. 2015). Compared to A1/D3, A1/D1 has slender microsclerotia and smaller spores (Banno et al. 2015; van Tran et al. 2013).

In oilseed rape, instead of producing wilting symptoms after colonization of the xylem, *V. longisporum* causes a dark unilateral striping of the stem during ripening of the crop (Eynck et al. 2007). The striping symptoms originate from the necrosis produced after the fungus leaves the xylem and colonizes the stem parenchyma. Yield losses depend on the cultivar, disease incidence, and weather conditions (Depotter et al. 2019; Zheng et al. 2019b). The symptoms caused by *V. longisporum* on oilseed rape in the greenhouse differ from those that occur under field conditions. With spore root-dip inoculation at the seedling stage in the greenhouse, oilseed rape showed chlorosis, vascular discoloration, and stunting (Eynck et al. 2007).

V. longisporum has become one the most important pathogens in oilseed rape (Depotter et al. 2016a) and has been found in most oilseed rape growing countries in Europe, as well as in Canada (Bokor et al. 2014; CFIA 2015; Depotter et al. 2016a; Gladders et al. 2011; Inderbitzin et al. 2011b; Spitzer and Matušinsky 2017; Steventon et al. 2002; Yu et al. 2016). According to previous studies, A1/D1 appeared to be the predominant lineage in oilseed rape (Depotter et al. 2017b; Inderbitzin et al. 2011b; Zou et al. 2020). Nevertheless, the strains used in those studies had either been isolated several decades ago or originated from a restricted range of geographical areas. Thus, information on the current distribution of lineages in this crop on a broader geographic scale is still missing.

Although *V. longisporum* seems to be a recently evolved species bearing a relatively high genetic homogeneity (Inderbitzin et al. 2011b), several studies have shown the presence of distinct genetic groups based on the geographic origin. Amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) analyses displayed genetic subgroups of *V. longisporum* strains from European oilseed rape fields (Fahleson 2003; Steventon et al. 2002). More recently, a genetic characterization of lineage A1/D1 based on microsatellites distinguished strains from oilseed rape in the UK from strains from north-central Europe (Depotter et al. 2017b). However, an assessment of the diversity within the main oilseed rape growing regions using recently isolated strains is still missing. In addition, there is still a lack of knowledge on the potential relationship between geographic origin, genetic subgroups, and pathogenicity within lineage A1/D1. This is particularly relevant with a poorly mobile soil-borne pathogen like *V. longisporum*, which is unable to spread over long distances by conidia.

Genetic and phenotypic characterization of plant pathogen populations is essential to establish effective breeding and management programs (Dung et al. 2019). Additionally, population genetic studies might elucidate the potential history of dispersion of a pathogen (Knight et al. 2019; Milgroom et al. 2016). Pathogenicity studies are vital to evaluate the agronomic importance of different genetic subgroups and reveal the relationship between specific genetic features and pathogenicity (Chen et al. 2017; Kumari et al. 2014). Thus, to characterize *V. longisporum* populations in oilseed rape growing regions, a lineage monitoring with broad geographic scale was conducted. The strains obtained from this monitoring were genetically analyzed, as well as assessed for their pathogenicity on oilseed rape in the greenhouse. The high throughput technology genotyping by sequence (GBS) was applied, which provides a high resolution genotypic analysis based on a high number of genome-wide stable markers and allows a deep insight into the population structure (Andrews et al. 2016; Milgroom et al. 2016).

2. Materials and methods

2.1. Isolation of *V. longisporum* from field plant samples

Samples from different locations in Europe and Canada were obtained through a network of collaborators. Segments of epidermal tissue excised from oilseed rape stubbles infected *V. longisporum* were disinfected by immersion in 70% ethanol for 30 s and rinsing twice with distilled water. Epidermis segments were then placed on synthetic nutrient-poor agar (SNA) medium with 200 ppm streptomycin. Petri dishes were incubated in the dark at room temperature for 3 to 5 days (Figure 1). A fragment of the growing colony of *V. longisporum* was transferred to potato dextrose agar (PDA) with 400 ppm streptomycin and incubated for two weeks. After confirmation of the species by microscopy, single spore cultures were produced. For long-term storage of the isolates, 150 µL of an aqueous suspension, containing spores and mycelial fragments harvested from the PDA medium, was transferred into sterilized Eppendorf tubes with filter paper discs, evaporated until dryness, and stored at -20 °C.

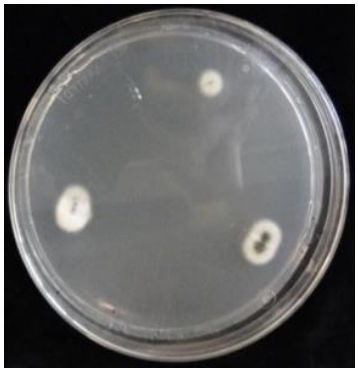

Figure 1. *Verticillium longisporum* colony growth on synthetic nutrient-poor agar medium five days after plating the infected epidermis segments of oilseed rape stubbles carrying microsclerotia.

2.2. Lineage characterization of the isolates

To assign the newly obtained isolates to one of the known lineages of *V. longisporum*, mycelium was produced by cultivation on potato dextrose broth (PDB) in Erlenmeyer flasks at room temperature for 10 days on a rotary shaker at 100 rpm. After vacuum filtration with a Büchner funnel, the mycelium was freeze-dried for 72 h. DNA was extracted following a modified cetyl trimethylammonium bromide (CTAB) extraction protocol (Brandfass and Karlovsky 2008). The quality of extracted DNA was verified by running the samples for 60 min at 3 V/cm on 1% agarose gel (Roth, Karlsruhe; Germany) stained with Midori Green (Nippon Genetics Europe GmbH, Düren; Germany) in 0.5x TBE (Tris-Borate-EDTA) buffer. DNA was visualized under UV light and documented with the software INTAS GelDoc (INTAS science imaging GmbH; Germany). DNA samples were stored at -20 °C until further use.

A multiplex-PCR was carried out according to Inderbitzin et al. (2013). The primers used in this study are listed in Table 1. The reference isolates used are listed in Table 2. Each PCR reaction had an end volume of 25 µL containing approximately 10 ng of DNA, 2 µL of primer mixture (0.5 µM for each primer, except 0.025 µM for primers VL-Df and VL-Dr) and 12.5 µM of GoTaq® G2 Hot Start Colorless Master Mix (Promega GmbH, Manheim; Germany). The PCR program consisted of 2 min initial denaturation step at 94 °C, 32 cycles of 10 s at 94 °C, 20 s at 65 °C, and 30 s at 72 °C, followed by final extension of 7 min at 72 °C. To analyze the products by agarose gel electrophoresis, 5 µL of the multiplex-PCR product and 1,000 bp ladder were loaded on 1.5% agarose gel stained with Midori Green (Nippon Genetics Europe GmbH, Düren; Germany) in 0.5x TBE buffer and run at 3 V/cm for 2 h.

Table 1. Primers used for lineage characterization of *Verticillium longisporum* with multiplex-PCR assay according to Inderbitzin et al. (2013)

Name	Sequence	Target loci	Amplicon size (bp)
VL-D1f	CCCCGGCCTTGGTCTGAT	*GPD* from D1	1,020
VL-AlfD1r	TGCCGGCATCGACCTTGG		
VL-A1f	AAGTGGAGCCCCGTATCTTGAAT	*EF* from A1	310
VL-A1r	CAACTGGCAACAGGGCTTGAAT		
VL-Df	CCGGTCCATCAGTCTCTCTG	*ITS* from *V. dahliae*	490
VL-Dr	CTGTTGCCGCTTCACTCG		

GPD= *glyceraldehyde-3-phosphate dehydrogenase*, *EF*= elongation factor 1-alpha, *ITS*= ribosomal internal transcribed spacer region.

Table 2. *Verticillium* reference isolates used for multiplex-PCR assay according to Inderbitzin et al. (2013).

Name	Species, linage	Origin	Collection	Host
VL40	*V. longisporum* A1/D1	Mecklenburg, Germany	[a]UGoe	*Brassica napus* (oilseed rape)
PD356	*V. longisporum* A1/D2	Illinois, USA	[b]UC Davis[b]	*Armoracia rusticana* (horseradish)
PD589	*V. longisporum* A1/D3	Gunma, Japan	UC Davis	*B. oleracea* (cabbage)
VD73 (PD637)	*V. dahliae*	Germany	UGoe	*Linum usitatissimum* (common flax)
PD327	*V. dahliae*	USA	UC Davis	*Capsicum annuum* Pepper

[a]UGoe= Georg-August University of Göttingen; [b]UC Davis= University of California, Davis.

2.3. Phylogenetic analysis

2.3.1. DNA extraction

For the phylogenetic analysis, DNA of 80 A1/D1 isolates from Europe and six from Canada were used to establish the GBS libraries (Appendix 2). Additionally, reference isolates were used in the analysis, which included seven isolates of the three *V. longisporum* lineages and one *V. dahliae* isolate (Table 3). DNA was extracted based on Xin and Chen (2012) with modifications. About 100 mg of lyophilized mycelium was ground with a 5 mm tungsten ball by using a MM400 mill (Retsch GmbH, Haan, Germany) three times at 28 hz for 1 min. Afterwards, 500 µL of freshly prepared lyticase buffer (100 mM potassium citrate, pH 8.0; 50 mM EDTA, pH 8.0; 1 M sorbitol; 5 mM TCEP; 200 U/mL lyticase) (Cat#L4025, Sigma; Germany) was added and mixed twice for 30 s at 20 hz. After incubation at 37 °C for 30 min, 125 µL of 5 M NaCl was added and mixed well. Immediately thereafter, 1,000 µL of extraction buffer (100 mM Tris, pH 8.0; 20 mM EDTA; 2% CTAB; 1.2 M NaCl; 5 mM TCEP) was added and incubated at 60 °C for 1 h.

Samples were cooled down for 5 min at room temperature and 1.5 mL of chloroform:isoamylalcohol (24:1, v/v) was added. After centrifugation at 3,000 x g for 15 min, the supernatant was transferred to a new tube with 2.2 mL of dilution buffer (100 mM Tris, pH 8; 20 mM EDTA; 2% CTAB) and incubated at 60 °C for 30 min. The mixture was centrifuged at 3,000 x g for 15 min, the supernatant was discarded and 2.2 mL of washing buffer (Tris-EDTA : ethanol, 3:7) was added to the pellet. The samples were kept at room temperature to soak for 30 min. Subsequently, the mixture was centrifuged for 15 min at 3000 x g, the pellet was re-suspended in 100 µL of high salt TE buffer (10 mM Tris, pH 8; 20mM EDTA; 1 M NaCl) with 1.1 µL RNaseA (50 µg/mL, Cat#R4875, Sigma; Germany) and incubated at 60 °C for 30 min. MagAttract suspension G (11 µl) (Cat# 1026901, Qiagen; Germany) and 100% ethanol (120 µl) were added and incubated at room temperature for 5 min. Using a magnetic rack, ethanol was removed by pipetting. Beads were washed three times with 400 µL washing buffer and air-dried at room temperature. For DNA re-suspension, 100 µL TE buffer (10mM Tris, 1 mM EDTA, pH8) was added and samples were incubated at 60 °C for 5 min. Finally, with the help of the magnetic rack, TE buffer was dissolved and DNA was transferred into a new tube for storage. The quality of the DNA was verified as described in section 2.2.

Table 3. Reference isolates from *Verticillium longisporum* A1/D1, A1/D2, and A1/D3 lineages, as well as *Verticillium dahliae,* used in the phylogenetic analysis.

Species, lineage	Identifiers in literature	Host	Collection site	Isolate origin	Reference
A1/D1	VL43	*Brassica napus* (oilseed rape*)*	Mecklenburg, Germany	[a]UGoe	Zeise and Tiedemann 2001 and 2002
	VL40	*B. napus* (oilseed rape*)*	Mecklenburg, Germany	UGoe	Zeise and Tiedemann 2001 and 2002
	VD11	*B. napus* (oilseed rape*)*	Sweden	[b]SUAS	Steventon et al. 2002
A1/D2	PD356	*Armoracia rusticana* (horseradish)	Illinois, USA	[c]UC Davis	Inderbitzin et al. 2011b
	PD402	*A. rusticana* (horseradish)	Illinois, USA	UC Davis	Inderbitzin et al. 2011b
A1/D3	PD614	unknown	Germany	[d]ISHC	Inderbitzin et al. 2011b
	PD589	*Brassica oleracea* (cabbage)	Gunma, Japan	UC Davis	Inderbitzin et al. 2011b
V. dahliae	VD73 (PD637)	*Linum usitatissimum* (common flax)	Germany	UGoe	Inderbitzin et al. 2011b

[a]UGoe= Georg-August University of Göttingen; [b]SUAS=Swedish University of Agricultural Sciences; [c]UC Davis= University of California, Davis; [d]ISHC= Horticulture Research International.

2.3.2. GBS analysis

After extraction, 10 ng of DNA diluted in TE buffer were sent to LGC Genomics GmbH (Berlin; Germany) for GBS analysis using the single enzyme digestion with *Ms*II, size selection and inline barcode addition with a custom PCR protocol followed by paired-end 150 bp sequencing by Illumina NextSeq 500 V2. Raw reads were obtained as Fastq files and imported into CLC Genomics Workbench v.9.0 software (Qiagen Bioinformatics, Aarhus; Denmark). The quality score was set to a limit of 0.05. The enzyme recognition sequence was removed from the 5' end of each raw sequence read after quality trimming. After trimming of Illumina adapters, reads with a length less than 20 bp were discarded. Libraries of the 94 isolates, consisting of paired-end trimmed reads, were aligned individually to the reference genome of the Swedish isolate *V. longisporum* VL1 downloaded from Ensembl Fungi (accession number GCA_001268145, also named VD11). Single nucleotide variants (SNVs) were called using the low frequency variant tools by filtering for a minimum low-frequency of 1%, a minimum coverage of five reads, a minimum count of three reads harbouring a variant, and minimum frequency of 20% variants. Further quality filters of neighborhood radius five, minimum central quality 20, and minimum neighborhood quality 15 were applied. Called SNVs for the 94 isolates were combined and further filtered using shell scripts. SNVs showing more than one allele were removed. A total of 94 genotypes with 11,879 SNVs were used for final data analysis.

2.3.3. Phylogenetic analysis

The phylogenetic analysis was carried out in R (4.0.2.). Data were stored as individual genotypes with the *adegenet* package. Hierarchal clustering using Nei´s distance was performed with the *poppr* package. For bootstrapping, 100 runs and a bootstrapping cut-off value of 70 were set.

2.4. Pathogenicity characterization of the A1/D1 lineage in the greenhouse

2.4.1. Materials and experimental design

Fourteen isolates representing the main A1/D1 genetic clusters were selected to characterize their pathogenicity on oilseed rape and clarify if different genetic clusters of lineage A1/D1 had different pathogenicity characteristics. Strains originating from Poland, Sweden, Germany, the UK (England), Latvia and Canada were selected. Additionally, the reference A1/D1 isolate VL43, characterized as aggressive, as well as the *V. longisporum* A1/D2 isolate PD402, considered non-aggressive on oilseed rape (Inderbitzin et al. 2011a; Novakazi et al. 2015), were included in the test for comparison.

To assess the pathogenicity of selected isolates on oilseed rape, winter oilseed rape cultivars `Falcon´ and `Express´ were used, which have been reported to be susceptible and moderately resistant to *V. longisporum,* respectively (Eynck et al. 2009). Seeds were pre-germinated in quartz sand at 18–24 °C and with a 14 h photoperiod (Horti-Lux HPS-400 Watt). Ten days after sowing, seedlings were removed from the quartz sand and washed thoroughly under running tap water. The spore suspension was obtained by incubating mycelial plugs in PDB in Erlenmeyer flasks on a shaker (100 rpm) at 22 °C in the dark for 10 days. The Erlenmeyer flasks were amended with 400 ppm streptomycin, 50 ppm chloramphenicol, and 50 ppm rifampicin to avoid bacterial contamination. After 10 days, the culture was filtered through a sterile sieve. The spore density was determined using a Thoma haemocytometer and diluted to 1 x 10^6 spores / mL. The spore suspension was applied as a 40 min root-dip inoculation. Roots of control seedlings were immersed in water.

Each treatment had 24 plants. Treated plants were transplanted into 7 x 7 x 8 cm pots filled with a substrate mixture of sand and steamed compost (1:3). Each pot had two plants with the same treatment and four pots were placed in one tray to avoid cross-contamination between treatments during irrigation. Each tray was considered one biological replicate. All trays were organized according to a completely randomized design and kept with the same light and temperature conditions as mentioned above. Due to greenhouse space limitations, 16 isolates were tested in six consecutive screening runs and each isolate was tested twice.

2.4.2. Disease assessment

Disease assessment was conducted at 7, 14, 21, and 28 days after inoculation (dpi). The assessment of yellowing and death of the leaves was done according to the 9-score assessment key described by Eynck et al. (2009) and the net area under the disease progress curve (AUDPC) was calculated accordingly. At 28 dpi,

plant height was measured from the cotyledons to the tip of the longest leaf to calculate the stunting effect caused by the disease.

2.4.3. Statistical analyses

The statistical analysis of the pathogenicity assessment was carried out with R (version 4.0.2.). Data of the two experimental replicates of each isolate were merged due to their high similarity. Two-way ANOVA was conducted for each parameter with a previous box-cox transformation. For post hoc analysis, a Tukey HSD test at a significance level of 0.05 was carried out. Pearson correlations between the parameters were also performed.

3. Results

3.1. *V. longisporum* isolation and lineage characterization

In total, 273 single spore isolates of *V. longisporum* were isolated from oilseed rape stubbles collected from 62 locations in eight countries between 2013 and 2017 (Table 4). Germany and Sweden were the countries from which the highest number of isolates was obtained, with 110 and 76 isolates from 20 and 17 locations, respectively. France and Poland followed, with 26 and 19 isolates from nine and three locations, respectively. In addition, there were 18 English isolates from three locations, five isolates from five different locations in Latvia and 15 Canadian isolates from two locations obtained. The highest number of isolates from one location was obtained from Schoningen and Fehmarn, which are both located in Germany. Multiplex-PCR products showed that all isolates had the same band size as the reference isolate PD638 (A1/D1), which indicated that all belong to lineage A1/D1 (Figure 2).

Table 4. Number of *Verticillium longisporum* isolates obtained from different locations from seven different countries between 2013 and 2017.

Country of origin	Number of locations	Year of sampling	Number of isolates
Canada	2	2017	15
Denmark	3	2016	3
UK (England)	3	2014-2016	18
France	9	2014-2016	26
Germany	20	2013-2016	110
Latvia	5	2016	5
Poland	3	2016	19
Sweden	17	2013, 2015	76
Total	62	**Total**	273

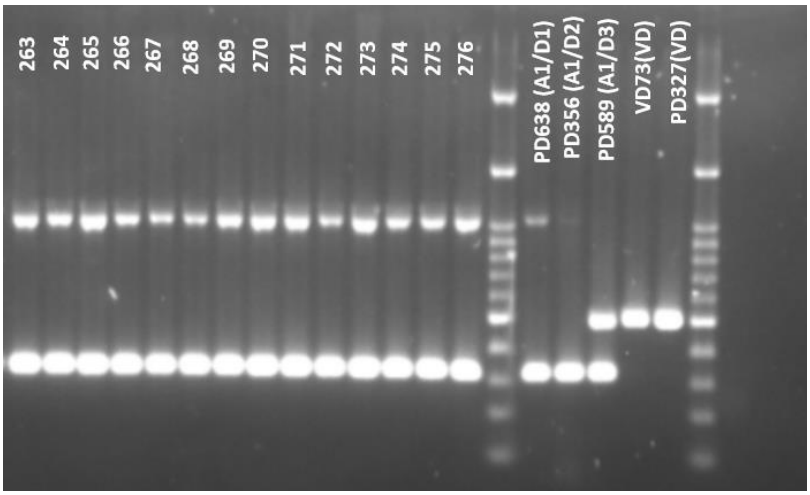

Figure 2. Multiplex-PCR to distinguish the *Verticillium longisporum* lineages A1/D1, A1/D2, and A1/D3 according to Inderbitzin et al. (2013). Three reference isolates corresponding to *V. longisporum* lineages were loaded (PD638, PD356, PD589) between the 1,000 bp ladders, followed by two *V. dahliae* reference isolates (VD73 and PD327). Lines 1 to 14 represent 14 different *V. longisporum* isolates, all exhibiting the band pattern of lineage A1/D1. VD= *V. dahliae.*

3.2. Phylogenetic analysis

A phylogenetic tree based on Nei´s genetic distance using 11,879 SVPs was constructed to illustrate the phylogenetic relationships between the three *V. longisporum* lineages (Figure 3). The first node of the resulting phylogeny separates two major clusters (A and B). Cluster A bifurcates in the *V. longisporum* A1/D2 and A1/D3 branch (A1) and the *V. dahliae* branch (A2). Cluster B contains isolates from the *V. longisporum* lineage A1/D1 group and it bifurcates in four main genetic groups, one of them (group four) consisting of a single French isolate (M76). Group one branches separately from the other groups. German isolates were distributed among groups one to three. Danish, Canadian, and Latvian isolates are only in group three, whereas English isolates were only present in group two. Group one and two had representatives from France, Germany, and England, with the addition of a Polish (M203) and Swedish isolate (M189). Isolates from the same field were found apart from each other in different groups, such as French isolates from Châtres (M76 and M77), German isolates from Fehmarn (M58 and M43), and Swedish isolates from Tomelilla (M189 and M191). Clustering was supported by robust bootstrapping values.

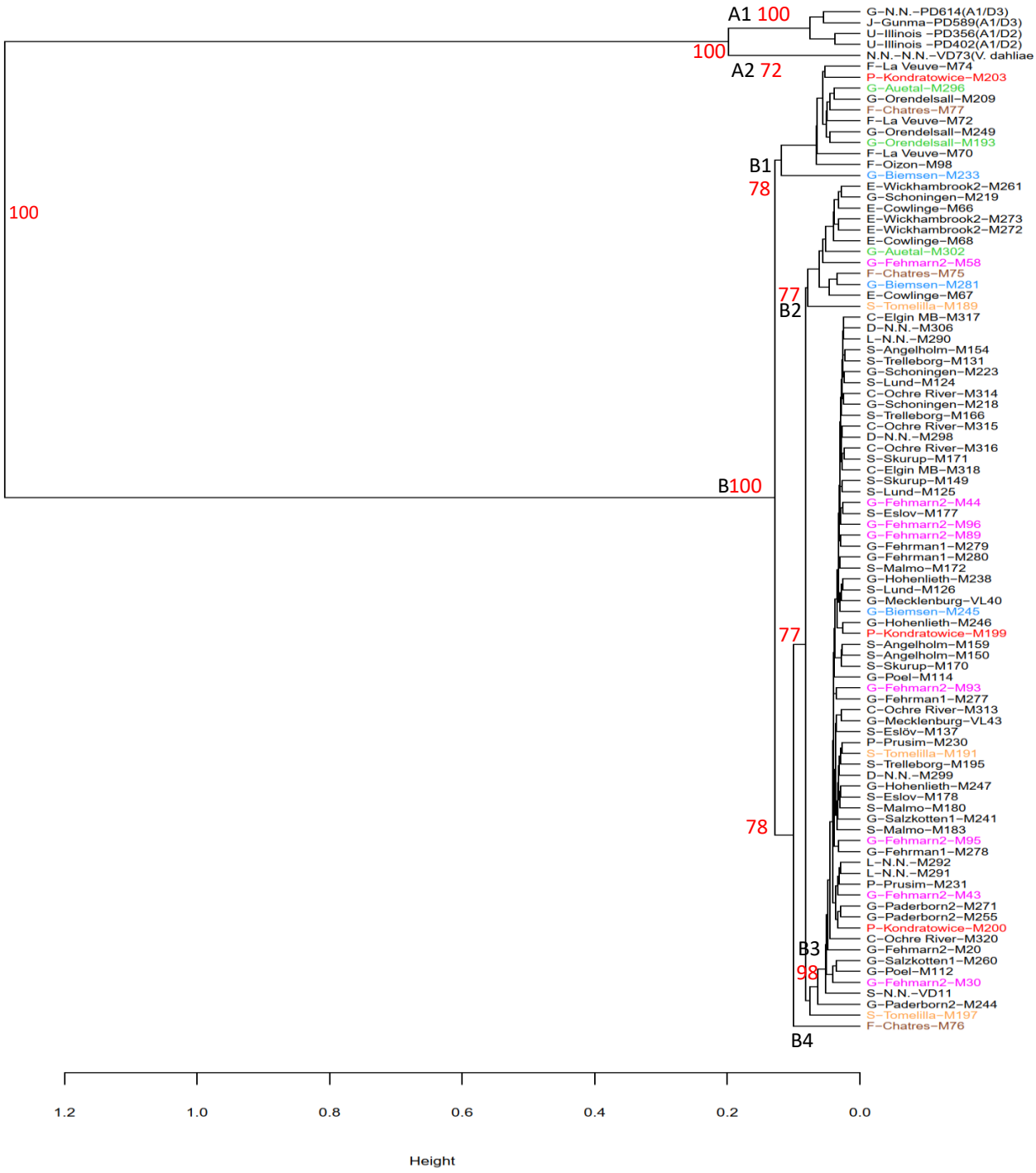

Figure 3. Dendrogram showing the genetic relationships between 89 European and Canadian isolates of V. *longisporum* lineage A1/D1, four isolates from lineages A1/D2 and A1/D3, and one *V. dahliae* isolate. The dendrogram was constructed based on 11,789 SVPs and Nei´s distance. The first node separated two major clusters (A and B). Cluster A bifurcates into the branch of *V. longisporum* lineages A1/D2 and A1/D3 (A1) and the branch of *V. dahliae* (A2). Cluster B contains the *V. longisporum* lineage A1/D1 group, which has four main groups (B1–B4). C= Canada; D= Denmark; E= England; F= France; G= Germany; J= Japan; L= Latvia; P= Poland; S= Sweden; U= USA; N.N= not known. Isolates from the same field are noted with the same color. Bootstrapping values of the main clusters are shown in red.

3.3. Pathogenicity characterization of the A1/D1 lineage in the greenhouse

Based on their geographic origin, a selection of 15 isolates from the main A1/D1 groups were used for the pathogenicity assessment (Table 5). A diverse level of pathogenicity was observed among the A1/D1 isolates. However, neither geographic origin nor genetic group explained the differences in net AUDPC and relative plant height (Figures 4 and 5). When isolates were either very aggressive or had a low pathogenicity, there were less differences between symptoms of the two cultivars. There was a positive strong correlation between AUDPC and plant height reduction (r_p=-0.87, P<0.0001) caused by all isolates in both cultivars. Nevertheless, some isolates that produced statistically similar symptoms on Falcon, such as E-W (261) and L (290), varied in their pathogenicity on Express. The three isolates F-V (72), P-K1 (199), and S-M (183) produced very mild symptoms that were statistically equal to those produced by the non-aggressive A1/D2 reference isolate PD402. The most aggressive isolates were G-S2 (219), S-T (195), and L (290) (Figure 4).

Table 5. *Verticillium longisporum* isolates (all lineage A1/D1 except PD402) used for virulence characterization on oilseed rape.

Isolate #	Code for pathogenicy test	Group in dendrogram	Country of isolation	Location	Year of Isolation
PD402	A1/D2	A1	USA	Illinois	1990s
VL43	VL43	B-3	Germany	Mecklenburg	1970s
M317	C- E (317)	B-3	Canada	Elgin MB	2017
M306	D (306)	B-3	Denmark	unknown	2016
M67	E-C (67)	B-2	UK (England)	Cowlinge	2014
M261	E-W (261)	B-2	UK (England)	Wickhambrook	2016
M72	F-V (72)	B-1	France	La Veuve	2014
M58	G-F (58)	B-2	Germany	Fehmarn	2013
M218	G-S1 (218)	B-3	Germany	Schoningen	2016
M219	G-S2 (219)	B-2	Germany	Schoningen	2016
M290	L (290)	B-3	Latvia	unknown	2016
M199	P-K1 (199)	B-3	Poland	Kondratowice	2016
M203	P-K2 (203)	B-1	Poland	Kondratowice	2016
M183	S-M (183)	B-3	Sweden	Malmö	2015
M195	S-T (195)	B-3	Sweden	Trelleborg	2015
M189	S-To (189)	B-2	Sweden	Tomelilla	2015

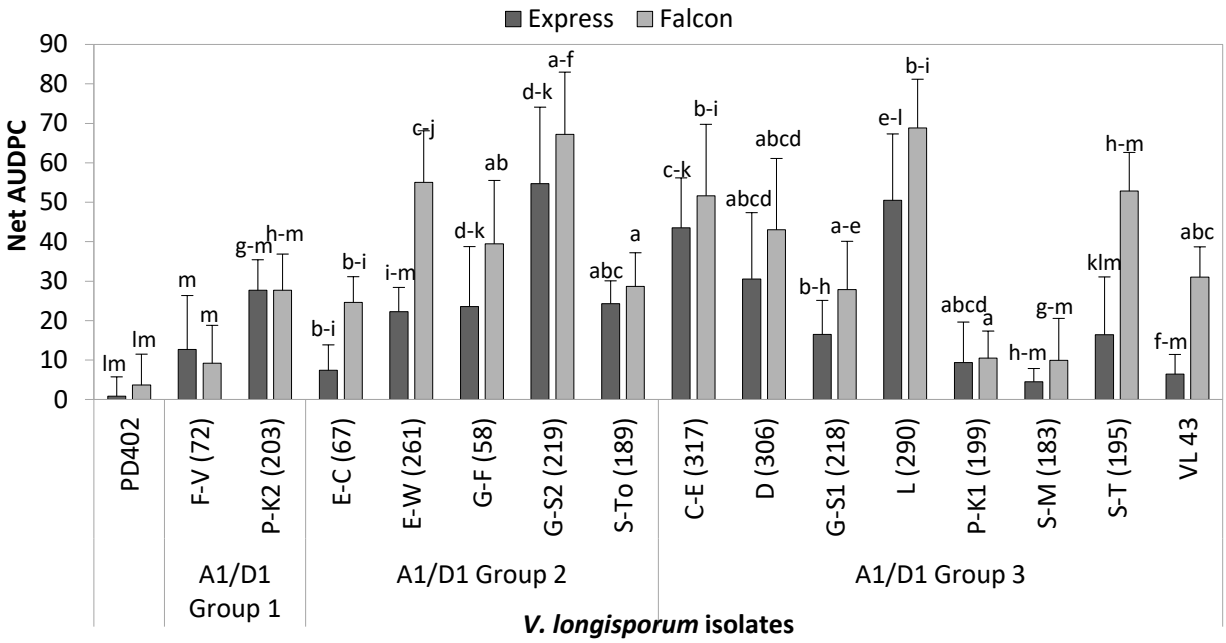

Figure 4. Net area under the disease progress curve (AUDPC) on moderately resistant oilseed rape cultivar Express and susceptible cultivar Falcon infected with *Verticillium longisporum* isolates (1.10^6 spores / mL) collected from oilseed rape fields. The non-aggressive isolate PD402 from *V. longisporum* lineage A1/D2 and aggressive isolate VL43 from lineage A1/D1 were used as references. Groups 1, 2, and 3 refer to different A1/D1 genetic groups based on phylogenetic analysis (see Figure 3). The first letter of the isolate code refers to the country of origin (C= Canada; D= Denmark; E= UK, England; F= France; G= Germany; L= Latvia; S= Sweden; P= Poland), the second letter indicates the location (C= Cowlinge; F= Fehmarn; E= Elgin MB; K= Kandrotowice; M= Malmö; S= Schoningen; T= Trelleborg; To= Tomelilla; V= La Veuve; W, Wichambrook). There were two isolates from Kandrotowice and Schoningen. Data points represent the mean of six biological replicates, each consisting of merged data from eight plants from two experimental repetitions. Error bars refer to the standard deviation. Different letters indicate significant differences (Tukey test, $P \leq 0.05$). When one treatment has more than five letters, they are shown as a range.

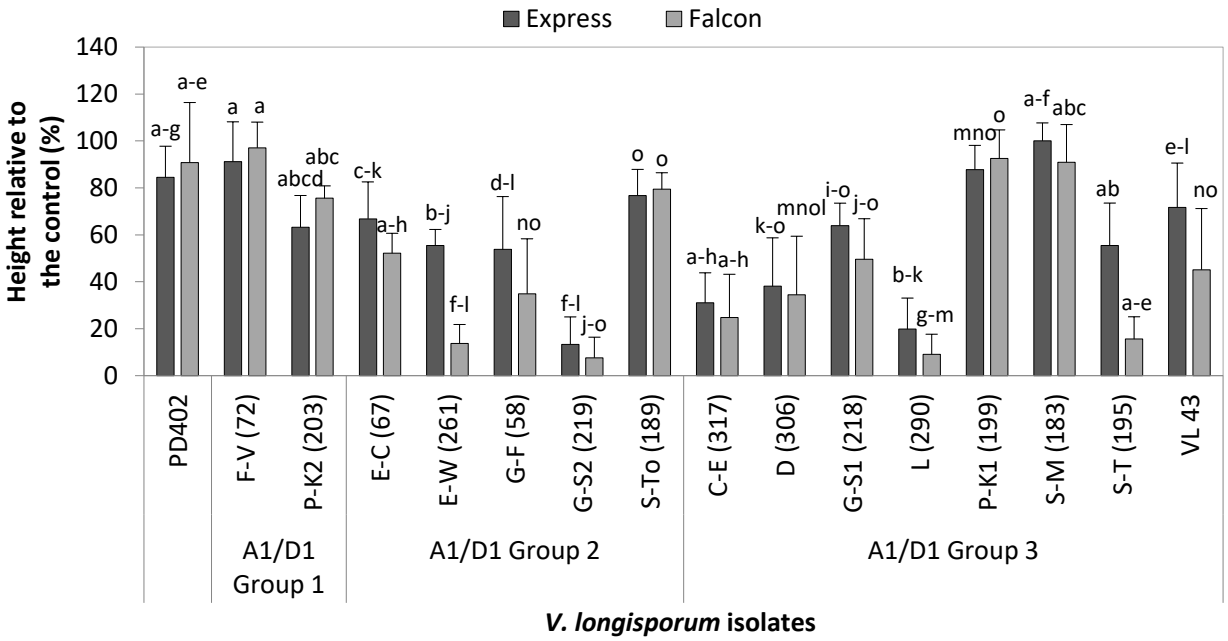

Figure 5. Plant height reduction in moderately resistant oilseed rape cultivar Express and susceptible cultivar Falcon infected with *Verticillium longisporum* isolates (1.10^6 spores / mL) collected from oilseed rape fields. The non-aggressive isolate PD402 from *V. longisporum* lineage A1/D2 and aggressive isolate VL43 from lineage A1/D1 were used as references. Groups 1, 2, and 3 refer to different A1/D1 genetic groups based on phylogenetic analysis (see Figure 3). The first letter of the isolate code refers to the country of origin (C= Canada; D= Denmark; E= UK, England; F= France; G= Germany; L= Latvia; S= Sweden; P= Poland), the second letter indicates the location (C= Cowlinge; F= Fehmarn; E= Elgin MB; K= Kandrotowice; M= Malmö; S= Schoningen; T= Trelleborg; To= Tomelilla; V= La Veuve; W, Wichambrook). There were two isolates from Kandrotowice and Schoningen. Data points represent the mean of six biological replicates, each consisting of merged data from eight plants from two experimental repetitions. Error bars refer to the standard deviation. Different letters indicate significant differences (Tukey test, $P \leq 0.05$). When one treatment has more than five letters, they are shown as a range.

4. Discussion

Based on a multiplex-PCR assay, collected strains of *V. longisporum* were assigned to three major lineages (Inderbitzin et al., 2011b). The monitoring conducted in European and Canadian oilseed rape fields with multiplex-PCR revealed that A1/D1 was the most relevant lineage, which confirmed that there is a high level of host specialization in *V. longisporum* field populations. To our knowledge, this is the first time that a *V. longisporum* lineage monitoring with such a high number of isolates and broad geographical scope has been carried out. The results are in tune with the work from Depotter et al. (2017) and Zou et al. (2020), where isolates collected from oilseed rape fields from England and Canada were also assigned to lineage A1/D1. Despite the dominance of A1/D1, Inderbitzin et al. (2011b) characterized several European isolates from oilseed rape collected in the 1980s as A1/D3. The lack of A1/D3 isolates in the present monitoring indicates that the increase of oilseed rape production in the last decades (Ismail et al. 2017) might have drifted the lineage distribution or that infection of oilseed rape by A1/D3 has always been merely anecdotal, while A1/D1 has invariably been the dominant lineage. There are other oilseed rape production regions with

a shorter *V. longisporum* history, such as Russia, the Czech Republic, or Slovakia (Bokor et al. 2014; Spitzer and Matušinsky 2017; Yu et al. 2016), that are still lacking a lineage monitoring. In a report from 2016, Yu et al. were not able to assign *V. longisporum* strains from Russian oilseed rape fields to any known lineage. Thus, it cannot be excluded that other hybridizations of *Verticillium* haploids may have happened and have been overseen in the studies so far because they took place in regions that were not monitored.

Phylogenetic relations of different *Verticillium* species have been addressed before by sequence comparison of the ITS region (van Tran et al. 2013) and five gene amplicons (Inderbitzin et al. 2011a). Similarly to those studies, the present phylogenetic analysis has revealed a closer relationship of *V. longisporum* A1/D2 and A1/D3 to *V. dahliae* than to *V. longisporum* lineage A1/D1. The phylogenetic analysis from Yu et al. (2016), based on two mitochondrial genes and the ITS region, reported that lineage A1/D3 clustered with *V. dahliae* and was separated from lineage A1/D2. However, the present study, similarly to Inderbitzin et al. (2011a), did not reveal differences in the genetic distance of the two lineages with *V. dahliae*.

Population genetic studies with *V. dahliae* based on microsatellites (Rafiei et al. 2018) and GBS (Dung et al. 2019) revealed genetic clusters related to the geographic origin. Genetic differences based on geographic origin have also been recorded for air-borne pathogens, such as *Colletotrichum truncatum* (Diao et al. 2015; Li et al. 2016) or *Cercospora beticola* (Knight et al. 2019). Depotter et al. (2017b) carried out a genetic characterization based on microsatellites of the *V. longisporum* lineage A1/D1, which resulted in separation of isolates from different *Brassica* crops into two groups: one consisting of isolates from the UK, Netherlands, the US, and Japan (West group), and another with representatives from Germany, France, Sweden, and Latvia (East group). In the present phylogenetic analysis, despite the lack of clear-cut genetic grouping of A1/D1 isolates based on country of origin, there were some broad clustering trends based on geographic regions. Swedish, Polish, Danish, Canadian, and Latvian isolates clustered together in group three, except for one Polish (M203) and one Swedish (M189) isolate. In group three, neither French nor English isolates were observed. Despite similar clustering trends with the work from Depotter et al. (2017), comparisons should be made with caution. Firstly, Depotter et al. (2017) used isolates from different *Brassica* hosts and based the genetic analysis on microsatellites. Moreover, although microsatellites present the advantage of being more polymorphic (Rafiei et al. 2018), GBS possesses a higher resolution (Milgroom et al. 2016) and is better suited for large spatial scales (Milgroom et al. 2016; Putman and Carbone 2014; Tsykun et al. 2017).

The study by Depotter et al. (2017) showed that English *Verticillium* isolates were genetically more diverse than all isolates from East Europe, but the continental European isolates used in that study had been isolated up to decades prior to the English ones. In the present study, German and French isolates seemed to have the highest genetic diversity, as they were the most broadly distributed among the A1/D1 clusters. Higher genetic diversity would be expected in Sweden, as this was the first country were the pathogen was detected on oilseed rape (Kroeker 1970). However, the more diverse climatic conditions in France and Germany may

have pushed the genetic diversity in those countries (Depotter et al. 2017b). *V. longisporum* has several potential hosts in the Brassicaceae family, and pathogenicity assays have shown that strains isolated from one host can be aggressive in different host species (Depotter et al. 2016a; Novakazi et al. 2015). The higher diversity of *Brassica* crops grown in Germany and France compared to Sweden (FAOSTAT 2018) might have increased the range of *Brassica* species acting as hosts and genetic reservoirs of the pathogen. The lineage A1/D1 has already been found in cabbage and cauliflower in Japan, China, and California, as well as in horseradish in Germany (Banno et al. 2015; Inderbitzin et al. 2011b; Yu et al. 2015). However, extensive *V. longisporum* lineage monitoring and comparative genetic analysis on different *Brassica* crops are still missing.

In the UK and Canada, the first reports of *V. longisporum* in oilseed rape are quite recent, from 2011 and 2014, respectively (CFIA 2015; Gladders et al. 2011). Separate clustering of Canadian and English isolates reported in this study might be an indication that the isolates introduced into these countries were of different origin. Being soilborne and soil-bound, *V. longisporum* should have a much shorter range of dissemination than airborne pathogens usually have. It is likely that the spread of soilborne inoculum requires the support of humans. Seed transmission of *V. dahliae* has been observed in cotton and spinach (Du Toit et al. 2005; Göre et al. 2011). In contrast, *V. longisporum* can rarely be transmitted by seed of winter oilseed rape under field conditions (Zheng et al. 2019a). However, seeds of spring type oilseed rape or other *Brassica* crops might have served as a carrier for long distance distribution.

This study revealed a high variability in pathogenicity within the lineage A1/D1 that could not be explained with the genetic subgrouping based on GBS (Figure 4 and 5). Similar studies with *V. dahliae* (Atallah et al. 2011; Bhat et al. 2003) and *V. albo-atrum* (Radišek et al. 2006) reported the difficulty of linking pathogenicity to genetic subgroups based on genome-wide genetic polymorphisms. This suggests several yet unknown highly variable loci to be involved in pathogenicity expression within the *V. longisporum* genome. The outcome also suggests that, in a single field, a highly diverse population structure of V. *longisporum* exists, which is indicated by the recovery of isolates from the same field positioned in different phylogenetic subgroups and with contrasting pathogenicity. In contrast, Del Mar Jiménez-Gasco et al. (2004) and Kolmer et al. (2013) showed, that *Fusarium oxysporum* and *Puccinia triticina*, respectively, exhibited a clonal genetic population structure and pathotypes correlated with the genetic groups inferred from DNA fingerprinting. This is often attributed to the asexual mode of fungal propagation, which can be associated with epidemics and invasion events. Clonal reproduction is an evolutionary short-term survival mechanism, ideally suited for rapid population expansion. However, it also results in genetic bottlenecks for pathogen populations, giving rise to small isolated fungal populations that lack adaptability to changes in their environment (Drenth et al. 2019). In the case of *Verticillium*, a long-term adaptability and survival strategy for this pathogen might have been the formation of interspecific polyploid hybrids, creating expanded population diversity allowing for

adaptation to diverse environments and hosts (Depotter et al. 2016b; Drenth et al. 2019). Besides hybridization, the role of non-sexual recombination in creating a genetically diverse population of *V. longisporum* might have previously been underestimated.

Three A1/D1 isolates from France, Poland, and Sweden produced very mild symptoms that were statistically similar to the symptoms produced by the non-aggressive isolate PD402 from lineage A1/D2. This contrasts with previous reports showing that lineage A1/D1 is usually highly aggressive on oilseed rape (Depotter et al. 2017a; Novakazi et al. 2015). The results of this study suggest that pathogenicity diversity of A1/D1 might be larger than previously thought. When collecting isolates for this study, the isolation method required high levels of microsclerotia infestation on the stubble epidermis to be successful (section 2.1.) The poor correlation between greenhouse symptoms and microsclerotia development on stubbles in the field revealed that saprophytic growth on senescent tissue does not reflect isolate pathogenicity or plant resistance, as previously suggested (Knüfer et al. 2017). Accordingly, Knüfer et al. (2017) found a significant correlation between greenhouse symptoms and the level of fungal colonization in the field at the beginning of ripening when fungal growth in plant tissue was assessed by qPCR.

The identification of locally diverse population structures of *V. longisporum* may further complicate the establishment of effective management strategies against *V. longisporum* in oilseed rape. The assessment of soil inoculum has proven to be an effective risk predictor against *V. dahliae* in spinach fields (Sapkota et al. 2016). However, the reported variability in pathogenicity of *V. longisporum* isolates within one field hampers reliable risk prediction based on soil inoculum. Moreover, the pathogenicity of recovered isolates was only tested under standard greenhouse conditions, but further external factors might influence disease severity. Chen et al. (2018) described how the wheat microbiome may affect the pathogenicity of *Fusarium graminearum*. It has been shown that elevated temperature increases the severity of *V .longisporum* infection (Siebold and Tiedemann 2013), and Onaga et al. (2017) reported that elevated temperature increases the expression of pathogenicity related genes of *Magnaporthe oryzae.* Thus, a deeper understanding of the underlying molecular mechanisms that define pathogenicity, as well as the impact of external factors that modulate them, is required to establish effective strategies for *V. longisporum* control in oilseed rape.

5. Author contributions

Marta Vega-Marín wrote the manuscript and performed the lineage monitoring and greenhouse assessment. Christian Obermeier performed Illumina data processing, SVP calling, and initial phylogenetic analysis. Marta Vega-Marin and Xiaorong Zheng further conducted the phylogenetic analysis. All authors were involved in designing the research, the revision of the manuscript, and the collective discussion of the results.

6. Acknowledgements

This work was financially supported by the German Federal Ministry for Food and Agriculture (BMEL) and the Agency for Renewable Resources (FNR). The sending of stubble samples by the following persons, institutions, and companies is highly appreciated: Alf Djurberg and Gunilla Berg from the Swedish Board of Agriculture, Gosia Jedryczka from the Institute of Plant Genetics of the Polish Academy of Sciences, Clinton Jurke from the Canola Council of Canada, Czech University of Life Sciences Prague, Limagrain, KWS, Deutsche Saatveredelung AG (DSV), Quintus, MU Agro, Syngenta, Saaten Union, SEGES, NPZ, and W. von Borries-Eckendorf. The authors acknowledge the technical assistance of Isabel Müller in the preparation of single spore cultures from plant material. The phenotypic assessment in the greenhouse was supported by Isabel Müller and Shilu Dahal, and the lab molecular work, including assistance in DNA extraction and multiplex-PCR, by Dagmar Tacke.

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8. Appendix

Appendix 1. *Verticillium longisporum* strains isolated from oilseed rape stubbles from different collection sites between 2013 and 2017 and used for lineage monitoring.

Country	Location	Year	Isolates
Canada	Elgin MB	2017	5
Canada	Ochre River	2017	10
Denmark	unknown 1*	2016	1
Denmark	unknown 2	2016	1
Denmark	unknown 3	2016	1
England	Cowlinge	2014	8
England	Wickhambrook 1	2014	4
England	Wickhambrook 2	2016	6
France	Châtres	2014	3
France	La Veuve	2014	5
France	Liverdy-en-Brie 1	2014	3
France	Liverdy-en-Brie 2	2014	4
France	Liverdy-en-Brie 2	2015	2
France	Oizon	2014	3
France	Pacy-sur-Armançon	2014	2
France	Presles-en-Brie 1	2016	1
France	Presles-en-Brie 2	2016	2
France	Verdun	2016	1
Germany	Auetal	2016	3
Germany	Barbis	2014	3
Germany	Biemsen	2016	5
Germany	Equord	2016	2
Germany	Fehmarn 1	2015	1
Germany	Fehmarn 1	2016	5
Germany	Fehmarn 2	2013	14
Germany	Fehmarn 2	2014	8
Germany	Fehmarn 2	2015	3
Germany	Fehmarn 2	2016	4
Germany	Groß Lengden	2016	2
Germany	Hohenlieth	2016	4
Germany	Klein Bülten	2016	1
Germany	Orendelsall	2016	3
Germany	Paderborn 1	2016	2
Germany	Paderborn 2	2016	3
Germany	Poel	2016	1
Germany	Poel	2015	3
Germany	Rolfshagen	2016	2
Germany	Salzkotten 1	2016	3
Germany	Salzkotten 2	2016	4
Germany	Salzkotten 3	2016	1

Germany	Schoningen	2016	31
Germany	Wibbecke	2014	2
Latvia	unknown 1	2016	1
Latvia	unknown 2	2016	1
Latvia	unknown 3	2016	1
Latvia	unknown 4	2016	1
Latvia	unknown 5	2016	1
Poland	Dąbrówka	2016	2
Poland	Kandratowice	2016	12
Poland	Prusim	2016	5
Sweden	Ängelholm	2015	4
Sweden	Eslöv	2015	4
Sweden	Helsingborg	2015	5
Sweden	Linköping	2015	7
Sweden	Lund	2015	9
Sweden	Malmö	2015	6
Sweden	Motala	2015	5
Sweden	Norsholm	2015	2
Sweden	Örebro	2015	1
Sweden	Simrishamn	2015	2
Sweden	Skänninge	2015	1
Sweden	Skurup	2015	5
Sweden	Svalöv	2013	10
Sweden	Tomelilla	2015	3
Sweden	Trelleborg	2015	4
Sweden	Vadstena	2015	7
Sweden	Ystad	2015	1
Total	62	Total	273

*Different numbers indicate different fields in the same location.

Appendix 2. *Verticillium longisporum* A1/D1 isolates used for phylogenetic analysis, and information on their host, location, and year of isolation. SOR= spring oilseed rape; WOR= winter oilseed rape.

Strain identifier	Lineage/ Species	Host	Country	Collection site	Year
M317, M318	A1/D1	SOR	Canada	Elgin MB	2017
M313-316, M320	A1/D1	SOR	Canada	Ochre River	2017
M298-299, M306	A1/D1	WOR	Denmark	Unknown 1,2,3	2016
M75-77	A1/D1	WOR	France	Châtres	2014
M70, M72, M74	A1/D1	WOR	France	La Veuve	2014
M98	A1/D1	WOR	France	Oizon	2014
M296, M302	A1/D1	WOR	Germany	Auetal	2016

M233, M245, 281	A1/D1	WOR	Germany	Biemsen	2016
M20, M30, M43-44, 58	A1/D1	WOR	Germany	Fehmarn 2	2013
M89, M93, M95-96	A1/D1	WOR	Germany	Fehmarn 2	2014
M277-280	A1/D1	WOR	Germany	Fehrman 1	2016
M238, M246-247	A1/D1	WOR	Germany	Hohenlieth	2016
M193, M209, M249	A1/D1	WOR	Germany	Orendelsall	2016
M244, M255, M271	A1/D1	WOR	Germany	Paderborn 2	2016
M112, M114	A1/D1	WOR	Germany	Poel	2015
M241, M260	A1/D1	WOR	Germany	Salzkotten 1	2016
M218-219, M223	A1/D1	WOR	Germany	Schoningen	2016
M290-292	A1/D1	WOR	Latvia	unknown 1, 2, 3	2016
M199-200, M203	A1/D1	WOR	Poland	Kondratowice	2016
M230-231	A1/D1	WOR	Poland	Prusim	2016
M150, M154, M159	A1/D1	WOR	Sweden	Ängelholm	2015
M137, M177	A1/D1	WOR	Sweden	Eslöv	2015
M124-126	A1/D1	WOR	Sweden	Lund	2015
M172, M180, M183	A1/D1	WOR	Sweden	Malmö	2015
M149, M170-171	A1/D1	WOR	Sweden	Skurup	2015
M189, M191, M197	A1/D1	WOR	Sweden	Tomelilla	2015
M131, M166, M195	A1/D1	WOR	Sweden	Trelleborg	2015
M66-68	A1/D1	WOR	UK, England	Cowlinge	2014
M272-273, M261	A1/D1	WOR	UK, England	Wickhambrook 2	2016

*Different numbers are used to describe different plots and/or fields.

Chapter III. Biocontrol potential of the non-aggressive *Verticillium longisporum* lineage A1/D2 against the aggressive lineage A1/D1 in oilseed rape

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Abstract

Verticillium longisporum is a soil-borne vascular pathogen of oilseed rape and other *Brassica* crops. The long survival of fungal resting structures in the soil and lack of effective curative measures make soil-borne vascular pathogens particularly difficult to control. The limited availability of control measures against them calls for the investigation of new control alternatives. Cross-protection refers to the use of non-aggressive or non-aggressive isolates of vascular pathogens as biocontrol agents. *V. longisporum* consists of three lineages that originated from three independent hybridization events of four haploid *Verticillium* parents. Previous pathogenicity tests in the greenhouse have shown that lineage A1/D2 is mostly non-aggressive on different *Brassica* and non-*Brassica* hosts. Thus, the cross-protection potential of the A1/D2 lineage against an aggressive *V. longisporum* isolate in oilseed rape was tested. A1/D2 did not inhibit the growth of the aggressive isolate *in vitro*. However, *in planta,* using root-dip inoculation, A1/D2 reduced disease symptoms when applied before, after, or at the same time as the aggressive isolate. Moreover, differences in the biocontrol efficacy of isolates were observed. These results indicate that the putative biocontrol mechanisms of A1/D2 following root-dip inoculation are competition for infection sites in the rhizosphere and induction of plant defense. However, the induction of SA, a signal known in basal and cultivar-related resistance, was not involved in the biocontrol mechanism. To the best of our knowledge, this is the first report of efficient biocontrol against *V. longisporum* in oilseed rape with a non-aggressive strain by root-dip inoculation. However, A1/D2 application with a seed coat, which is the most practical and best feasible application method, failed to confirm the biocontrol effect observed with root-dip inoculation. Confocal microscopy analysis revealed that seed coating led to insufficient A1/D2 hyphal establishment on the roots compared to root-dip inoculation, which may explain the lack of a biocontrol effect with a seed coat and illustrates the importance of the application method for efficacy of a biocontrol agent.

1. Introduction

Soilborne vascular aggressive fungi are amongst the most destructive plant pathogens. The long survival of their fungal resting structures in the soil and the lack of effective curative measures make them very difficult pathogens to control (Deketelaere et al. 2017). Verticillium wilts are widely distributed vascular diseases responsible for immense economic losses in more than 200 crops. They are caused by soilborne monocyclic pathogens that survive in the soil as microsclerotia, resting mycelium, or chlamydospores**.** *Verticillium longisporum* is aggressive in *Brassica* horticultural crops, as well as in oilseed rape (Depotter et al. 2015). In oilseed rape, instead of producing wilting symptoms, *V. longisporum* causes a dark unilateral striping on the stem during ripening of the crop (Eynck et al. 2007). The striping symptoms originate from necrosis produced after the fungus leaves the xylem and colonizes the parenchyma, which leads to premature ripening of the crop. The symptoms caused by *V. longisporum* in oilseed rape in the greenhouse differ from those that occur under field conditions. After root-dip inoculation at the seedling stage in the greenhouse, plants show chlorosis, vascular discoloration, and stunting (Eynck et al. 2007). Despite symptoms being distinctly different in the field, Knüfer et al. (2017) showed a close correlation between the severity of greenhouse symptoms and level of fungal colonization in the field at the beginning of ripening.

Yield losses caused by *V. longisporum* have been recorded by Dunker et al. (2008) in individual plants under controlled conditions. In the field, yield losses depend on the cultivar, disease incidence, and weather conditions (Depotter et al. 2019; Zheng et al. 2019c). *V. longisporum* has been found in most oilseed rape growing countries, including Canada, Czech Republic, France, Germany, Russia, Sweden, Slovakia, Poland and the UK (Bokor et al. 2014; CFIA 2015; Depotter et al. 2015; Gladders et al. 2011; Inderbitzin et al. 2011a; Spitzer and Matušinsky 2017; Steventon et al. 2002; Yu et al. 2016). The worldwide demand for biodiesel is driving oilseed rape production and shortening crop rotations, which contributes to an increase in disease incidence (Depotter et al. 2015; Ismail et al. 2017).

Integrated disease management with a combination of different cultural control methods remains the only strategy available against vascular pathogens. Chemical soil treatment is strongly restricted due to environmental and health concerns. The limited effectiveness of the available control measures against vascular pathogens calls for the investigation of new alternatives, such as the use of microorganisms as biocontrol agents (Deketelaere et al. 2017; Yadeta and Thomma 2013). Several studies have shown the biocontrol potential of various fungi and bacteria against vascular pathogens (Angelopoulou et al. 2014; Dong et al. 2006; Markakis et al. 2008; Sant et al. 2010; Scheffer et al. 2008; Stinson et al. 2003). One biocontrol approach against vascular pathogens is using non-pathogenic isolates from the same species or from other vascular pathogens. This type of biocontrol strategy has been described as cross-protection (Deketelaere et al. 2017; Tyvaert et al. 2014). These protective isolates might be weak pathogens (non-aggressive) in the host

plant or non-pathogenic in a strict sense (Qin et al. 2008). In cross-protection, protective isolates typically share the same ecological niche as the pathogen and display several biocontrol modes of action (Deketelaere et al. 2017). These include competition for space in the rhizosphere (Pantelides et al. 2009), plant growth promotion (Shittu et al. 2009), or induction of plant defense (Angelopoulou et al. 2014; García et al. 2011). The rapid activation of defense responses upon pathogen infection after a treatment with certain compounds or organisms is called priming (Conrath et al. 2002). A priming effect has previously been reported for fungal biocontrol agents against soil-borne pathogens (Begum et al. 2010; El-Mougy and Abdel-Kader 2008; Tonelli et al. 2011).

There has been extensive research on the biocontrol potential of non-pathogenic *Fusarium* isolates against Fusarium wilts in different crops (Elmer 2004; Fravel et al. 2003; Freeman et al. 2002; Kaur et al. 2010; Nel et al. 2006; Sajeena et al. 2020; Silva and Bettiol 2005). Additionally, some reports have shown that non-pathogenic *Fusarium* isolates conferred protection against Verticillium wilts (Angelopoulou et al. 2014; Pantelides et al. 2009; Veloso and Díaz 2012; Zhang et al. 2015). Similarly, non-pathogenic *V. dahliae* isolates reduced Verticillium wilt in tomato, cotton, and strawberry (Diehl et al. 2013; Shittu et al. 2009; Zhu et al. 2013) and *V. tricorpus* isolate protects lettuce against *V. dahliae* (Qin et al. 2008). So far, two studies have shown significant biocontrol effects of a non-pathogenic *V. isaacii* isolate against *V. longisporum* (França et al. 2013; Tyvaert et al. 2014).

The scarce research on non-pathogenic and non-aggressive *Verticillium* isolates as biocontrol agents against *V. longisporum* calls for the screening of new biocontrol candidates. *V. longisporum* consists of three genetically distinct lineages that originated from three independent hybridization events of four haploid *Verticillium* parents. Two of these parents, D2 and D3, correspond to *V. dahliae*. The parents A1 and D1 are unknown *Verticillium* species. While isolates from the lineage A1/D1 and A1/D3 have a wider geographic distribution and host range, A1/D2 has so far only been found in horseradish in Illinois and Ontario (Inderbitzin et al. 2011b; Yu et al. 2016). Pathogenicity tests in the greenhouse revealed that A1/D1 was the most aggressive lineage on oilseed rape, while A1/D2 was the least aggressive on all tested *Brassica* and non-*Brassica* crops (Novakazi et al. 2015). Thus, due to its consistent non-aggressiveness, A1/D2 was considered a candidate for effective cross-protection.

The ultimate success of a biocontrol agent in the field mostly depends on its establishment capacity and competition ability (Alabouvette et al. 2009; Lopisso et al. 2017). Consequently, the mode of application of the biocontrol agent is critical for effective control. Moreover, its application has to be practically feasible and economically justified, which is particularly critical in large field crops. Biocontrol agents are typically directly added to the soil or applied to the seeds, leaves, or roots. In arable crops, the best application method for a biocontrol agent is as seed coat (Alabouvette et al. 2009; Rocha et al. 2019).

In the present study, the potential of A1/D2 isolates as biocontrol agents against an aggressive A1/D1 isolate was investigated on oilseed rape. To this end, an *in vitro* and *in planta* assay with root-dip inoculation were used to screen biocontrol candidates within the lineage A1/D2. The best performing isolate from those assays was tested as a seed coat, with the aim to assess its potential efficacy in the field. Because salycilic acid (SA) is involved in *V. longisporum* resistance (Zheng et al. 2019a), its role in the biocontrol effect exhibited by A1/D2 was investigated, as well as whether priming is involved in the biocontrol mechanism. Finally, differences in root colonization by A1/D2 upon different application methods were investigated with confocal microscopy.

2. Methods

2.1. Fungal isolates and inoculum production

V. longisporum isolates were obtained from the fungal isolate collection of the Division of Plant Pathology and Crop Protection of the Georg-August University of Göttingen. To assess the biocontrol potential of the non-aggressive lineage A1/D2 against an aggressive A1/D1 isolate on oilseed rape, three isolates from A1/D2 were selected. The selected A1/D1 isolate (VL43) was collected from a diseased oilseed rape plant in Germany in the 1990s (Zeise and Tiedemann 2001 and 2002). VL43 has been extensively used in oilseed rape research and its pathogenicity on this crop is well documented (Depotter et al. 2017; Eynck et al. 2007; Eynck et al. 2009a; Novakazi et al. 2015; Zheng et al. 2019a; Zheng et al. 2019b). The three A1/D2 isolates were obtained from horseradish in the USA and are non-aggressive on oilseed rape (Depotter et al. 2017; Novakazi et al. 2015) (Table 1).

Table 1. *Verticillium longisporum* isolates used in this study.

Identifier	Identifiers in literature	Host	Sampling site	Collection	Reference
VL43	PD638, VL43	*Brassica napus* (oilseed rape)	Mecklenburg, Germany	[a]UGoe	Zeise and Tiedemann 2001 and 2002
A1/D2a	PD402	*Armoracia rusticana* (horseradish)	Illinois, USA	[b]UC Davis	Inderbitzin et al. 2011b
A1/D2b	PD730	*A. rusticana* (horseradish)	Illinois, USA	UC Davis	Inderbitzin et al. 2011b
A1/D2c	PD356	*A. rusticana* (horseradish)	Illinois, USA	UC Davis	Inderbitzin et al. 2011b

[a]UGoe= Georg-August University of Göttingen, [b]UC Davis= University of California, Davis

2.1.1. Spore inoculum

For the preparation of spore inoculum, 1 mL of spore suspension, which had previously been stored axenically at -80 °C in 25% aqueous glycerol, was added into potato dextrose broth (PDB) in Erlenmeyer flasks with 400 ppm streptomycin, 50 ppm chloramphenicol, and 50 rifampicin. PDB flasks were incubated on a shaker (100 rpm) at 22 °C in the dark. After 10 days, the culture was filtered through a sterile sieve. The spore density

was determined using a Thoma haemocytometer and diluted to 1 x 10^6 spores / mL. The spore suspension was applied as a 30 min root-dip inoculation. The spore suspension was applied either as a 30 min root-dip inoculation or as a seed coat.

2.1.2. Microsclerotia inoculum

Sand-rye flour (SRF) medium (2 kg Quartz sand, 142 g rye flour, and 190 mL water) was prepared to produce microsclerotia inoculum. SRF medium was autoclaved two times in plastic bags (55 x 33.5 cm). After cooling, three agar plugs from two-week-old fungal potato dextrose agar (PDA) cultures, as well as 20 mL sterile tap water, were added to each plastic bag. Bags were stored in the dark at room temperate for up to eight weeks and kneaded a couple of times per week to promote fungal growth throughout the whole medium. After sufficient production of microsclerotia, visible through the appearance of black grains inside the SRF medium, the bags were opened and their content was scattered onto plastic trays (50 x 32 x 6.5 cm). The trays were stored in a drying chamber at 25 °C for three to five days until moisture evaporated. For separation of the microsclerotia from the SRF medium, the mixture was poured in a sieve shaker and partitioned by particle sizes of 0–100 µm, 100–315 µm, 315–500 µm und >500 µm. The sieved microsclerotia were stored in the dark at 4 °C until application as a soil amendment or as seed coat.

2.2. Antagonistic potential of A1/*D2 in vitro*

To evaluate the *in vitro* antagonism between A1/D2 and the aggressive isolate VL43, a dual culture assay was conducted. The effect of A1/D2 on the *in vit*ro growth of VL43 was measured, as well as the effect of VL43 on growth of A1/D2. Agar plugs (8-mm diameter) were taken from two-week-old fungal colonies grown on PDA. One agar plug of VL43 was placed on PDA at 3 cm from each A1/D2 isolate. As a control, each isolate was plated against itself. Four Petri dishes per combination were prepared, and the experiment was repeated three times. Additionally, to assess the potential role that volatile metabolites might have on fungal growth, each plating combination was conducted both with paraffin-sealed and non-sealed Petri dishes. Petri dishes were kept completely randomized in a dark climate chamber at 23 °C for three weeks. The radius of colonies was measured at 7 and 14 days post-inoculation (dpi). At each time point, the difference between the growth of the isolate when plated against itself and when plated against another isolate was calculated. At 21 dpi, images of colony morphology were taken.

2.3. Biocontrol potential of A1/D2 with root-dip inoculation

2.3.1. Experimental design

To evaluate the potential biocontrol effect of the non-aggressive *V. longisporum* lineage A1/D2 *in planta*, a greenhouse screening with oilseed rape seedlings was conducted. Seeds of the susceptible oilseed rape cultivar 'Falcon' (Eynck et al. 2009b) were pre-germinated in quartz sand at 18–24 °C and with a 14 h

photoperiod (Horti-Lux HPS-400 Watt). Ten days after sowing, seedlings were removed from the quartz sand and washed thoroughly under running tap water. After the removal of sand particles, seedlings were dipped in a spore solution prepared as described in section 2.1.1. Roots of control seedlings were immersed in water.

Three inoculation methods (pre-inoculation, co-inoculation and post-inoculation) were used, with which A1/D2 isolates were inoculated before, at the same time or after the aggressive isolate VL43. An inoculation scheme of each method is shown in Table 2. For the pre-inoculation method, seedlings were first inoculated with an A1/D2 isolate or water. At 7 dpi, seedlings were removed from the soil, and the roots were briefly washed before treatment with water or VL43. For co-inoculation, a non-aggressive A1/D2 isolate was inoculated at the same time as VL43. With the post-inoculation approach, VL43 or water were inoculated and at 7 dpi, seedlings were treated with water or an A1/D2 isolate. To avoid cross-contamination, seedlings inoculated with different treatments in the first week were treated separately in the second week.

Table 2. Procedure of inoculation methods used to assess the biocontrol potential of isolates from the non-aggressive *Verticillium longisporum* lineage A1/D2 against the aggressive isolate VL43 on oilseed rape seedlings of cultivar Falcon via root-dip inoculation. The second treatment was done seven days after the first one.

Inoculation method	1st treatment	2nd treatment
Pre-inoculation	Water	Water
	Water	VL43
	A1/D2	VL43
Co-inoculation	Water	X
	VL43	X
	A1/D2+VL43	X
Post-inoculation	Water	Water
	Water	A1/D2
	VL43	A1/D2

The three treatments (water, VL43, and A1/D2+VL43) of each inoculation method consisted of 24 plants. Treated plants were transplanted into 7 x 7 x 8 cm pots filled with a soil mixture of sand and steamed compost (1:3). Each pot had two plants with the same treatment and four pots were placed in one tray to avoid cross-contamination between treatments during irrigation. Each tray was considered one biological replicate. All trays were organized according to a completely randomized design and kept with the same light and temperature conditions as previously mentioned. The experiment was repeated twice.

2.3.2. Disease assessment

Disease assessment was conducted at 7, 14, 21, and 28 dpi. In the case of the pre- and post-inoculation methods, assessment started after the second inoculation. The evaluation of yellowing and death of leaves was performed according to the 9-score assessment key described by Eynck et al. (2009b), and the net area under the disease progress curve (AUDPC) was calculated accordingly. At 28 dpi, plant height was measured

from the cotyledons to the tip of the longest leaf to calculate the height reduction caused by disease in relation to the control.

2.4. Potential priming effect of A1/D2

2.4.1. Experimental design

To elucidate a potential priming effect of A1/D2 in oilseed rape against VL43, the effect of inoculation with A1/D2 on SA production of cultivar Falcon. Seedlings were root-dip inoculated following the pre-inoculation method described in section 0. The four treatments were water-water, A1/D2b-water, water-VL43, and A1/D2b-VL43. Aerial parts of the plants were sampled at 6 and 10 dpi. For each treatment and sampling day, 5 samples of 10 pooled plants were harvested. The trays were arranged in a completely randomized design. The experiment was performed twice in the same conditions as described in section 0. Samples were immersed in liquid nitrogen immediately after collection to halt any metabolic processes and stored in a freezer at – 22 °C.

2.4.2. Free SA extraction

Frozen samples of plant tissue were homogenized with a 5 mm tungsten ball by using a MM400 mill (Retsch GmbH, Haan, Germany) at 25 hz for 1 min. Free SA was extracted according to Kamble and Bhargava (2007) with modifications. Ground plant tissue (100 mg) was suspended in 1.5 mL of acetone, shaken vigorously and centrifuged (5500 rpm) at 4 °C for 45 min. This step was repeated twice and the two supernatants were merged and evaporated in a speed vacuum centrifuge at 30 °C. The residue was dissolved in 1 mL demineralized water, and 1 mL ethyl acetate was subsequently added. The upper phase was transferred and evaporated at 35 ◦C. The residue was dissolved again in 200 µL of high-performance liquid chromatography (HPLC) grade methanol.

2.4.3. HPLC

Samples were centrifuged for 2 min at 5000 rpm to precipitate unsolvable particles before loading them into HPLC vials. A HPLC-fluorescence system consisting of a ProStar 410 autosampler, a Prostar 210 binary pump system (Varian, Darmstadt; Germany) with a 10 W SS head, a LiChrospher ® 100 RP18 reverse phase column (particle size 5 µm) inside a LiChroCART ® 125-1 HPLC cartridge (Merck KGaA, Darmstadt; Germany) at 30 °C, and a Varian ProStar 363 fluorescence detector was used. For fluorescence detection, an excitation wavelength of 315 nm and emission wavelength of 405 nm were used. Each sample was analyzed for 27 min under a bi-mobile phase with (A) 20 mM sodium acetate pH 5.0 and (B) methanol with a flow rate of 1 mL / min with the following protocol: 0.0–2.0 min 10 % B, 2.0–10.0 min from 10% B to 30 % B, 10.0–13.0 min from 30% B to 98% B, 13.0–18.0 min 98% B, 18.0–21.0 min from 98% B to 10% B, and 21.0–25.0 min 10 % B. A dilution series from 100 nM to 20 µM of SA dissolved in HPLC-grade methanol was used as

a standard. The free SA was quantified using the Galaxie Chromatography Workstation software (Varian, 2002, Darmstadt; Germany).

2.5. Biocontrol potential of A1/D2 as a seed coat

The best performing A1/D2 isolate from the previous assays (sections 2.2 and 0) was selected to test two seed coating methods.

2.5.1. Seed coating

For the seed coating with an A1/D2 isolate, seeds of the spring oilseed rape cultivar `Licosmos´ were surface sterilized by soaking in 70% ethanol for 2 min. Afterwards, they were washed with sterile water and stirred in 1% sodium hypochlorite + 1% Tween 80 solution for 15 min. Following disinfection, seeds were washed three times with sterilized water. Seeds were coated either with microsclerotia or a spore suspension. For the microsclerotia seed coating, preparation of the liquid formulation followed the procedure by Lopisso et al. (2017) with modifications. First, a sticker solution containing methylcellulose (MC) (MFCD00081763, Sigma-Aldrich; USA) was prepared by dissolving 1 % methylcellulose in distilled and boiled water. The solution was mixed on a magnetic stirrer for two hours. When the solution reached room temperature, 60 mg microsclerotia of 0–100 µm in size were dissolved in a 100 mL MC solution. For the seed coating, 2 g of oilseed rape seeds were mixed with 2.5 mL of the microsclerotia-MC solution in 15 mL Falcon tubes. Mixing was performed by vigorously shaking the tubes for 1 min by hand and vortexing them five times for 1 min. Afterwards, coated seeds were distributed apart from each other to prevent clumping and dried under a laminar flow cabinet. Seeds of the control treatment were coated with MC alone.

For the seed coating with spores, a spore suspension was prepared as described in section 2.1.1. The sterilized seeds were left in an A1/D2 spore suspension (10 mL spore suspension/g seeds) for 30 min, and the suspension was stirred manually three times during the incubation period. Afterwards, they were dried under a laminar flow cabinet. Seeds of the control treatment were coated with water.

2.5.2. Microsclerotia soil amendment

VL43 was applied into the soil as a microsclerotia soil amendment. For that, microsclerotia of size 315–500 µm were added at a concentration of 800 mg microsclerotia / kg soil. Microsclerotia were mixed manually in the soil by adding 50 mL of water per kg of soil.

2.5.3. Experimental design and evaluation of disease development

Treated seeds were directly transplanted into 7 x 7 x 8 cm pots filled with a soil mixture of sand and compost (1:3) and each pot had just one plant. For this assessment, 32 plants divided in four trays were used per treatment (control, VL43 soil amendment alone or combined with A1/D2 seed coating). Each tray was

regarded as one biological replicate and all trays were organized according to a completely randomized design. Each seed coating method was tested in a climate chamber and greenhouse. In the climate chamber, conditions were the same as in section 0. In the greenhouse, the temperature range was 15–25 °C. Disease evaluation was conducted by weekly assessing the plant height (cm) from 28 to 84 dpi. For that, plants were measured from the hypocotyl to the highest point of the plant (including leaves, flowers, and pods). Necrotic dead plants were denoted with a zero.

2.6. Microscopic assessment of different A1/D2 applications on oilseed rape roots

To assess root colonization ability of A1/D2 with different application methods, hyphal colonization of the external layers of the roots was assessed by confocal microscopy at 14 dpi.

2.6.1. Experimental design

Double surface sterilized Falcon seeds (see section 0) were sown in trays containing quartz sand. For the assay with root-dip inoculation, six seedlings per treatment were uprooted and root-dip inoculated with A1/D2b as described in section 2.1.1. For the seed coating method, seeds were either coated with spores or microsclerotia as described in section 0. Sand was selected as substrate to ensure the harvest of complete roots without any adhered material. Pots were watered every other day and fertilized twice per week with the full nutrient solution “Flory Basisdünger” (EUFLOR GmbH, Schermbeck; Germany). The experiment was conducted twice with the same conditions as in section 0. Two weeks after treatment application, plants were carefully uprooted and roots were briefly washed. The differentiation zone of the main root was investigated by selecting, with light microscopy, two sections that presented hyphae. Then, intensity of hyphal colonization was investigated with confocal microscopy.

2.6.2. Staining and confocal microscopy.

The entire roots were stained with a sequential double staining method. Samples were first immersed in 50 $\mu g.mL^{-1}$ Wheat Germ Agglutinin, Alexa Fluor® 488 Conjugate (W11261, Molecular probes; USA) and vacuum-infiltrated for 30 min in the dark at room temperature. Subsequently, samples were vacuum-infiltrated for 20 min with 10 $\mu g.mL^{-1}$ propidium iodide (2470810 Carl Roth GmbH, Karlsruhe; Germany). Roots were immediately dipped twice in distilled water, mounted on 50% glycerol and covered with a coverslip (0.16 mm thickness). Microscopic examinations were performed using a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Manheim; Germany). A two-channel analysis was carried out, with a 488 nm wavelength to excite Alexa Fluor and 500–530 nm to receive the emission. In the case of propidium iodide, a 550–560 nm wavelength range was used for excitation and 608–680 nm to receive the emission.

2.7. DNA quantification

For quantification of the potential biocontrol effect of the A1/D2 lineage against systemic colonization of VL43, qPCR analysis was carried out. In the case of the root-dip experiments, stems of the eight plants from each tray were pooled together and considered one biological replicate. For the seed coating experiments, since plants were bigger, the hypocotyl of three randomly selected plants per tray were used. Each experimental repetition had four biological replicates. In the case of the root-dip inoculation experiments, only reduction of the VL43 systemic colonization by the best performing A1/D2 isolate was assessed. Plants were lyophilized and ground to fine powder with liquid nitrogen. Total DNA was extracted from 50 mg of ground sample using the cetyltrimethylammonium bromide (CTAB) method (Brandfass and Karlovsky 2008). After DNA extraction, the pellet was dissolved overnight in 200 µL TE buffer (10mM Tris, 1 mM EDTA, pH8) at 4 °C. The quality of the extracted DNA was verified by running the samples 60 min at 3 V/cm on 1% agarose gel (Roth, Karlsruhe; Germany) stained with Midori Green (Nippon Genetics Europe GmbH, Düren; Germany) in 0.5x TBE (Tris-Boric acid-EDTA) buffer. DNA was visualized under UV light and documented with the software INTAS GelDoc (Intas Science Imaging Instruments GmbH, Göttingen; Germany).

A CFX384 Thermocycler (Bio-Rad, Rüdigheim; Germany) with 384 well microplates (SARSTEDT AG & Co. KG, Nümbrecht; Germany) was used for the amplification and quantification of *V. longisporum* DNA using ß-tubulin forward (5'-GCAAAACCCTACCGGGTTATG-3') and reverse (5'-AGATATCCATCGGACTGTTCGTA-3') primers (Debode et al. 2011). The amplification reaction had a total volume of 10 µL, which consisted of 5 µL of qPCRBIO SyGreen Mix (Nippon Genetics Europe GmbH, Düren; Germany), 0.4 µM of each primer, and 10 ng of template DNA. The PCR program consisted of a 3 min initial denaturation step, 40 cycles of 5 s at 95 °C, 15 s at 68 °C, and 15 s at 72 °C, followed by a final extension of 2 min at 72 °C. Each sample had three technical replicates and data was analyzed using software BioRad CFX Maestro 1.1 (Bio-Rad laboratories, Inc.; USA).

2.8. Statistical analyses

Statistical analyses were carried out with R (version 4.0.2.). A t-test or Wilcoxon Rank Sum test were performed for pair-wise comparisons of the *in vitro* and qPCR assays. Because the root-dip experiments were repeated twice, each inoculation method (pre-inoculation, co-inoculation, and post-inoculation) was analyzed with a linear mixed model, where experimental replicates were the random factor. In the seed coating and priming experiments, a one-way ANOVA or Kruskal-Wallis test were carried out. For the parametric statistical tests, a box-cox transformation was performed if necessary, based on the variance homogeneity. For post hoc analyses, Tukey HSD or pairwise Wilcoxon Rank Sum tests at a significant level of 0.05 were performed.

3. Results

3.1. Antagonistic potential of A1/D2 *in vitro*

For the dual culture assay *in vitro*, the effect of A1/D2 on growth of VL43 was assessed, as well as the impact of VL43 on the growth of the A1/D2 isolates. Regardless of the plating combination, the colonies halted their growth toward each other right before contact and advanced further in unoccupied spaces in the Petri dish (Figure 1). In both sealed and non-sealed Petri dishes, A1/D2a and A1/D2c grew slower when plated against VL43 than when plated against themselves (Table 3). However, in sealed Petri dishes, the growth of A1/D2b was not affected when plated against VL43. The growth of VL43 was negatively affected by A1/D2b at 7 dpi in non-sealed plates, but not at 14 dpi. Moreover, in sealed plates, VL43 grew faster when plated against A1/D2b and A1/D2c than when plated with itself.

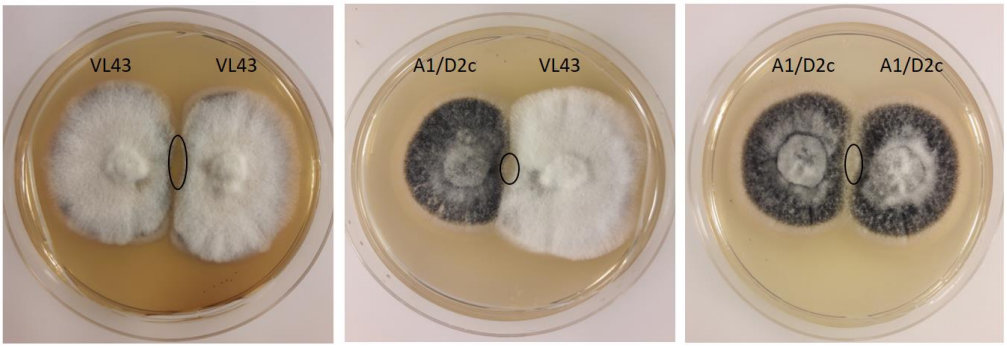

Figure 1. Dual culture on PDA of the *Verticillium longisporum* aggressive isolate VL43 and the non-aggressive isolate A1/D2c from lineage A1/D2 21 days post inoculation (center). The control treatments, which consisted of each isolate plated against itself, are also shown (right and left). The colonies halted their growth toward each other right before contact (circle).

Table 3. Dual culture to assess the antagonism between *Verticillium longisporum* lineage A1/D2 (isolates A1/D2a, A1/D2b and A1/D2c) and aggressive isolate VL43. *In vitro* growth (cm) on PDA is expressed as the growth difference between each isolate when plated against another isolate and when plated against itself. The experiment was performed in both sealed and non-sealed Petri dishes. Data points are the mean of three biological replicates consisting of merged data from four Petri dishes with the corresponding standard deviation. * indicates a significant growth difference compared to the control (t-test, $P \leq 0.05$). dpi= days post inoculation.

Strain A	**Strain B**	**Growth difference with control (cm)**			
		Sealed Petri dishes		Non-sealed Petri dishes	
		7 dpi	14 dpi	7 dpi	14 dpi
VL43	A1/D2a	-0.79 ± 1.14	-0.13 ± 1.21	1.45 ± 1.22*	0.29 ± 1.29
	A1/D2b	-0.33 ± 0.81	0.79 ± 0.76*	-1.04 ± 1.05*	0.95 ± 0.78
	A1/D2c	0.21 ± 0.86	1.17 ± 0.50*	-1.29 ± 1.84	0.58 ± 1.64
A1/D2a	VL43	-0.71 0.81	-1.70 ± 0.74*	-0.45 ± 0.66	- 1.65 ± 0.86*
A1/D2b		-0.38 1.12	-0.46 ± 1.20	0.05 ± 1.20	- 0.33 ± 1.30
A1/D2c		-1.17 0.62	-1.75 ± 0.99*	0.08 ± 1.01	-1.25 ± 0.95*

3.2. Biocontrol potential of A1/D2 with a root-dip inoculation

3.2.1. Effect of A1/D2 root-dip inoculation on VL43 visual symptoms

To assess the biocontrol effect of A1/D2 on VL43, oilseed rape seedlings were root-dip inoculated with an A1/D2 isolate before (pre-inoculation) or after (post-inoculation) VL43, as well as the same time as VL43 (co-inoculation). The co-inoculation of VL43 with A1/D2 isolates led to a relative plant height that was up to 30 % higher than the plant height of seedlings only treated with VL43 (Figure 2 and 3). With pre-inoculation of A1/D2b and A1/D2c, the relative height of the seedlings was up to 15% higher than when only treated with VL43. Pre-inoculation with A1/D2a did not cause a biocontrol effect on plant height. The biocontrol effect caused by A1/D2 isolates on the AUDPC was very similar with the co- and pre-inoculation methods, which caused, on average, a symptom reduction of 50%. With both inoculation methods, A1/D2a was the isolate with the lowest biocontrol effect against VL43.

The post-inoculation assay led to the highest AUDPC symptoms and showed the least biocontrol effect. The only A1/D2 isolate that caused a significant biocontrol effect with this inoculation method was A1/D2b, which led to a significant height recovery of approximately 20%. However, post-inoculation with A1/D2b did not lead to a reduction of AUDPC. Additionally, post-inoculation with A1/D2a caused an AUDPC 40% higher than when VL43 was inoculated alone.

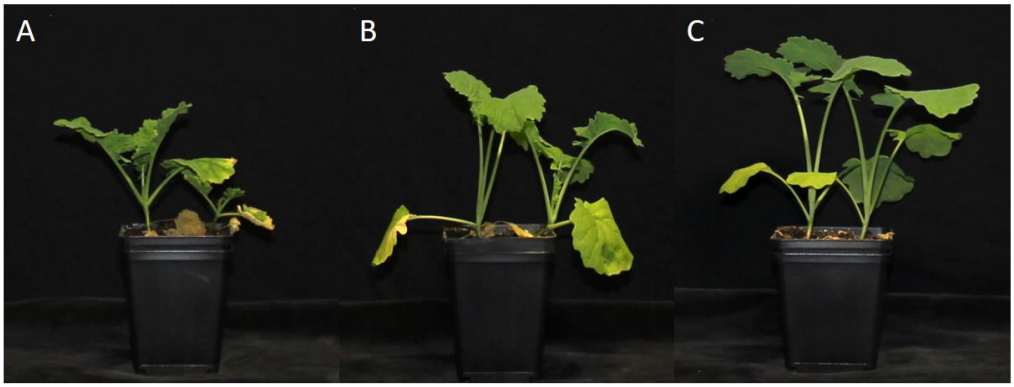

Figure 2. Oilseed rape seedlings (cultivar Falcon) root-dip inoculated (1.10^6 spores / mL) with the aggressive *Verticillium longisporum* isolate VL43 (A) showed severe disease symptoms (height reduction, leaf yellowing) 28 days post inoculation compared to control plants (C). Pre-inoculation with an isolate from the *V. longisporum* non-aggressive lineage A1/D2 one week before inoculation with VL43 partially protected the plants (B), as it resulted in less stunting and leaf symptoms in comparison to plants only treated with VL43 (A).

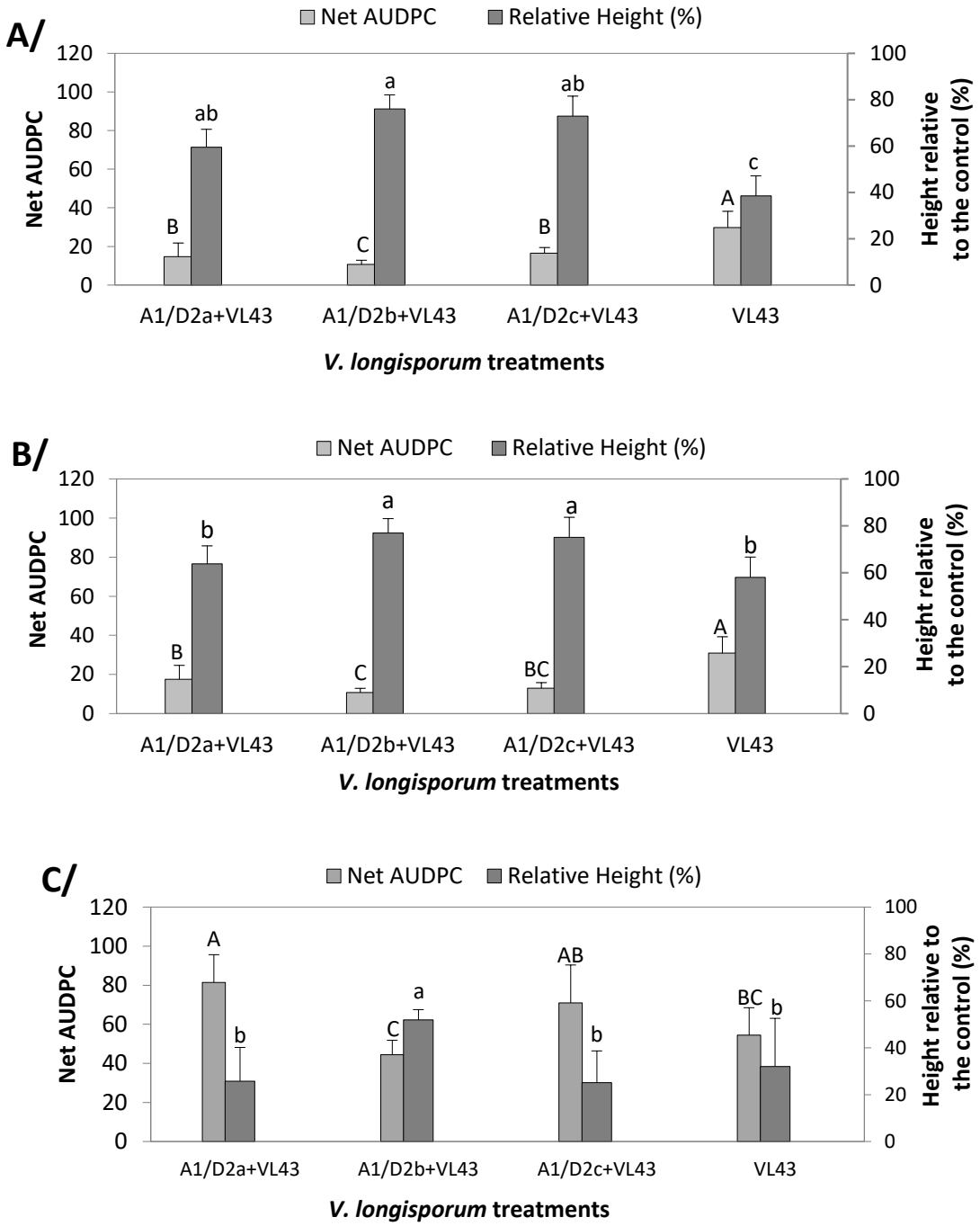

Figure 3. Assessment of biocontrol effect of the non-aggressive *Verticillium longisporum* lineage A1/D2 (isolates A1/D2a, A1/D2b and A1/D2c) against the aggressive isolate VL43 after root-dip inoculation (1.10^6 spores / mL) on oilseed rape seedlings (cultivar Falcon). Disease symptoms are expressed as net area under the disease progress curve (AUDPC) and plant height reduction relative to the control. A1/D2 isolates were pre- (A), co- (B), or post- (C) inoculated in relation to VL43. Data points represent the mean of six biological replicates, each consisting of merged data from eight plants. Error bars refer to standard deviation. Different letters indicate significant differences (Tukey test, $P \leq 0.05$).

3.2.2. Effect of A1/D2 root-dip inoculation on VL43 systemic colonization

The impact of A1/D2b applied as a root-inoculation (pre-, co-, and post-inoculation methods) on the systemic plant colonization of VL43 was assessed by measuring the fungal DNA at 28 dpi in the stems. Co-inoculation

with A1/D2b and VL43 resulted in a significant reduction of fungal DNA, from 94 ng DNA/ g plant to 14 ng (Figure 4). The other two inoculation methods of A1/D2b did not result in a significant reduction of fungal DNA in the stems.

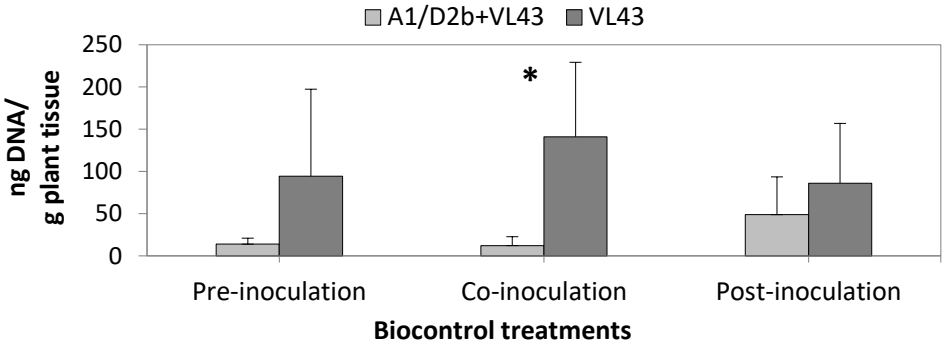

Figure 4. Biocontrol assessment of the non-aggressive *Verticillium longisporum* lineage A1/D2 (isolate A1/D2b) on systemic colonization of the aggressive isolate VL43 after root-dip inoculation (1.10^6 spores / mL) on oilseed rape seedlings (cultivar Falcon). The systemic colonization was measured as fungal DNA in the stem tissue at 28 days post inoculation. The isolate A1/D2b was pre-, co-, or post-inoculated in relation to VL43. Data points represent the mean of eight biological replicates, each consisting of eight merged stem samples. Error bars refer to standard deviation.* indicates a significant pairwise comparison (Wilcoxon rank sum test, $P \leq 0.05$).

3.3. Analysis of the potential priming effect caused by A1/D2

The potential priming effect of A1/D2 against VL43 in oilseed rape was assessed by quantifying the free SA production at 6 and 10 dpi. There were no significant differences in the levels of SA between the treatments. Additionally, the differences between each experiment repetition at 10 dpi did not allow merging of the data. Those differences were particularly high in the mix (A1/D2b+VL43) and water treatment. At 6 dpi, the treatment with the lowest free SA content was the mix treatment. At 10 dpi, it was the A1/D2b treatment (Figure 5).

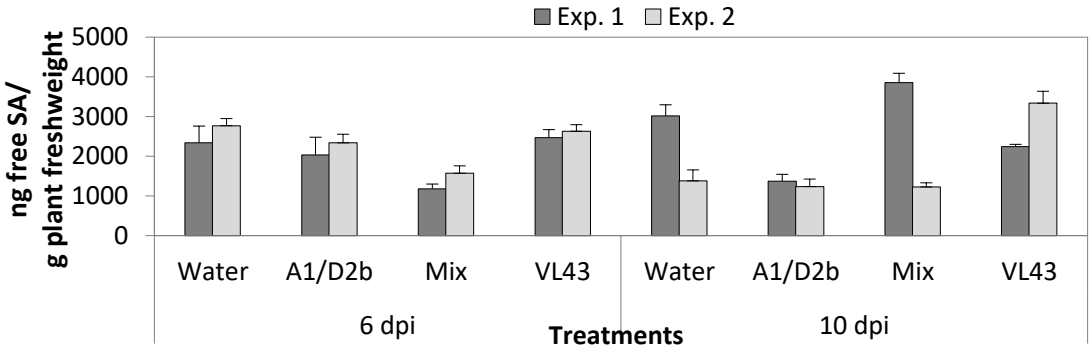

Figure 5. Free salycilic acid (SA) quantification in aerial parts of oilseed rape seedlings (cultivar Falcon) at 6 and 10 days post inoculation after a root-dip inoculation (1.10^6 spores / mL) of the *Verticillium longisporum* aggressive isolate VL43, preceded by a protective treatment one week prior with an non-aggressive isolate from *V. longisporum* lineage A1/D2 (Mix). The two separated treatments (A1/D2b, VL43) and the control (water) were also quantified for comparison. The experiment was performed twice (Exp. 1, Exp. 2). Data points represent the mean of five biological replicates, each consisting of ten merged plant samples. Error bars refer to standard deviation. There were no significant differences between treatments (Anova, $P \geq 0.05$).

3.4. Biocontrol potential of A1/D2 as a seed coat

3.4.1. Effect of A1/D2 seed coat on VL43 visual symptoms

The biocontrol potential of A1/D2b against VL43 was tested on spring oilseed rape by applying A1/D2b as a seed coat. None of the two seed coating treatments (spore suspension and microsclerotia) had a biocontrol effect on VL43 (Figure 6 and 7), both in the greenhouse and climate chamber. In the climate chamber, VL43-treated plants were up to 40 cm smaller than the control. In contrast, in the greenhouse, VL43-treated plants displayed a significant height reduction of up to 20 cm.

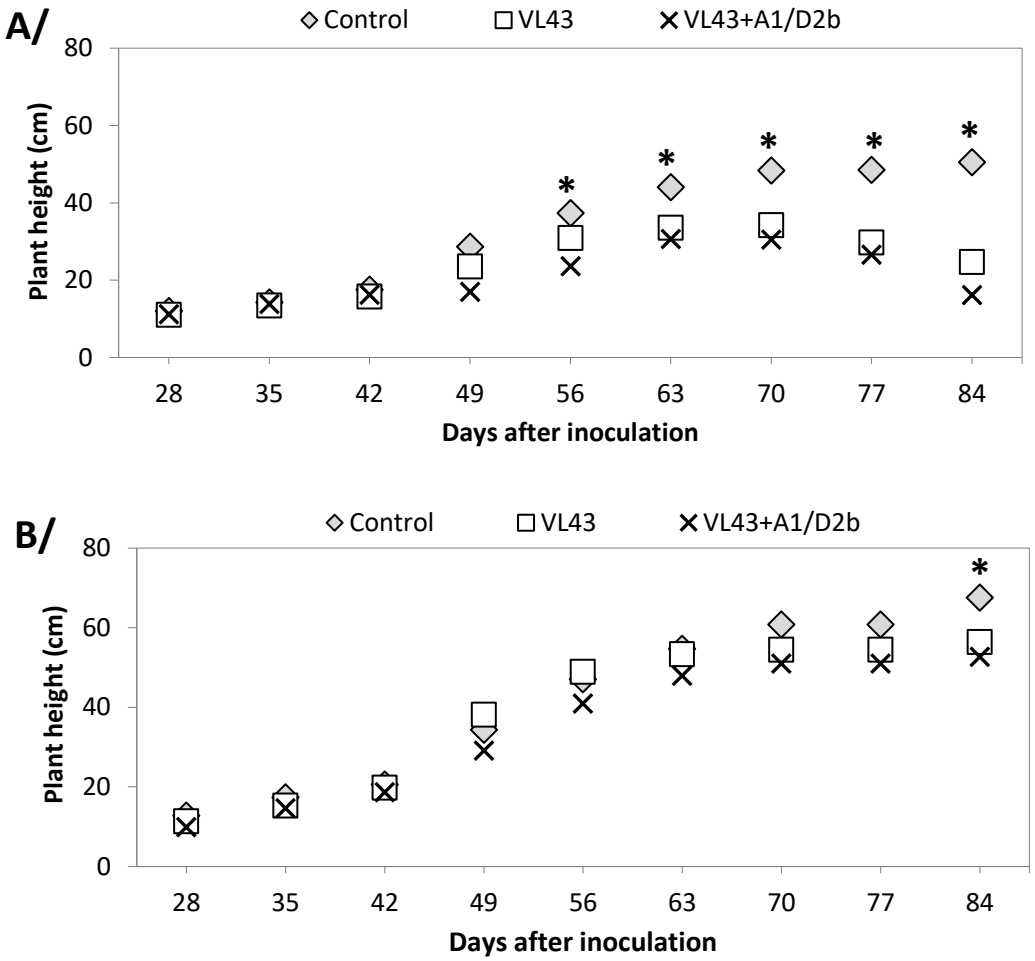

Figure 6. Biocontrol assessment of the *Verticillium longisporum* non-aggressive lineage A1/D2 (isolate A1/D2b) against the aggressive isolate VL43 on oilseed rape spring (cultivar Licosmos). VL43 was inoculated as a soil amendment (800 mg microsclerotia / kg soil), and A1/D2b as a seed coat (0.6 mg microsclerotia / mL sticking solution). For disease assessment, plant height was measured from 28 to 84 days post inoculation. The experiment was conducted in a climate chamber (A) and greenhouse (B). The seed coat did not have a biocontrol effect against VL43. Data points represent the mean of four biological replicates, each consisting of merged data from six plants.* indicates significant differences between the treatments and control (A: Pairwise Wilcox test, B: Tukey test, $P \leq 0.05$).

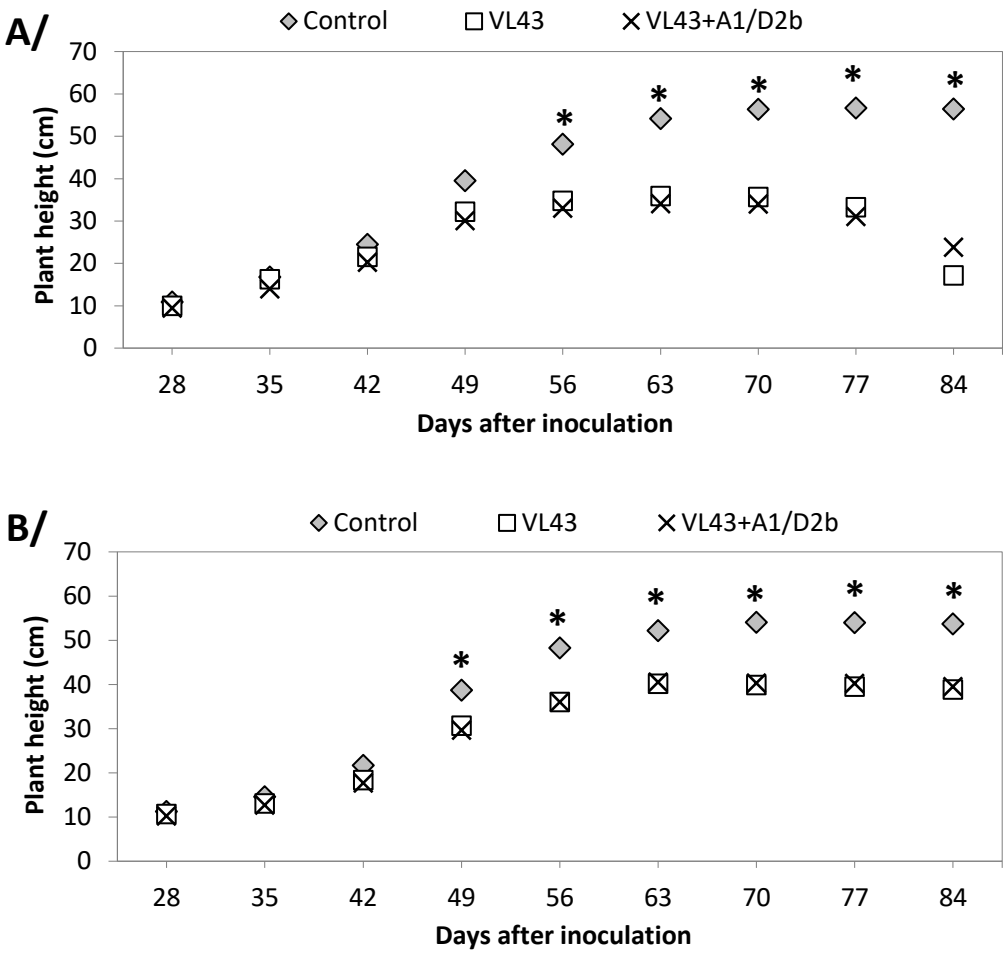

Figure 7. Biocontrol assessment of the *Verticillium longisporum* non-aggressive lineage A1/D2 (isolate A1/D2b) against the aggressive isolate VL43 on oilseed rape spring (cultivar Licosmos). VL43 was inoculated as soil amendment (800 mg microsclerotia / kg soil), and A1/D2b as seed coat (1.10^6 spores / mL). For disease assessment, plant height was measured from 28 to 84 days post inoculation. The experiment was conducted in a climate chamber (A) and in the greenhouse (B). The seed coat did not have a biocontrol effect against VL43. Data points represent the mean of four biological replicates, each consisting of merged data from six plants.* indicates significant differences between the treatments and control (A: Pairwise Wilcox test, B: Tukey test, $P \leq 0.05$).

3.4.2. Effect of A1/D2 seed coat on VL43 systemic colonization

Both seed coating methods of A1/D2b in spring oilseed rape did not lead to any significant reduction in hypocotyl colonization by VL43 at 84 dpi (Figure 8). This lack of biocontrol effect was seen both in the greenhouse and climate chamber. The amount of DNA detected in the hypocotyls ranged between 100 and 300 ng DNA/g tissue plant.

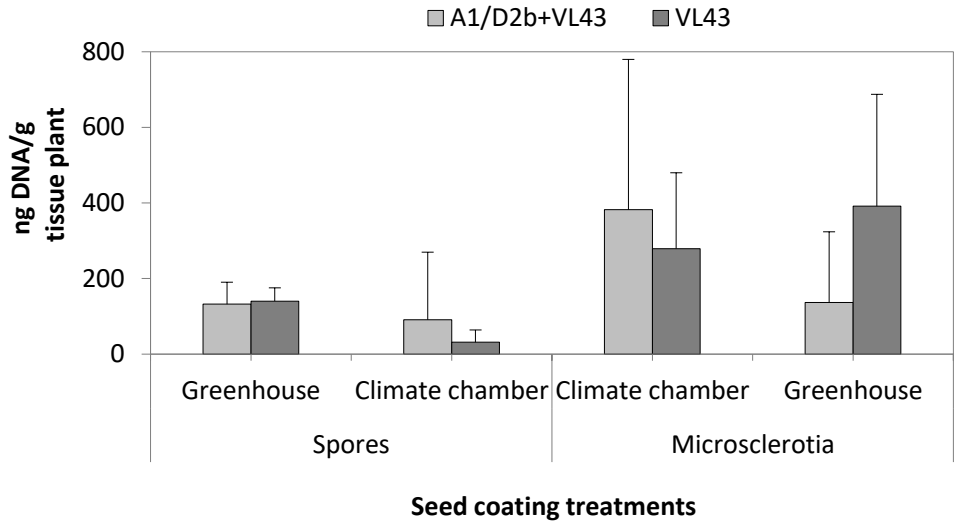

Figure 8. Assessment of the biocontrol effect of the non-aggressive *V. longisporum* lineage A1/D2 (isolate A1/D2b) on systemic colonization by the aggressive isolate VL43 on spring oilseed rape (cultivar Licosmos) at 84 days post inoculation. VL43 was inoculated as soil amendment (800 mg microsclerotia / kg soil), and A1/D2b as spore (1.106 spores / mL) or microsclerotia (0.6 mg microsclerotia / mL sticking solution) seed coat. Systemic colonization was assessed by quantifying the fungal DNA in the hypocotyl. The experiments were conducted in the climate chamber and greenhouse. Data points represent the mean of four biological replicates, each consisting of three merged hypocotyl samples. Error bars refer to standard deviation. There were no significant differences between treatments (Kruskal-Wallis, $P \geq 0.05$).

3.5. Microscopic assessment of different applications methods of an A1/D2 isolate on oilseed rape roots

The root colonization ability of A1/D2b applied as a root-dip inoculation or seed treatment (microsclerotia and spore suspension) was assessed by confocal microscopy. This assay confirmed that the three application methods allowed hyphal colonization of the roots at 14 dpi (Figure 9). A1/D2b hyphae were clearly observed after root-dip inoculation and spore seed coating, but the microsclerotia seed coat led to fungal colonization without typical hyphal morphology. Instead, fungal structures presented a crumbly morphology, which was also observed in the spore seed coating treatment. Root-dip inoculation resulted in the most intense hyphal coverage of the roots, as well as in extensive hyphal growth in the rhizosphere.

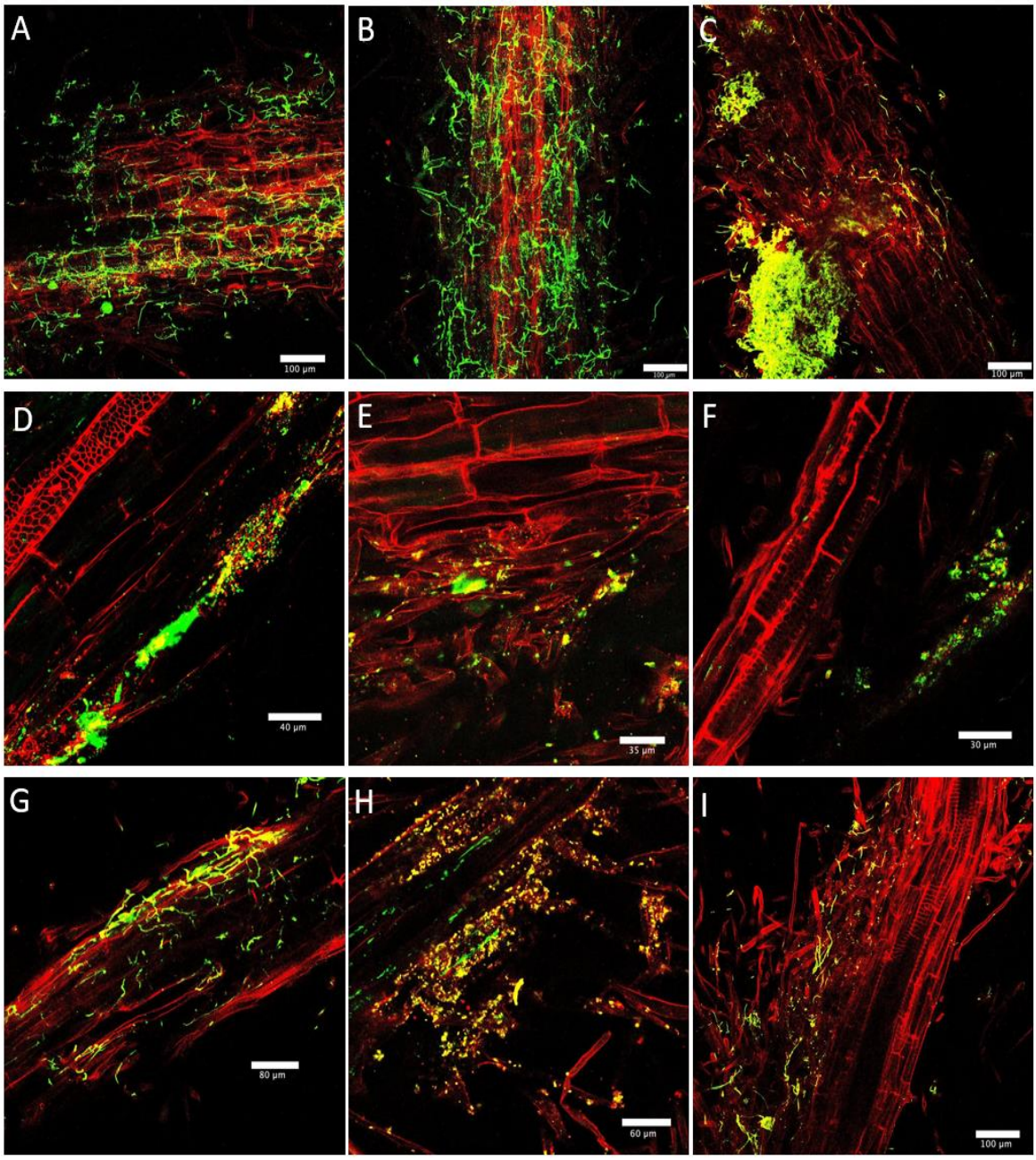

Figure 9. Confocal microscopic analysis of the external root colonization pattern of the non-aggressive *Verticillium longisporum* lineage A1/D2 (isolate A1/D2b) on oilseed rape seedlings cultivar Falcon at 14 days post inoculation. A1/D2b was applied with a root-dip inoculation (1.10^6 spores / mL) (A-C), as a microsclerotia seed coat (800 mg microsclerotia / kg soil) (D-F) and as a spore seed coat (1.10^6 spores / mL) (G-I). Root-dip inoculation led to extensive hyphal growth in the rhizosphere (A-C). With the microsclerotia seed treatment, fungal structures lacked the typical hyphal morphology and, instead, had a crumbly appearance (D-F). This morphology was also seen with the spore seed coating treatment (H), but this treatment also resulted in typical looking hyphae (G, I) Hyphae (green) were stained with Alexa Fluor and roots (red) with propidium iodide. Bar A-C= 100 µm, D-F= 40, 35, and 30 µm, G-I= 80, 60 and 100 µm.

4. Discussion

4.1. Biocontrol potential of A1/D2 *in vitro*

In vitro assays are often used as a first approach to investigate the biocontrol effect of microorganisms because of their simplicity (Wong et al. 2020). However, due to the artificial conditions, the information that *in vitro* tests provide, should be taken with caution (Deketelaere et al. 2017). The *in vitro* assessment in this study did not reveal any antibiotic antagonism, such as overgrowth (Carrero-Carrón et al. 2016) or intense inhibition zones (Alström 2000). Instead, the colonies halted their growth right before contact and advanced further in unoccupied spaces on the Petri dish (Figure 1 and Table 3). Similarly, previous reports have shown that non-pathogenic Verticillium isolates that lacked *in vitro* antagonism against *V. dahliae* were able to protect the plant (García et al. 2011; Shittu et al. 2009). Conversely, it has been reported that antibiosis was behind the reduction of Verticillium wilt in cotton by a non-pathogenic *F. oxysporum* isolate (Zhang et al. 2015).

Regardless of the combination and isolate, the two colonies of the dual culture assay grew towards each other and stopped right before contact, leaving a small area without fungal growth between them (Figure 1). Such a growth pattern has been described for *Alternaria solani* and different biocontrol agents, and has been associated with diffusible metabolites (Lahlali and Hijri 2010). The growth differences of VL43 in sealed and non-sealed plates when plated against A1/D2b and A1/D2c indicates a different volatile composition between isolates (Alfiky 2019). Volatile or diffusible compounds produced *in vitro* are not necessarily involved in the biocontrol mechanism *in planta*, as reported by Li et al. (2014). Howevber, García et al. (2011) suggested that *in vitro* metabolites from biocontrol agents might act as elicitors of plant defense.

4.2. Biocontrol potential of A1/D2 using root-dip inoculation

A1/D2 isolates reduced symptoms of oilseed rape seedlings when they were pre- or co-inoculated with VL43 (Figure 2 and Figure 3). The biocontrol effect of protective isolates against vascular diseases typically requires pre-inoculation (Alabouvette et al. 2009; Tyvaert et al. 2014). Conversely, co-inoculation of the biocontrol agent with the aggressive isolate normally does not result in biocontrol, as it has been reported for non-aggressive isolates of *V. tricorpus* and *V. albo- atrum* against *V. dahliae* on potato (Robinson et al. 2007). However, a successful biocontrol effect against *V. dahliae* with both inoculation strategies has been reported for *V. tricorpus* in lettuce (Qin et al. 2008), as well as for non-aggressive isolates of *V. dahliae* and *G. nigrescens* in cotton and tomato (Shittu et al. 2009; Zhu et al. 2013). A successful biocontrol with co-inoculation indicates that the biocontrol agent is a potent competitor for infection sites and space in the root. This mode of action has been confirmed for a non-pathogenic *Fusarium* isolate against *V. dahliae* in pepper (Pantelides et al. 2009). In contrast to the observations of Zhu et al. (2013) and Qin et al. (2008), the present study revealed a higher disease reduction with co-inoculation than with pre-inoculation of A1/D2 isolates.

This might be related to the higher expression of VL43 symptoms in the co-inoculation assay. With pre-inoculation, the seedlings were older when inoculated with the aggressive isolate. Consequently, they were bigger and more robust, which might have made them less susceptible to the pathogen (Sharabani et al. 2013).

The biocontrol effect observed for A1/D2b with post-inoculation implies that other biocontrol mechanisms besides competition for space in the rhizosphere are involved. By the time of post-inoculation with A1/D2b, VL43 was already well established in the vascular system, as aggressive isolates start invading the vascular system 60 hours after inoculation (Eynck et al. 2007). The isolate A1/D2b was the isolate with the highest biocontrol effect using pre- and co- inoculation. Additionally, it was the only A1/D2 isolate whose *in vitro* growth was not negatively affected by VL43. These observations indicate that A1/D2b has the strongest biocontrol potential among the three A1/D2 isolates tested and may have biocontrol properties that the other two isolates do not possess. In contrast, post-inoculation of A1/D2a worsened the AUDPC in comparison to the single VL43 treatment. This might indicate that A1/D2a is the least non-aggressive among the three A1/D2 isolates and, therefore, not a good biocontrol candidate.

Biocontrol mechanisms that could be involved after the vascular establishment of the aggressive isolate include plant growth promotion, antibiosis, or induction of plant defense. At this stage of colonization stage, a direct antagonistic mechanism through antibiosis would only be possible if A1/D2b was able to strongly colonize the vascular system (Deketelaere et al. 2017). Shittu et al. (2009) reported that a non-pathogenic *V. dahliae* isolate was able to colonize the vascular system. However, when *Verticillium* isolates produce low or no symptoms in the host, restricted systemic colonization is typically observed (Eynck et al. 2007; Knüfer et al. 2017; Zhang et al. 2016; Zhou et al. 2006). This is in tune with a previous assessment, which showed that DNA from A1/D2c was not present in the aerial parts of oilseed rape at 21 dpi (Depotter et al. 2017). Thus, intense vascular systemic colonization in oilseed rape by the lineage A1/D2 is not likely. Depotter et al. (2017) has reported that A1/D2 does not induce plant growth promotion in oilseed rape. Consequently, induction of plant defense is the most plausible mechanism of A1/D2b applied post-inoculation. Such a biocontrol mechanism has been confirmed for two protective *F. oxysporum* isolates, which upregulate pathogenesis-related proteins (PR) upon inoculation in eggplant and *Arabidopsis* (Angelopoulou et al. 2014; Veloso and Díaz 2012).

Reduced systemic colonization of a plant pathogen due to the biocontrol effect of a protective isolate has been reported in previous studies (Shittu et al. 2009; Tyvaert et al. 2014). Similarly, the qPCR analysis showed that co-inoculation with VL43 and A1/D2b led to a significant reduction of fungal colonization in the stems (Figure 4). Due to the high variability of each biological replicate in the amount of fungal DNA, a higher number of samples is needed to verify a significant reduction in systemic colonization with pre- and post-inoculation application of the biocontrol agent.

4.3. Potential priming effect of A1/D2

The A1/D2b biocontrol effect via root-dip inoculation suggests that induction of plant defense might be involved in the biocontrol. SA putatively is the translocating signal in systemic acquired resistance (Conrath 2006). Moreover, Zheng et al. (2019a) showed that induction of SA at early stages of infection is required for basal and cultivar-related resistance *in oilseed rape against V. longisporum*. Priming has been reported as the biocontrol mechanism of non-pathogenic *F. oxysporum isolates against F. oxysporum* f. sp. *lycopersici in tomato (Aimé et al. 2008) and Verticillium wilt in pepper (Veloso et al. 2016).* In this study, free SA was quantified to assess whether A1/D2b protects seedlings against VL43 by directly inducing defense reactions or through priming. Our results (Figure 5) indicate that neither VL43 nor A1/D2b have an effect on SA production in the susceptible cultivar Falcon. Similarly, Zheng et al. (2019a) reported a lack of early induction of SA in Falcon by VL43. These results reveal that the induction of SA is not involved in the biocontrol mechanism of A1/D2b and suggest that other pathways of plant defense rather than components of basal resistance are activated or enhanced by A1/D2b. Among these are salicylate and jasmonate dependent defense pathways, production of defense-related enzymes, or accumulation of lignin, which have been reported for different biocontrol agents against Verticillium species (reviewed by Deketelaere et al. 2017).

4.4. Seed coating and microscopic assessment

Biocontrol assessments should preferably mimic natural conditions as much as possible (Deketelaere et al. 2017; Stadler and Tiedemann 2014). Consequently, the biocontrol effect of A1/D2b against VL43 was also tested on a spring oilseed rape cultivar in unsterile greenhouse conditions, enabling plant development until flowering without artificial vernalization required for winter cultivars. Additionally, the aggressive isolate VL43 was inoculated as a microsclerotia soil amendment and A1/D2b as a seed coat. However, in this study, both seed coating treatments (spore suspension and microsclerotia) did not result in a reduction of VL43-induced symptoms, neither in the greenhouse nor in the climate chamber (Figure 6 and 7). This result is similar to previous studies that reported no biocontrol effect against *V. dahliae* when protective isolates were applied as a seed coat (Angelopoulou et al. 2014; Lopisso et al. 2017).

For successful biocontrol with a seed coating, the protective isolate must colonize the rhizosphere during seed germination (El-Mougy and Abdel-Kader 2008). With confocal microscopy, we were able to confirm that A1/D2b was colonizing the roots when applied as a seed coat (Figure 9). Nevertheless, the microscopic assessment showed that A1/D2b root colonization was significantly lower when A1/D2b was applied as a seed treatment than by root-dip inoculation. Lower hyphal colonization of the roots might lead to lower competition ability and induction of plant defense by the protective strain, which might explain the absence of biocontrol effect with a seed treatment. This is in agreement with the results from Bao and Lazarovits

(2001), who showed that physical plant defense reactions on the roots occurred only in the root section where the biocontrol agent was present.

The role of temperature in disease severity of *V. longisporum* has been reported by Zheng et al. (2019c). At cooler night temperatures in the greenhouse, disease severity was lower than in the climate chamber, which might have transiently inhibited the potential biocontrol effect. Besides height reduction, VL43-treated plants often showed premature ripening and pod shedding. The development of a disease key including these symptoms could improve the assessment of the potential biocontrol effect of a protective isolate in spring oilseed rape.

The high level of disease in the climate chamber might have been compensated by higher concentrations of the biocontrol agent. The importance of concentration for biocontrol efficacy has already been shown by Lopisso et al. (2017) and Tyvaert et al. (2014). Besides concentration, the formulation of the biocontrol agent is essential for its efficacy and survival (Rocha et al. 2019). Low hyphal colonization of the roots after seed coating with spores may be attributed to a low survival rate of spores when applied with this method. Crumbled hyphal structures on the root might have been dead spores or only partially germinated microsclerotia (Figure 9). Formulating spores of the biocontrol agent with nutrients has been proposed as a method to increase the viability and competition of biocontrol agents when applied as a seed coat (Alabouvette et al. 2009).

4.5. Conclusions

To the authors´ knowledge, this is the first report of biocontrol against *V. longisporum* in oilseed rape with a non-aggressive isolate by root-dip inoculation. Due to its biocontrol effect when applied in a pre-, co- and post-inoculation manner, A1/D2b appears to be a promising biocontrol candidate. The results from this study suggest that this isolate might induce plant defense and compete with the pathogen for infection sites in the rhizosphere. The induction of SA, a signal of basal and cultivar-related resistance, is not involved in this biocontrol mechanism. No biocontrol effect was recorded if the biocontrol agent was applied as a seed coat, which illustrates the importance of the application method for efficacy of a biocontrol agent.

5. Author contributions

Marta Vega-Marín conducted the research. Both authors were involved in the design of the research, discussed collectively the results, and wrote the manuscript.

6. Acknowledgements

This work was financially supported by the German Federal Ministry for Food and Agriculture (BMEL) and the Agency for Renewable Resources (FNR). The authors acknowledge the technical assistance of Isabel Müller, Leonard Sundermann, Virginia Casasnovas, and Shilu Dahal with the phenotypic assessments. Thanks are also extended to Dagmar Tacke for her assistance with DNA extraction and qPCR analysis. Dr. Antonia Wilch, Alice Eseola, and Dima Alnajar provided indispensable guidance and assistance on the confocal microscopy assessment.

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8. Appendix

Appendix 1. Biocontrol assessment of *Verticillium longisporum* non-aggressive lineage A1/D2 (isolate A1/D2b) against aggressive isolate VL43 on oilseed rape spring cultivar Licosmos. VL43 was inoculated as soil amendment (800 mg microsclerotia / kg soil) and A1/D2b as a seed coat (0.6 mg microsclerotia / mL sticking solution). For disease assessment, plant height was measured from 28 to 84 dpi. The experiment was conducted in a climate chamber. The seed coat did not have a biocontrol effect against VL43. Data points represent the mean of four biological replicates, each consisting of merged data from six plants, and the corresponding standard deviation. Different letters indicate significant differences between the treatments and the control (Pairwise Wilcox test, $P \leq 0.05$).

Treatments	28 dpi	35 dpi	42 dpi	49 dpi	56 dpi	63 dpi	70 dpi	77 dpi	84 dpi
Control	11.9a ±0.65	14.2a ±0.4	17.5a ±1.8	28.6a ±2.8	37.3a ±2.6	44a ±3.8	48.3ab ±5.1	48.5a ±3.1	50.5a ±3.9
VL43	11a ±0.71	13.3a ±0.6	15.7a ±2.3	23.5a ±6.1	30.8a ±6.4	33.5a ±7.8	34.2c ±8.8	29.7b ±10.4	24.7b ±7.5
VL43+ A1/D2	11.2a ±0.7	13.8a ±0.1	16.2a ±1.6	16.9a ±1.4	23.6b ±3.7	30.6b ±6	30.4c ±6.3	26.54b ±12.3	16.1b ±9.4

Appendix 2. Biocontrol assessment of the Verticillium longisporum non-aggressive lineage A1/D2 (isolate A1/D2b) against the aggressive isolate VL43 on oilseed rape spring cultivar Licosmos. VL43 was inoculated as soil amendment (800 mg microsclerotia / kg soil) and A1/D2b as seed coat (0.6 mg microsclerotia / mL sticking solution). For disease assessment, plant height was measured from 28 to 84 dpi. The experiment was conducted in the greenhouse. The seed coat did not have a biocontrol effect against VL43. Data points represent the mean of four biological replicates, each consisting of merged data from six plants, and the corresponding standard deviation. Different letters indicate significant differences between the treatments and the control (Tukey test, $P \leq 0.05$).

Treatments	28 dpi	35 dpi	42 dpi	49 dpi	56 dpi	63 dpi	70 dpi	77 dpi	84 dpi
Control	12.8a ±0.8	17.4a ±1.1	20.6a ±1.6	34.3a ±4.9	47.1a ±4.3	54.7a ±3	60.8a ±4.2	64.1a ±4.7	67.6a ±5.3
VL43	11.4ab ±0.3	15.3ab ±0.7	19.8a ±1.6	38.2a ±2.9	48.9a ±4.7	53.3a ±4.8	54.5a ±6.2	55.4ab ± 6.3	56.4b ±6
VL43+ A1/D2	9.9b ±1.5	14.6b ± 1.1	18.6a ±1.4	29.1a ±6.9	40.9a ±4.8	47.9a ±4.7	51a ±4.2	59.3ab ± 4.4	52.7b ±4.3

Appendix 3. Biocontrol assessment of the *Verticillium longisporum* non-aggressive lineage A1/D2 (isolate A1/D2b) against the aggressive isolate VL43 on oilseed rape spring cultivar Licosmos. VL43 was inoculated as soil amendment (800 mg microsclerotia / kg soil), and A1/D2b as seed coat (1.10^6 spores / mL). For the disease assessment, plant height was measured from 28 to 84 dpi. The experiment was conducted in a climate chamber. The seed coat did not have a biocontrol effect against VL43. Data points represent the mean of four biological replicates, each consisting of merged data from six plants, and the corresponding standard deviation. Different letters indicate significant differences between the treatments and the control (Pairwise Wilcox test, $P \leq 0.05$).

Treatments	28 dpi	35 dpi	42 dpi	49 dpi	56 dpi	63 dpi	70 dpi	77 dpi	84 dpi
Control	10.9a ±0.8	16.8a ±1	24.5a ±3	39.5a ±3.9	48.1a ±3.1	54.2a ±3.4	56.3a ±3.3	56.6a ±3.5	56.5a ±3.3
VL43	10a ±0.2	16.2b ±1.8	21.6a ±2.1	32.2a ±3.2	34.7b ±3.4	35.8b ±3.6	35.6b ±4	33.3b ±3	17.1b ±12.2
VL43+ A1/D2b	9.4a ±0.9	14b ±2	20.2a ±3.2	30a ±5.7	33ab ±6.5	34.1b ±6.1	34b ±6.9	31.1b ±9.2	23.8b ±10

Appendix 4. Biocontrol assessment of the *V. longisporum* non-aggressive lineage A1/D2 (isolate A1/D2b) against the aggressive isolate VL43 on oilseed rape spring cultivar Licosmos. VL43 was inoculated as soil amendment (800 mg microsclerotia / kg soil) and A1/D2b as seed coat (1.10^6 spores / mL). For the disease assessment, plant height was measured from 28 to 84 dpi. The experiment was conducted in the greenhouse. The seed coat did not have a biocontrol effect against VL43. Data points represent the mean of four biological replicates, each consisting of merged data from six plants, and the corresponding standard deviation. Different letters indicate significant differences between the treatments and the control (Tukey test, $P \leq 0.05$).

Treatments	28 dpi	35 dpi	42 dpi	49 dpi	56 dpi	63 dpi	70 dpi	77 dpi	84 dpi
Control	11.3a ±0.8	14.6a ±1.4	21.7a ±2.5	38.7a ±3.8	48.3a ±3.9	52.2a ±4.6	54a ±4.3	54a ±4.3	53.7a ±2.8
VL43	10.6a ±0.2	13a ±0.4	18.3a ±2.2	30.6b ±3.7	36b ±5.2	40.1b ±4.6	39.8b ±5.4	39.5b ±5.4	38.8b ±6.1
VL43+ A1/D2b	10.3a ±0.6	12.7a ±1	17.7a ±3.2	29.6b ±5	36ab ±4.9	40.5b ±2.8	40.3b ±5.2	40.2b ±5.2	39.5b ±5.4

Chapter IV: Interactions of the aggressive *Verticillium longisporum* lineage A1/D1 and non-aggressive A1/D2 lineage with roots of oilseed rape

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Abstract

Verticillium longisporum is a soil-borne fungal vascular pathogen of oilseed rape and other Brassica crops. It consists of three lineages that originated from three independent hybridization events of four haploid *Verticillium* parents. *V. longisporum* lineage A1/D2 has only been found in horseradish in the USA and previous pathogenicity tests have revealed that it is mostly non-aggressive on different *Brassica* and non-*Brassica* hosts. The aim of this study was to investigate the interaction of the non-aggressive *V. longisporum* lineage A1/D2 in oilseed rape in comparison to the aggressive A1/D1 lineage to characterize the contrasting patterns of infection and colonization underlying the aggressive and non-aggressive types of interaction with roots of oilseed rape. The infection phenotype and fungal growth, assessed by qPCR of fungal DNA, revealed low pathogenicity and restricted systemic colonization by lineage A1/D2. In the external root colonization assessment by confocal microscopy, early sporulation, disorganized superficial hyphal growth, and hyphal coiling of A1/D2 was detected, which indicates oilseed rape is not the preferential host of A1/D2. In contrast, A1/D1 showed organized hyphal growth tight to the root surface, as well as hyphal swellings indicating points for penetration. Colonization of the root vascular system colonization was studied with electron microscopy, which revealed lower hyphal colonization, higher intensity of vascular responses, and lower plant cell wall degradation in A1/D2 treated plants in comparison to A1/D1 treated plants. The results indicate that the low rate of root surface penetration and xylem colonization by A1/D2, as well as a higher intensity of plant defense reactions upon A1/D2 infection in comparison to A1/D1, might explain the low pathogenicity of the lineage A1/D2.

1. Introduction

Verticillium wilts are widely distributed vascular diseases responsible for important economic losses in more than 200 hosts. They are caused by monocyclic pathogens that survive in the soil as microsclerotia, resting mycelium, or chlamydospores (Inderbitzin et al. 2011a). The genus comprises ten species, most of which are not host-specific and have a relatively wide geographic range. *V. longisporum* is aggressive in Brassica

horticultural crops as well as in oilseed rape (Inderbitzin et al. 2011b). Since its first description in in a horseradish plant from a German field in the 1960s (Stark 1961), *V. longisporum* has spread to other European countries, Japan, China, Russia, the USA, and Canada (CFIA 2015; Inderbitzin et al. 2011a; Yu et al. 2016; Yu et al. 2015).

In oilseed rape, instead of producing wilting symptoms, *V. longisporum* causes a dark unilateral striping on the stem during the ripening of the crop. The striping symptoms originate from necrosis produced after the fungus leaves the xylem and colonizes the parenchyma, which leads to premature ripening of the crop. After harvest, microsclerotia develop in the stem and roots and are released into the soil after the crop decomposes (Depotter et al. 2016; Pegg and Brady 2002). Yield losses caused by *V. longisporum* have been shown by Dunker et al. (2008) in individual plants under controlled conditions. In the field, yield losses depend on the cultivar, disease incidence, and weather conditions (Depotter et al. 2019; Zheng et al. 2019). The symptoms caused by *V. longisporum* in oilseed rape in the greenhouse differ from those that occur under field conditions. After root-dip inoculation of seedlings in the greenhouse, plants show chlorosis, vascular discoloration, and stunting (Eynck et al. 2007). The absence of such symptoms in the field is caused by the delay in fungal colonization that plants, when sown as winter crop,experience due to low soil temperatures (Zheng et al. 2019). Despite symptoms being distinctly different in the field, Knüfer et al. (2017) showed a direct correlation between the severity of greenhouse symptoms and level of fungal colonization in the field at the beginning of ripening.

V. longisporum is the only diploid species of the genus *Verticillium* (Inderbitzin et al. 2011a). It is amphidiploid and the amount of DNA, which may vary between different isolates, is about 1.8-fold of haploid *Verticillium* species (Collins et al. 2003; Steventon et al. 2002). By means of phylogenetic analysis based on five protein coding genes and the internal transcribed spacer region, Inderbitzin et al. (2011b) concluded that *V. longisporum* consists of three genetically homogenous lineages (A1/D1, A1/D2, and A1/D3) that originated from three independent hybridization events involving four haploid *Verticillium* parents. Two of the parents, D2 and D3, correspond to *V. dahliae*, whereas A1 and D1 are unknown *Verticillium* species.

The lineage A1/D2 has the narrowest host and geographic range, as it has only been reported in horseradish in Illinois and Ontario (Inderbitzin et al. 2011b; Yu et al. 2016). In contrast, A1/D3 and A1/D1 have been isolated from cabbage, oilseed rape, radish, and cauliflower in Japan, the USA, and Europe (Banno et al. 2015; Depotter et al. 2017b; Inderbitzin et al. 2011b). Within a *Verticillium* species, differences in pathogenicity and host range are common among isolates (Berbegal et al. 2010; Bhat et al. 2003; Bhat and Subbarao 1999; Douhan and Johnson 2001; Gurung et al. 2015; Iglesias-Garcia et al. 2013; Korolev et al. 2008; Novakazi et al. 2015). A1/D1 is the most aggressive lineage on oilseed rape, whereas A1/D2 is the least aggressive in all tested crops and A1/D3 isolates displayed a more diverse pathogenicity (Novakazi et al. 2015).

When non-aggressive *Verticillium* isolates produce mild symptoms in the host, qPCR analysis has shown restricted systemic fungal colonization (Eynck et al. 2007; Depotter et al. 2017a; Knüfer et al. 2017; Zhang et al. 2016; Zhou et al. 2006). Depotter et al. (2017a) reported that A1/D2 was unable to accumulate fungal biomass in the aerial parts of two oilseed rape cultivars. Microscopic assessments about the penetration of the pathogen and morphological changes in the plant (Eynck et al. 2007; Eynck et al. 2009a; Vallad and Subbarao 2008) indicated typical morphological changes inside the vessels including different depositions, such as gum or gels, which lead to vessel occlusion (Fradin and Thomma 2006). Previous microscopic studies have reported differences in root penetration and internal colonization between non-aggressive and aggressive isolates of vascular pathogens (Eynck et al. 2007; Jiménez-Fernández et al. 2013). Similarly, a microscopic study detected differential responses of aggressive *V. dahliae* and non-aggressive *V. albo-atrum* isolates to the morphological changes in the xylem that occurred upon infection in tomato (Gold and Robb 1995).

The aim of this study was to investigate colonization and penetration of the non-aggressive *V. longisporum* lineage A1/D2 on roots of oilseed rape in order to characterize this interaction in contrast to the aggressive lineage A1/D1. To this end, the two types of interactions were phenotypically assessed on oilseed rape in the greenhouse. By means of electron and confocal microscopy, differences between the two lineages in the infection process were investigated by assessing the external and internal root colonization patterns. Finally, the systemic spread of both lineages was quantified by qPCR.

2. Methods

2.1. Fungal isolates and inoculum production

V. longisporum isolates were obtained from the fungal isolate collection of the Division of Plant Pathology and Crop Protection of the Georg-August University of Göttingen (Table 1). To assess the interaction of the aggressive A1/D1 and non-aggressive A1/D2 lineages with oilseed rape, three A1/D2 isolates and one A1/D1 isolate were selected. The A1/D1 isolate VL43 was collected from a diseased oilseed rape plant in Germany (Zeise and Tiedemann 2001 and 2002). The three A1/D2 isolates were obtained from horseradish (Inderbitzin et al. 2011b). For the preparation of spore inoculum, 1 mL of spore suspension, which had previously been stored axenically at -80 °C in 25% aqueous glycerol, was added into potato dextrose broth (PDB) in Erlenmeyer flasks with 400 ppm streptomycin, 50 ppm chloramphenicol, and 50 rifampicin. PDB flasks were incubated on a shaker (100 rpm) at 22 °C in the dark. After 10 days, the culture was filtered through a sterile sieve. The spore density was determined using a Thoma haemocytometer and diluted to 1 x 10^6 spores / mL. The spore suspension was applied as a 30 min root-dip inoculation.

Table 1. *Verticillium longisporum* isolates used in this study.

Identifier	Identifiers literature	Host	Sampling site	Collection	Reference
VL43	PD638, VL43	*Brassica napus* (oilseed rape)	Mecklenburg, Germany	[a]UGoe	Zeise and Tiedemann 2001 and 2002
A1/D2a	PD402	*Armoracia rusticana* (horseradish)	Illinois, USA	[b]UC Davis	Inderbitzin et al. 2011b
A1/D2b	PD730	*A. rusticana* (horseradish)	Illinois, USA	UC Davis	Inderbitzin et al. 2011b
A1/D2c	PD356	*A. rusticana* (horseradish)	Illinois, USA	UC Davis	Inderbitzin et al. 2011b

[a]UGoe= Georg-August University of Göttingen; [b]UC Davis= University of California, Davis

2.2. Pathogenicity assessment in the greenhouse

Pathogenicity of *V. longisporum* lineages A1/D1 and A1/D2 on oilseed rape was assessed in the greenhouse. Seeds of the susceptible oilseed rape cultivar 'Falcon' (Eynck et al. 2009b) were pre-germinated in trays with quartz sand for 10 days at 18–24 °C and with a 14 h photoperiod (Horti-Lux HPS-400 Watt). Ten days after sowing, seedlings were removed from the sand and washed thoroughly under running tap water. After the removal of sand particles, seedlings were dipped in a spore suspension prepared as described in section 2.1. The roots of control seedlings were immersed in water. Each treatment (A1/D2a, A1/D2b, A1/D2c, VL43, and water) consisted of 24 plants. Treated plants were transplanted into 7 x 7 x 8 cm pots filled with a soil mixture of sand and steamed compost (1:3). Each pot had two plants and four pots were placed in one tray to avoid cross-contamination between treatments during irrigation. Each tray was considered one biological replicate. All trays were organized according to a completely randomized design and kept at the same light and temperature conditions as previously mentioned. The experiment was repeated twice.

Disease assessments were conducted at 7, 14, 21, and 28 days post inoculation (dpi). The evaluation of yellowing and death of leaves was performed according to the 9-score assessment key described by Eynck et al. (2009b) and the net area under the disease progress curve (AUDPC) was calculated accordingly. At 28 dpi, plant height was measured from the cotyledons to the tip of the longest leaf to calculate the height reduction caused by disease in relation to the control. Additionally, for the dry biomass assessment, plants were harvested at 28 dpi and dried at 50 °C for 48 h to determine the dry weight of roots and shoots (stems and leaves).

2.3. Quantification of systemic fungal colonization of the plant

For the quantification of fungal DNA, oilseed rape Falcon seedlings were inoculated and grown as described in section 2.2. Plants from one tray were pooled together as a biological replicate. Each treatment consisted of four biological replicates and the experiment was repeated twice. The stems of inoculated and control plants were lyophilized and ground to fine powder in liquid nitrogen. Total DNA was extracted from 50 mg of

ground sample using the cetyltrimethylammonium bromide (CTAB) method (Brandfass and Karlovsky 2008). After DNA extraction, the pellet was dissolved overnight in 200 µL TE buffer (10mM Tris, 1 mM EDTA, pH8) at 4 °C. The quality of the extracted DNA was verified by running the samples for 60 min at 3 V/cm on 1% agarose gel (Roth, Karlsruhe; Germany) stained with Midori Green (Nippon Genetics Europe GmbH, Düren; Germany) in 0.5x TBE buffer (Tris-Boric acid-EDTA). DNA was visualized under UV light and documented with the software INTAS GelDoc (Intas Science Imaging Instruments GmbH, Göttingen; Germany).

A CFX384 Thermocycler (Bio-Rad, Rüdigheim; Germany) with a 384 well microplate (SARSTEDT AG & Co. KG, Nümbrecht; Germany) was used for the amplification and quantification of *V. longisporum* DNA using ß-tubulin forward (5'-GCAAAACCCTACCGGGTTATG-3') and reverse (5'-AGATATCCATCGGACTGTTCGTA-3') primers (Debode et al. 2011). The amplification reaction had a total volume of 10 µL, which consisted of 5 µL qPCRBIO SyGreen Mix (Nippon Genetics Europe GmbH, Düren; Germany), 0.4 µM of each primer, and 10 ng template DNA. The PCR program consisted of a 3 min initial denaturation step, 40 cycles of 5 s at 95 °C, 15 s at 68 °C, and 15 s at 72 °C, followed by a final extension of 2 min at 72 °C. Each sample had three technical replicates and data was analyzed using the software BioRad CFX Maestro 1.1 (Bio-Rad laboratories, Inc.; USA).

2.4. Study of external root colonization with confocal microscopy

2.4.1. Experimental design and inoculation procedure

For the assessment of external root colonization of A1/D1 and A1/D2 isolates, an *in vitro* system was used to prevent soil particles from disrupting fungal colonization patterns when harvesting the roots. Falcon seeds were surface sterilized by immersion in 70% ethanol for 2 min. Afterwards, they were washed with sterile water and stirred in 1% sodium hypochlorite + 1% Tween 80 solution for 15 min. Following disinfection, seeds were washed three times with sterilized water and left to germinate for 24 hours on moist sterilized filter paper. Then, seeds were plated on square Petri dishes (10 x 10 x 2 cm) with a modified plant nutrient medium (Appendix 1) covered with a nylon membrane (Sefar Nitex 03-70/33; pore size 70 µm, Sefar GmbH; Switzerland) to prevent roots from growing into the medium (Johnson et al. 2011). Petri dishes were incubated vertically in a growth chamber at 20–23 °C and with a 14 h photoperiod (220 $\mu mol.m^{-1}s^{-1}$). Three days after plating the germinated seeds on the medium, roots were sprayed for 2 s with the spore suspension (section 2.1) of each isolate. Seedlings were sampled at 2, 3, and 7 dpi. Three Petri dishes were used for each time point, so that 12 plants were examined per treatment and sampling day. The differentiation and elongation zone of the main root were investigated by first selecting two sections of each zone where hyphal growth was visible with light microscopy. The root cap of the main root was also observed. Additionally, the presence of microsclerotia and spores in each sample was recorded. The experiment was repeated twice.

2.4.2. Staining for confocal microscopy

The entire root system was stained with a sequential double staining method. Samples were first immersed in 50 µg.mL^{-1} Wheat Germ Agglutinin, Alexa Fluor® 488 Conjugate (W11261, Molecular probes; USA) and vacuum-infiltrated in the dark at room temperature for 30 min. Subsequently, samples were vacuum-infiltrated for 20 min with 10 µg.mL^{-1} propidium iodide (2470810 Carl Roth GmbH, Karlsruhe; Germany). Roots were immediately dipped twice in distilled water, mounted on 50% glycerol, and covered with a coverslip (0.16 mm thickness). Microscopic examinations were performed using a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Manheim; Germany). Two-channel analysis was carried out with a 488 nm wavelength to excite Alexa Fluor and 500–530 nm to receive the emission. In the case of propidium iodide, a 550–560 nm wavelength range was used for excitation and 608–680 nm to receive the emission.

2.5. Study of internal root colonization by transmission electron microscopy (TEM)

2.5.3. Experimental design and inoculation procedure

Surface-sterilized Falcon seeds (section 2.4.1) were sown in 9 x 9 x 10 cm pots containing autoclaved quartz sand. One week after sowing, six seedlings per treatment were uprooted and root-dip inoculated (section 2.2) with VL43, A1/D2a, or water. Sand was selected as the substrate to ensure a complete harvest of roots without any adhering material while mimicking physical conditions. Pots were watered every other day and fertilized twice per week with full nutrient solution (Flory Basisdünger, EUFLOR GmbH, Schermbeck; Germany). The experiment was repeated twice and kept for four weeks in a controlled chamber at 23 °C/20 °C (day/night) and with a 14h photoperiod using white bulbs (220 µmol.m^{-1}s^{-1}).

2.5.4. Specimen preparation for electron microscopy

Four weeks after inoculation, root sections of each seedling (1 cm) were cut 1 cm below the hypocotyl. The fixative solution was prepared before cutting the samples but never longer than two hours before fixation. Sodium cacodylate (SC) (11654, Electron Microscopy Sciences; USA) stock buffer was diluted to 0.1 M with distilled water. Then, glutaraldehyde (16220, Electron Microscopy Sciences; USA) was diluted to 2.5% with 0.1 M SC. Six root fragments of each treatment were immediately transferred to an 1.5 mL Eppendorf tube with fixative and left at room temperature for 1 h. Old fixative was removed, fresh fixative was added, and tubes were stored overnight at 4 °C. Then, fixative was gently removed from each tube and samples were washed with 0.1 M SC three times for 10 min at room temperature. Osmium tetroxide (11654, Electron Microscopy Sciences; USA) was diluted to 1% with 0.1 M SC and pipetted into each tube. Tubes were left closed for two hours at room temperature. After two hours, OsO_4 solution was removed and samples were immediately washed with 0.1 M SC three times for 10 min.

After SC washes, successive dehydration with ethanol (9065.3, Carl Roth GmbH, Karlsruhe; Germany) started with 30% and 50% ethanol consecutive steps of one hour each at room temperature, followed by a dehydration step at 4 °C overnight with 90% ethanol. The next day, samples were dehydrated with 100% ethanol twice for 30 min at room temperature. Afterwards, two incubations with propylene oxide (20412, Electron Microscopy Sciences; USA) of 15 min each were carried out. Resin was prepared following the manufacturer's instructions (Araldite, Embed 812 Epon-812, Electron Microscopy Sciences; USA). When the propylene oxide was discarded, a 1:1 mixture of resin and propylene oxide was pipetted into the tubes and they were left overnight on a rotary shaker. The next day, 1:1 resin and propylene oxide mixture was replaced by a 1:3 mixture and left on the rotary shaker for six hours at room temperature. After changing the mixture to 100% resin, the tubes were left on the rotary shaker overnight and then polymerized for 24 hours at 60 °C on a silicone mold (CASP-SCI-G, Science Services GmbH, München; Germany)

For the preparation of sections for transmission electron microscopy, resin blocks were trimmed by hand with a razor blade (E71962, Science Services GmbH, München; Germany). To select areas of interest for ultrathin cuts, semi-thin sections (99 µm) for light microscopy were cut with a diamond knife (Diatome AG, Bienne; Switzerland) using an ultramicrotome (Ultracut E, Reichert-Jung; Austria). For light microscopy, semi-thin sections were stained with 0.1% toluidine blue for 5 min at 60 °C. Ultra-thin sections (70–90 nm) were placed on 150 mesh grids coated with Formvar and Carbon (EFCF150, Science Services GmbH, München; Germany). Between four and six samples were prepared per treatment and experimental repetition. For each sample, at least two ultra-thin sections were cut. Finally, samples were contrasted with UranyLess (E22409, Electron Microscopy Sciences; USA) and lead citrate (E22410, Electron Microscopy Science; USA) with 1 min incubation each and three washing steps between the incubations. Transmission electron microscopy was performed on a Zeiss EM 902 microscope (Carl Zeiss AG, Oberkochen; Germany).

2.6. Statistical analyses

Statistical analyses were carried out with R (version 4.0.2). Analyses of phenotypic assessments were carried out with a linear mixed model, where experimental replicates were the random factor. A box-cox transformation was performed, if necessary, based on the variance homogeneity. For fungal DNA quantification, the non-parametric Kruskal-Wallis test was selected. For post hoc analyses, a Tukey HSD test or Pairwise Wilcoxon Rank Sum test at a significance level of 0.05 were used

3. Results

3.1. Pathogenicity assessment in the greenhouse

V. longisporum A1/D2 isolates produced minimal symptoms on oilseed rape seedlings in comparison with VL43, with no significant differences between the three A1/D2 isolates (Figure 1). They induced a net AUDPC

of up to 5 and a height relative to the control between 91 to 96%. In contrast, the aggressive VL43 isolate induced a net AUDPC of 30 and a relative height of 60%. In addition to symptom development, dry biomass was assessed. There were no significant differences between the A1/D2 treatments in the effects on aerial (stem and leaves) and root dry biomass at 28 dpi and between plants inoculated with A1/D2 isolates and the control (Figure 2). However, root-dip inoculation of A1/D2b and A1/D2c caused a significant root biomass reduction of ca. 30% in comparison to the control. The aggressive isolate VL43 significantly reduced the aerial biomass by one third and root biomass by one tenth in comparison to the control.

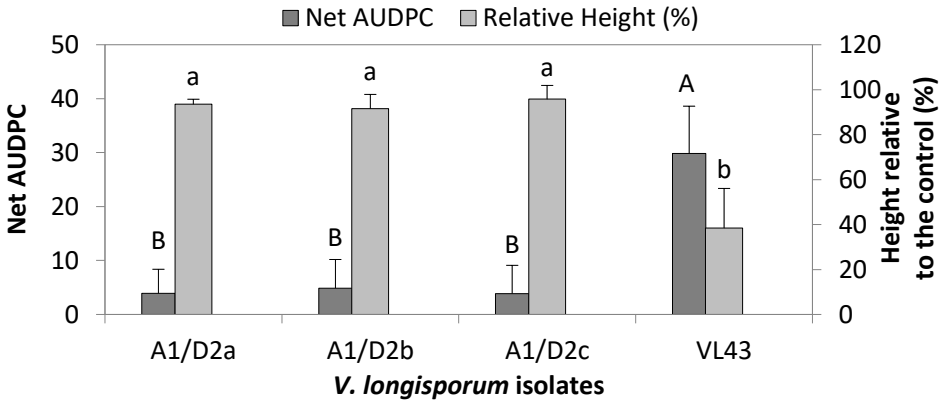

Figure 1. Net area under the disease progress curve (net AUDPC) and plant height relative to the mock-inoculated control of oilseed rape seedlings (cultivar Falcon) after root-dip inoculation (1.10^6 spores / mL) with *Verticillium longisporum* isolate VL43 from lineage A1/D1 and three isolates from lineage A1/D2 (A1/D2a, A1/D2b, and A1/D2c) at 28 days post inoculation. Data points represent the mean of six biological replicates, each consisting of merged data from eight plants. Error bars refer to the standard deviation. Different letters indicate significant differences (Tukey test, $P \leq 0.05$).

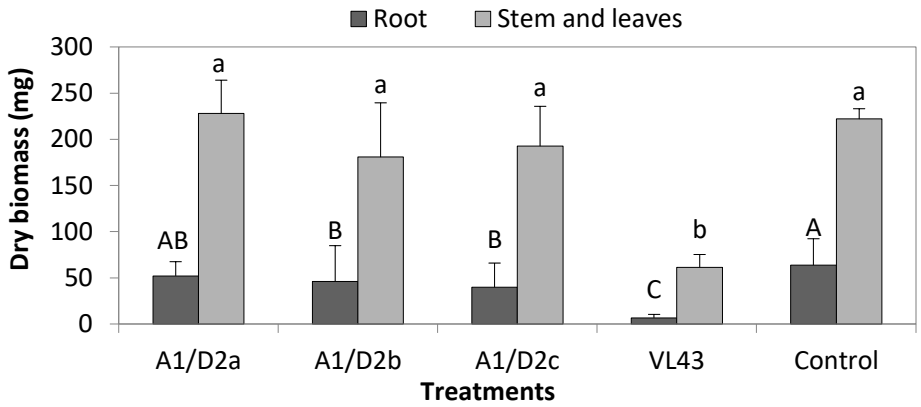

Figure 2. Dry biomass of roots and aerial tissues (stem and leaves) of oilseed rape seedlings (cultivar Falcon) after root-dip inoculation (1.10^6 spores / mL) with *Verticillium longisporum* isolate VL43 from lineage A1/D1, three isolates of lineage A1/D2 (A1/D2a, A1/D2b, and A1/D2c), and water (control) 28 days post inoculation. Data points represent the mean of six biological replicates, each consisting of merged data from eight plants. Error bars refer to the standard deviation. Root biomass was analysed separately from stem and leaf biomass. Different letters indicate significant differences (Tukey test, $P \leq 0.05$).

3.2. Quantification of systemic fungal colonization of the plant

The amount fungal DNA in oilseed rape stems at 28 dpi was quantified with qPCR. There was less than 1 ng of fungal DNA per g of plant tissue detected in A1/D2-inoculated samples and there were no significant differences in the amount of DNA detected between samples treated with the three A1/D2 isolates. In contrast, the amount of DNA detected in stem samples from VL43 infected plants was 30 times higher than in A1/D2-treated plants (Figure 3).

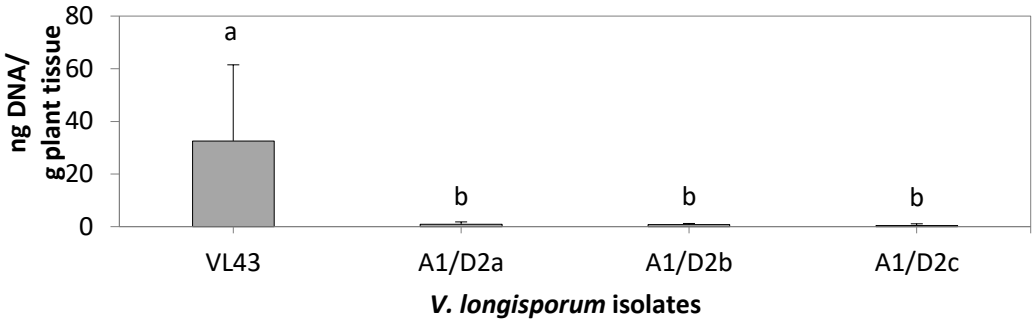

Figure 3. Plant colonization of *Verticillium longisporum* isolate VL43 of lineage A1/D1 and three isolates of lineage A1/D2 (A1/D2a, A1/D2b, and A1/D2c) in oilseed rape seedlings (cultivar Falcon) after root-dip inoculation (1.10^6 spores / mL). Colonization was measured as fungal DNA in stem tissue 28 days post inoculation. Data points represent the mean of eight biological replicates, each consisting of eight stem samples. Error bars refer to the standard deviation. Different letters indicate significant differences (Pairwise Wilcoxon Rank Sum, $P \leq 0.05$).

3.3. External root colonization

The main external root colonization patterns observed for VL43 and A1/D2 isolates on oilseed rape seedlings are summarized in Table 2. All isolates had their first contact with the plant on root hairs. In contrast to VL43, A1/D2 isolates showed early sporulation, hyphal coiling, and superficial mycelial growth. However, only one A1/D2 (isolate A1/D2a) presented those three colonization patterns simultaneously. VL43 was the only isolate that produced net-like hyphal layer on the root surface. All isolates produced microsclerotia and/or thickened hyphae.

3.3.1. Root hair colonization

Regardless of the isolate, the first contact of hyphae with roots was on root hairs occurring at 2 dpi. From the root hairs, hyphae grew towards the surface of the main root. In all samples, hyphae also grew from root hair to root hair (Figure 4). A1/D2a and A1/D2b hyphae extensively grew in the rhizosphere in a disorganized manner (Figure 5). Such growth was not observed for A1/D2c or for VL43.

Table 2. External root colonization patterns of *Verticillium longisporum* isolate VL43 of lineage A1/D1 and lineage A1/D2 (A1/D2a, A1/D2b, and A1/D2c) on oilseed rape seedlings (cultivar Falcon). dpi=days post inoculation.

Colonization patterns	A1/D2a	A1/D2b	A1/D2c	VL43
Early sporulation (3 dpi)	Yes	No	Yes	No
First contact at root hairs (2 dpi)	Yes	Yes	Yes	Yes
Growth parallel to the epidermal junctions (3, 7 dpi)	Yes	Yes	Yes	Yes
Hyphal coiling (3, 7 dpi)	Yes	Yes	No	No
Microsclerotia formation (7 dpi)	No	Yes	Yes	Yes
Net-like hyphal growth (3, 7 dpi)	No	No	No	Yes
Extensive outward mycelial growth (3, 7 dpi)	Yes	Yes	No	No
Root cap colonization (7 dpi)	Yes	No	Yes	Yes
Thickened hyphae (7 dpi)	Yes	No	Yes	No

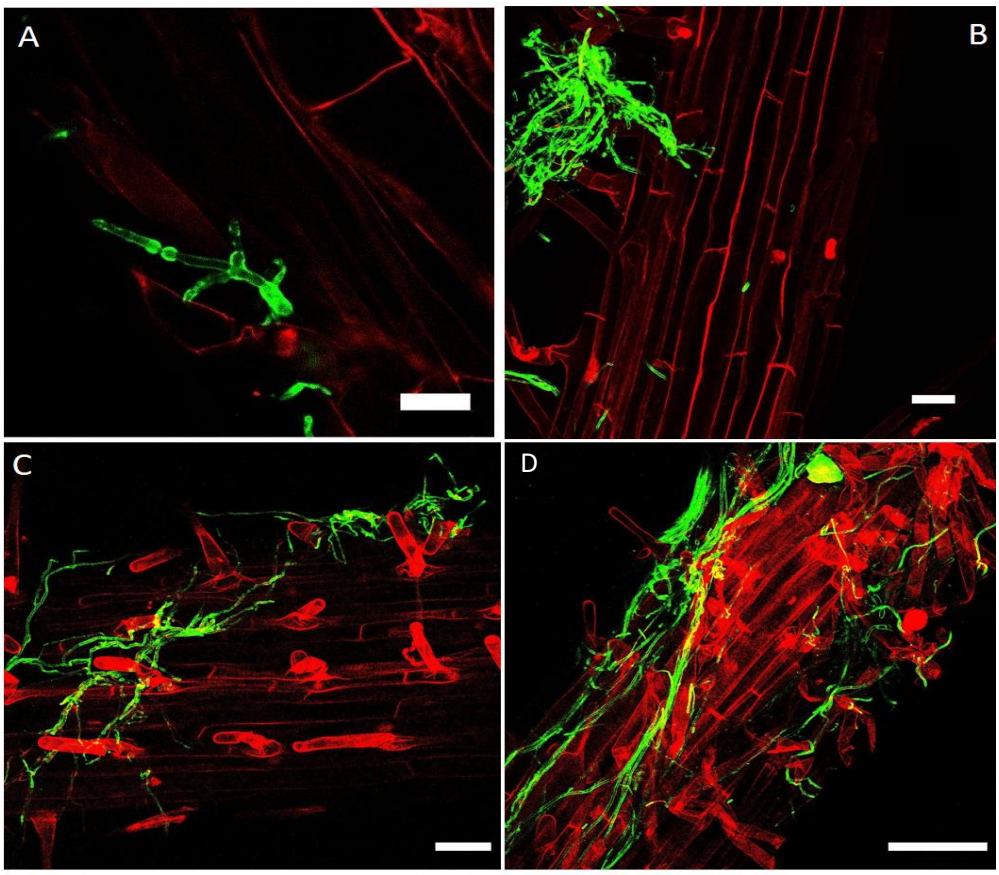

Figure 4. Confocal microscopic analysis of the hyphal growth of *Verticillium longisporum* lineage A1/D1 (isolate VL43) and lineage A1/D2 (isolates A1/D2a and A1/D2c) on the root surface of oilseed rape seedlings (cultivar Falcon). A) Hyphal elongation of VL43 around root hair at 2 days post inoculation (dpi). B) Hyphal growth of A1/D2c from root hairs towards the root surface at 3 dpi. C) Hyphal growth of A1/D2a from root hair to root hair at 2 dpi D) Hyphal growth of VL43 from root hair to root hair at 2 dpi. Hyphae (green) were stained with Alexa Fluor and roots (red) with propidium iodide. Bar A= 20.2 µm, B= 30 µm, C= 50 µm, D= 100 µm.

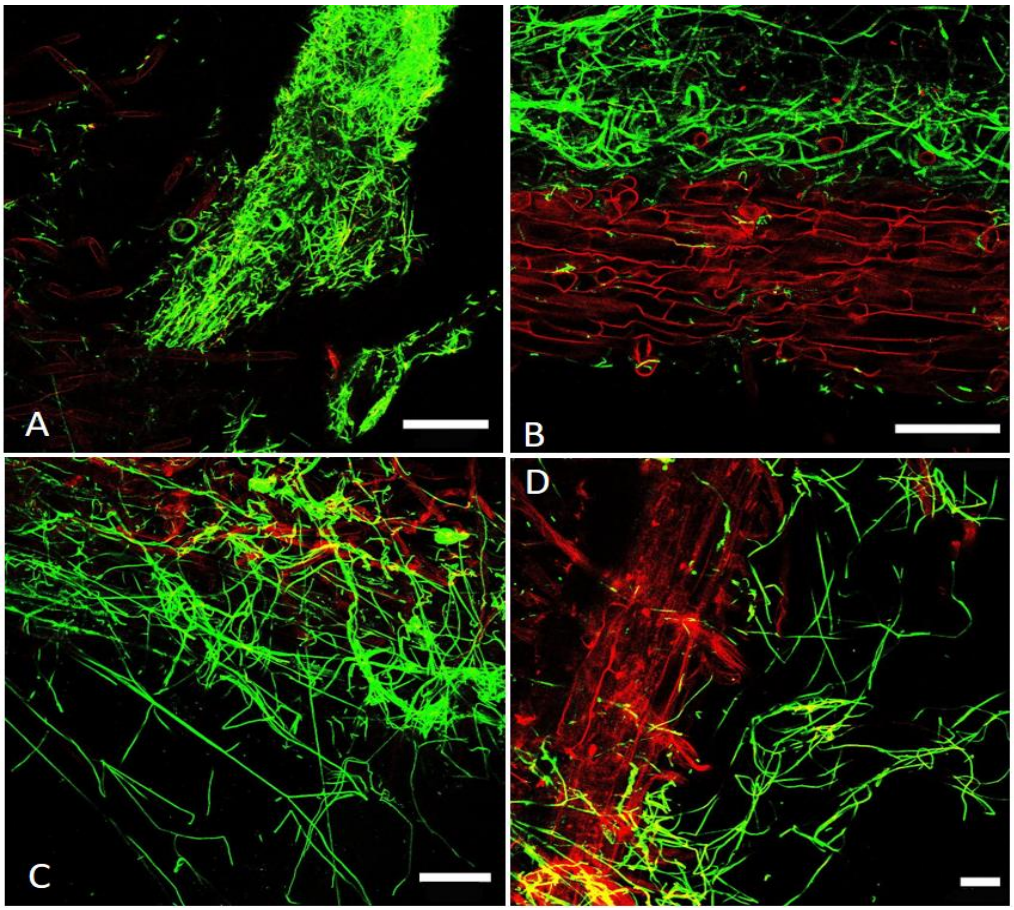

Figure 5. Confocal microscopic analysis of *Verticillium longisporum* lineage A1/D2 hyphal growth in the rhizosphere of roots of oilseed rape seedlings (cultivar Falcon) at 7 days post inoculation. A, B) Isolate A1/D2a. C, D) Isolate A1/D2b. Hyphae (green) were stained with Alexa Fluor and roots (red) with propidium iodide. Bar A-C= 100 µm, D= 50 µm.

3.3.2. Colonization patterns on the root surface

All isolates colonized the root extensively at 3 dpi and displayed hyphal growth parallel to the intercellular junctions at the differentiation zone. VL43 hyphae colonized the root surface in a net-like manner along the elongation and differentiation zones. In addition, VL43 hyphae were in close contact with the root. The tight net-like pattern was observed in one third of VL43-treated samples (Figure 6). A1/D2a and A1/D2c were both able to colonize the root cap but with less close contact to the root surface than VL43 (Figure 7).

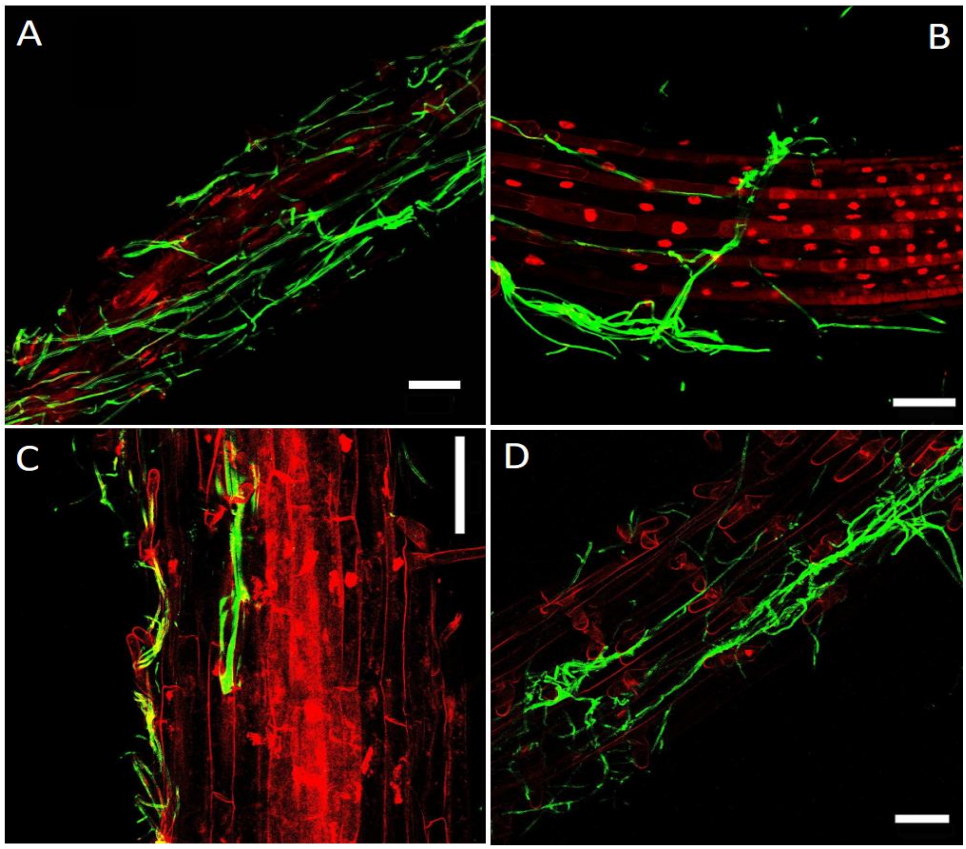

Figure 6. Confocal microscopic analysis of colonization patterns of *Verticillium longisporum* lineage A1/D1 (isolate VL43) and lineage A1/D2 (isolate A1/D2a) on roots of oilseed rape seedlings (cultivar Falcon). A) Net-like hyphal growth of VL43 on elongation zone at 3 days post inoculation (dpi). B) Net-like hyphal growth of VL43 on elongation zone at 7 dpi. C) VL43 hyphal growth parallel to epidermal cell junctions of the differentiation zone at 3 dpi. D) Hyphal growth of A1/D2a parallel to epidermal cell junctions of the differentiation zone at 3 dpi. Hyphae (green) were stained with Alexa Fluor and roots (red) with propidium iodide. Bar A= 50.2 µm, B-D= 50 µm, C=100 µm.

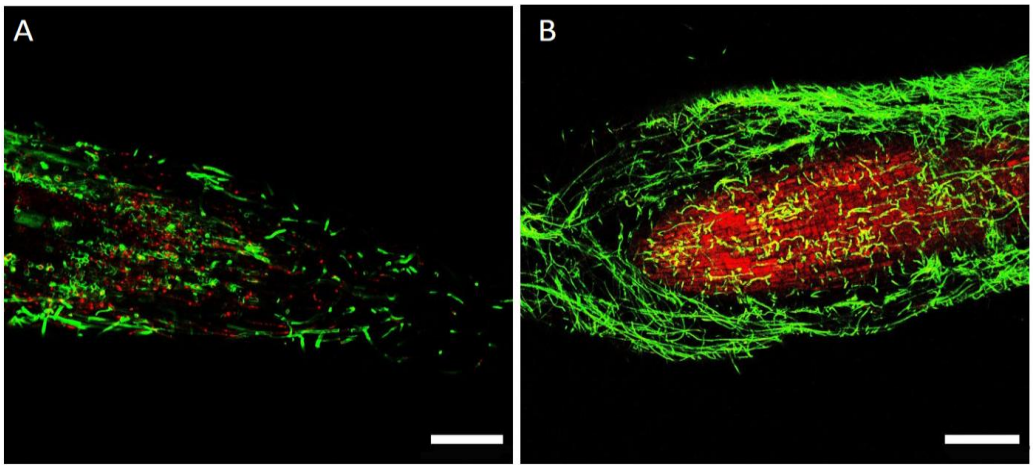

Figure 7. Confocal microscopic analysis of the root cap of oilseed rape seedlings (cultivar Falcon) colonized by *Verticillium longisporum* lineage A1/D1 (A, isolate VL43) and lineage A1/D2 (B, isolate A1/D2a) at 7 days post inoculation. Hyphae (green) were stained with Alexa Fluor and roots (red) with propidium iodide. Bar A= 50 µm, B= 100 µm.

3.3.3. Differences in sporulation and penetration differences between isolates

A1/D2a and A1/D2c produced conidiophores at 3 dpi. Additionally, groups of spores from A1/D2c and single spores from A1/D2a were observed at 3 dpi in all samples. In contrast, VL43 and A1/D2b only produced spores at 7 dpi. VL43 started forming penetration pegs at 3 dpi, which consisted of a hyphal tip enlargement at the epidermal cell walls of roots (Figure 8).

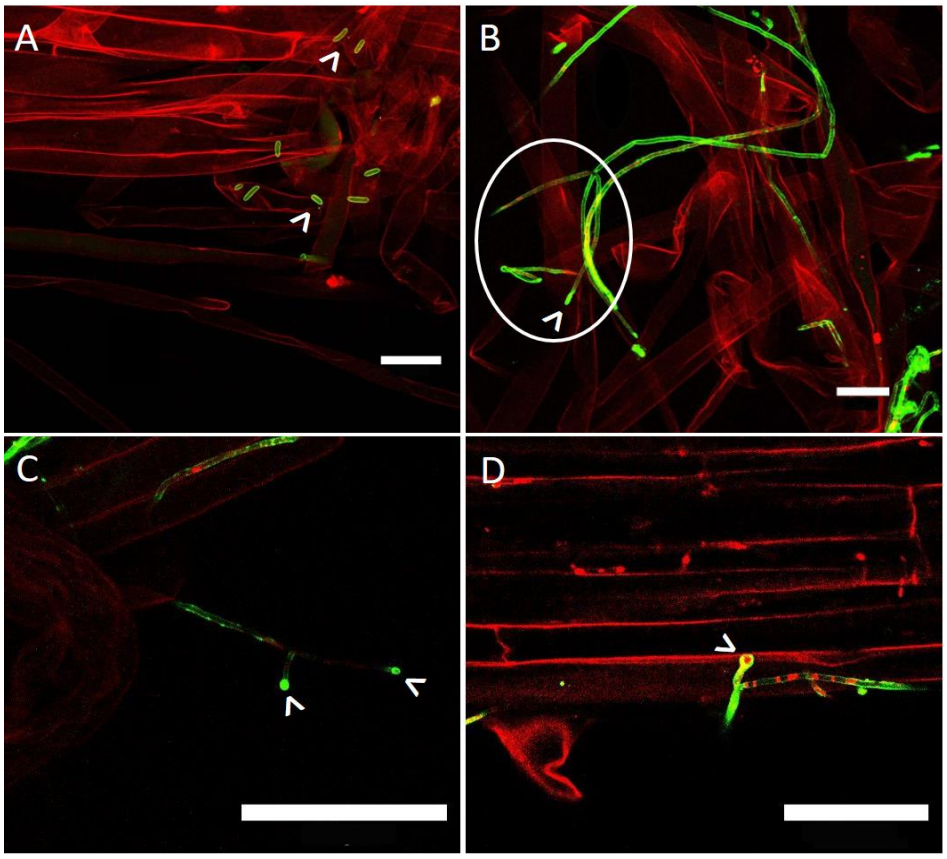

Figure 8. Confocal microscopic analysis of sporulation and root penetration by *Verticillium longisporum* lineage A1/D1 (isolate VL43) and lineage A1/D2 (isolates A1/D2a and A1/D2c) on roots of oilseed rape seedlings (cultivar Falcon). A) A1/D2c spores (arrow) around the root surface at 3 days post inoculation (dpi). B) Spore formation (arrow) of A1/D2c at conidiophore tip (circle) at 3 dpi. C) Spore formation (arrow) of A1/D2a at conidiophore tip at 3 dpi. C) Penetration peg (arrow) of VL43 at the epidermal cell wall of the root at 3 dpi. Hyphae (green) were stained with Alexa Fluor and roots (red) with propidium iodide. Bar A= 30 µm, B-C= 20 µm, D=50 µm.

3.3.4. Hyphal coiling, hyphal thickening and microsclerotia formation

A1/D2a and A1/D2b produced coiled hyphae in all samples, either on the surface of the main root or close to root hairs, at 3 dpi. A1/D2b, A1/D2c, and VL43 produced microsclerotia at 7 dpi. VL43 formed microsclerotia at the base of root hairs and on the root surface, whereas A1/D1b and A1/D2c also formed microsclerotia at the tip of root hairs. Although A1/D2a did not produce microsclerotia, thickened hyphae were observed at 7 dpi. In A1/D2c-treated samples, thickened hyphae and microsclerotia were observed (Figure 9).

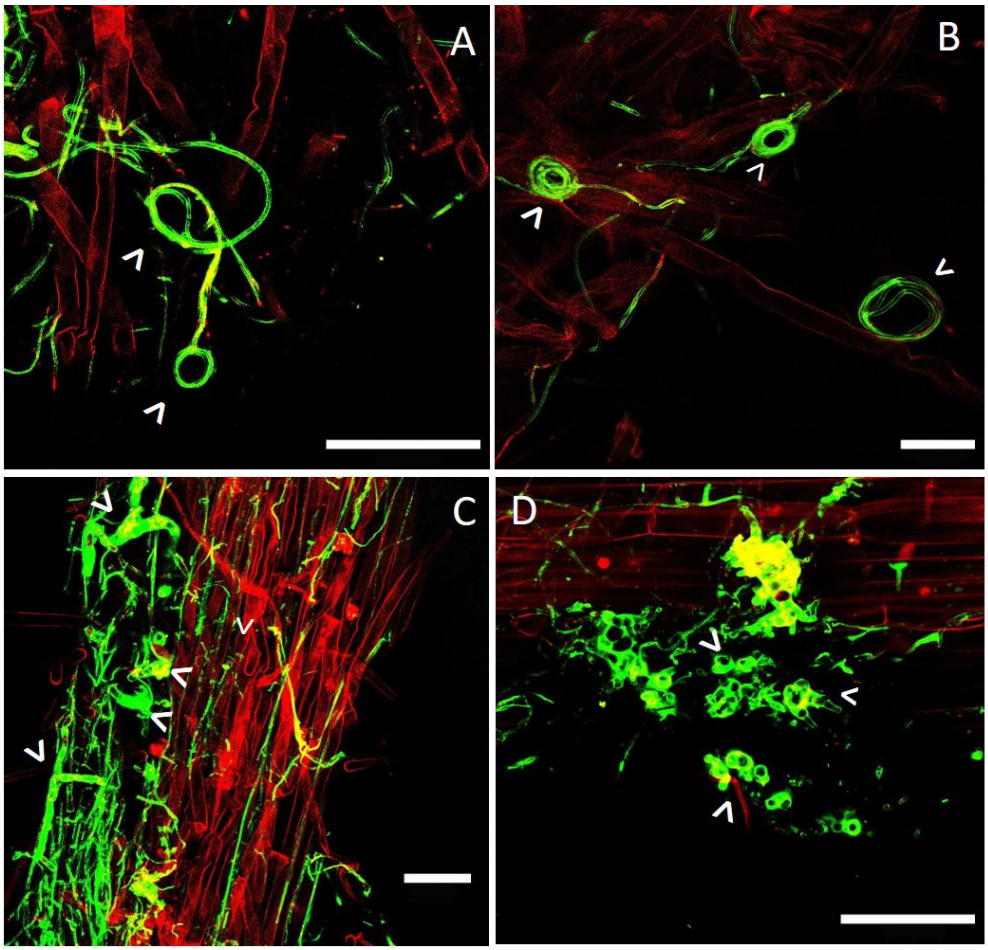

Figure 9. Confocal microscopic analysis of colonization patterns of *Verticillium longisporum* lineage A1/D1 (isolate VL43) and lineage A1/D2 (isolates A1/D2a and A1/D2b) on roots of oilseed rape seedlings (cultivar Falcon). A) Hyphal coiling (arrow) of A1/D2a at 3 days post inoculation (dpi). B) Hyphal coiling (arrow) of A1/D2a at 7 dpi. C) A1/D2a thickened hyphae (arrow) at 7 dpi. D) A1/D2b microsclerotia (arrow) at 7 dpi. Hyphae (green) were stained with Alexa Fluor and roots (red) with propidium iodide. Bar A-D= 100 µm, B=50 µm, C= 60 µm.

3.4. Internal root colonization

3.4.1. Hyphal colonization of vessels

Hyphae from VL43 and A1/D2a could be seen inside the root vascular bundle at 28 dpi and presented similar morphology. Hyphae were round or elongated, and hyphal walls were either in direct contact with the vessel lumen or covered by granular or fibrillar layers of electron-dense material (Figure 10). Hyphae with and without layers of electron-dense material were observed in close proximity to each other in the same vessel. The thickness of the layers was variable, but the thickest layers were found in VL43 hyphae. The hyphae interior also varied in appearance. In some cases, it was filled with electron-dense granular material and, in others, structures resembling vacuoles pushed the cytoplasm towards the hyphal wall. Additionally, half of the VL43-treated samples displayed hyphae with degraded cell walls (Figure 10d).

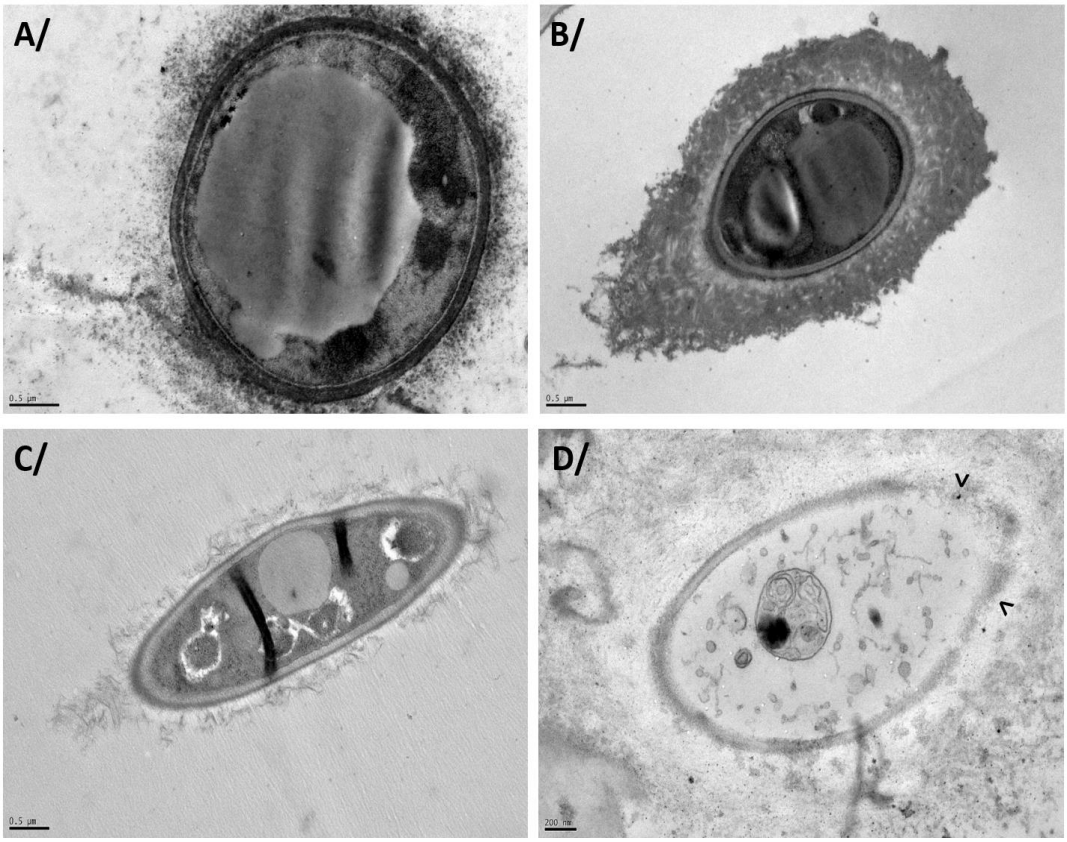

Figure 10. *Verticillium longisporum* hyphae from lineage A1/D1 (isolate VL43) and lineage A1/D2 (isolate A1/D2a) in the xylem lumen in roots of oilseed rape seedlings (cultivar Falcon) 28 days post inoculation. Hyphae were coated by granular or fibrillar layers of different thickness (A-C). VL43 hyphae occasionally displayed degraded walls (arrows) (D). Observations were made with transmission electron microscopy on longitudinal sections of stems fixed with Uranyless and lead citrate. Bars A-C = 0.5 µm, D= 200 nm.

VL43 and A1/D2a hyphae were either free in the lumen (Figure 10) or attached to the vessel walls (Figure 11). In some cases, hyphae were connected to the vessel walls by granular or fibrillar material. Hyphae typically grew along the vessel axis (Figure 11d). While VL43 hyphae were found in all samples, only 25% of samples inoculated with A1/D2a had hyphae in the vascular bundle vessels. VL43 hyphae colonized vessels extensively and many hyphae could be seen in close proximity to each other. In contrast, hyphal colonization in A1/D2a-treated samples was lower and the distribution scattered.

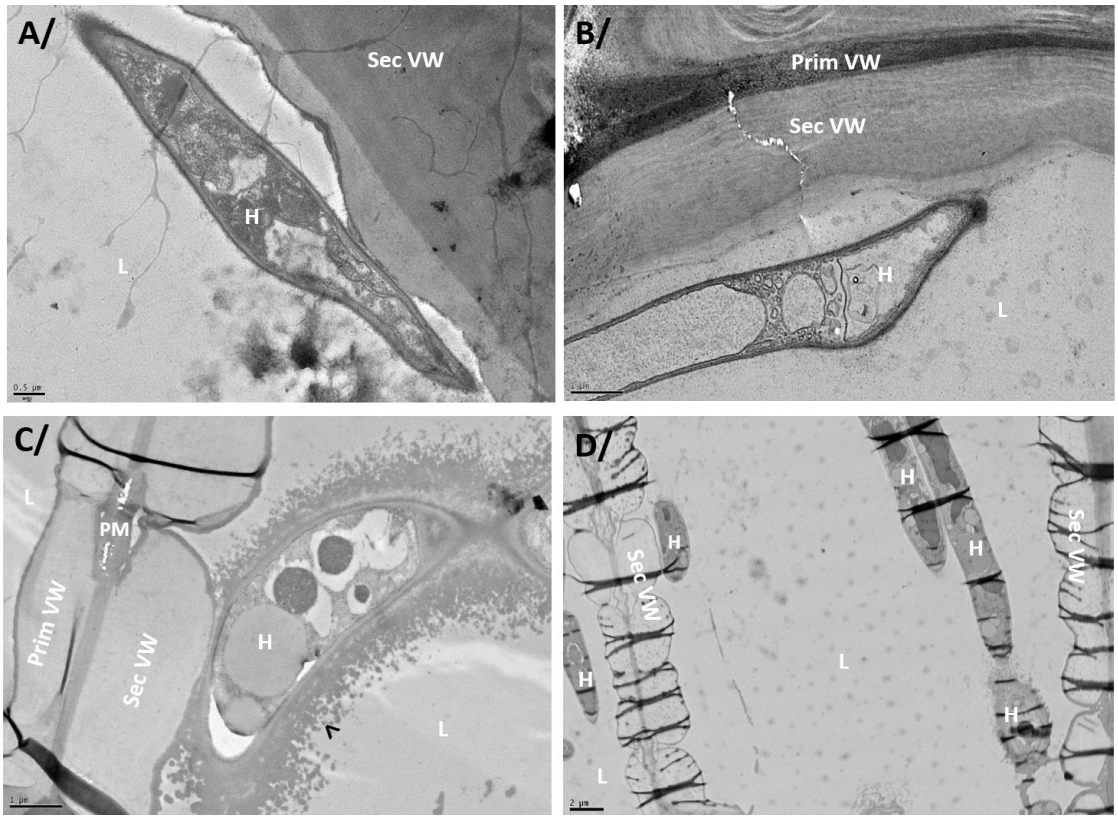

Figure 11. Longitudinal growth of *Verticillium longisporum* hyphae along secondary xylem vessel walls in roots of oilseed rape seedlings (cultivar Falcon) 28 days post inoculation. Hyphae of lineage A1/D1 (isolate VL43) and lineage A1/D2 (isolate A1/D2a) were directly attached to the secondary vessel walls (A, B) or by electron-dense material (C, arrow head). VL43 hyphae extensively colonized xylem vessels (D). Observations were made with transmission electron microscopy on longitudinal sections of stems, fixed with Uranyless and lead citrate. H=Hypha, L= vascular lumen, Prim VW= primary vessel wall, Sec VW= secondary vessel wall, PM= pit membrane, arrow heads= granular layer around hypha. Bars A = 0.5 µm, B-C= 1 µm, D= 2 µm.

3.4.2. Attempted hyphal penetration through vessel walls

Hyphae from VL43 and A1/D2a were observed directed towards vessel pits. Additionally, VL43 hyphae were found inside the pit membrane (Figure 12). Nevertheless, successful penetration through the pit was never observed. Transverse hyphal growth of VL43 through secondary wall openings was observed on two sections of one sample (Figure 13), for which the hyphae adapted their shape to the opening space. The secondary wall openings lacked the typical pit membrane structure, so that the transverse growth was either between two tracheids or between a tracheid and a neighbouring parenchyma cell.

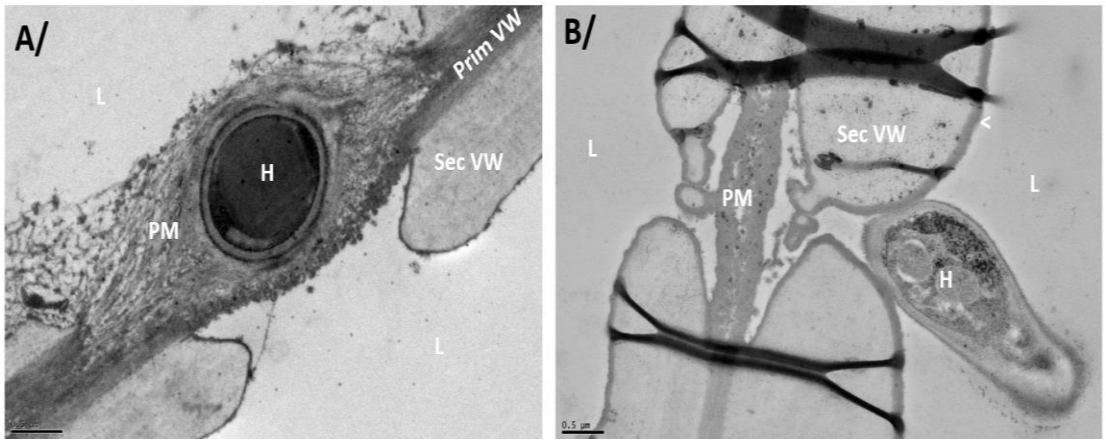

Figure 12. *Verticillium longisporum* movement attempts through pit membranes in the root xylem of oilseed rape seedlings (cultivar Falcon) at 28 days post inoculation. A) Hyphae from lineage A1/D1 (isolate VL43) inside a pit membrane. B) Hyphae from A1/D2 lineage (isolate A1/D2a) directed toward the pit membrane. Complete transverse hyphal growth through the pit membranes was never observed. Observations were made with transmission electron microscopy and longitudinal sections of stems, fixed in Uranyless and lead citrate. H= Hypha, L= vascular lumen, Sec VW= secondary vessel wall, Prim VW= primary vessel wall, PM= pit membrane, arrow= electro-dense coating. Bars= 0.5 µm.

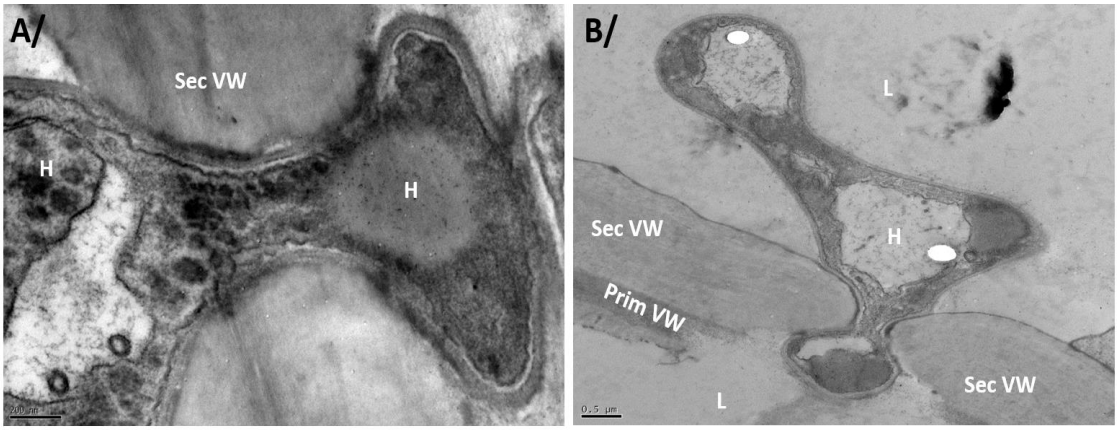

Figure 13. Transverse movement of hyphae of *Verticillium longisporum* lineage A1/D1 (isolate VL43) through secondary wall openings (A, B) in roots of oilseed rape seedlings (cultivar Falcon) 28 days post inoculation. Hyphae adapted their shape to the secondary wall opening. Observations were made with transmission electron microscopy on longitudinal sections of stems, fixed with Uranyless and lead citrate. H=Hypha, Sec VW= secondary vessel wall, Prim VW= primary vessel wall. Bars= 0.5 µm

3.4.3. Ultrastructural changes in the plant

Following infection, various ultrastructural changes were observed inside the xylem vessels. A1/D2a- and VL43-treated samples presented pit membranes with granular and loose appearance, as well as degraded secondary walls (Figure 14b-c). These changes also occurred without near vicinity of hyphae. Morphological changes of pit membranes and secondary walls were present in all VL43-treated samples, but only in about 50% of the A1/D2a-treated samples, including the samples where no hyphae were detected. Degradation of secondary walls in VL43-treated samples was so intense that it sometimes led to a collapse of vessels. In about 50% of VL43-treated samples and about 75% of A1/D2a-treated samples, electron-dense material was

coating the secondary walls and pits (Figure 14a, Figure 15a-c). The coating was absent around heavily degraded secondary walls and pit membranes (Figure 14b-c). In A1/D2a-treated samples, the wall coating was thicker than in VL43-treated samples and occasionally consisted of several layers of electron-dense material that expanded towards the lumen (Figure 15a-b). The coating of vessel walls was also seen without near vicinity of hyphae, as well as in A1/D2b-treated samples where no hyphae were observed. Finally, there were no clear differences between treatments on the cytoplasm appearance of xylem-neighbouring cells or electron-dense deposits in the xylem lumen.

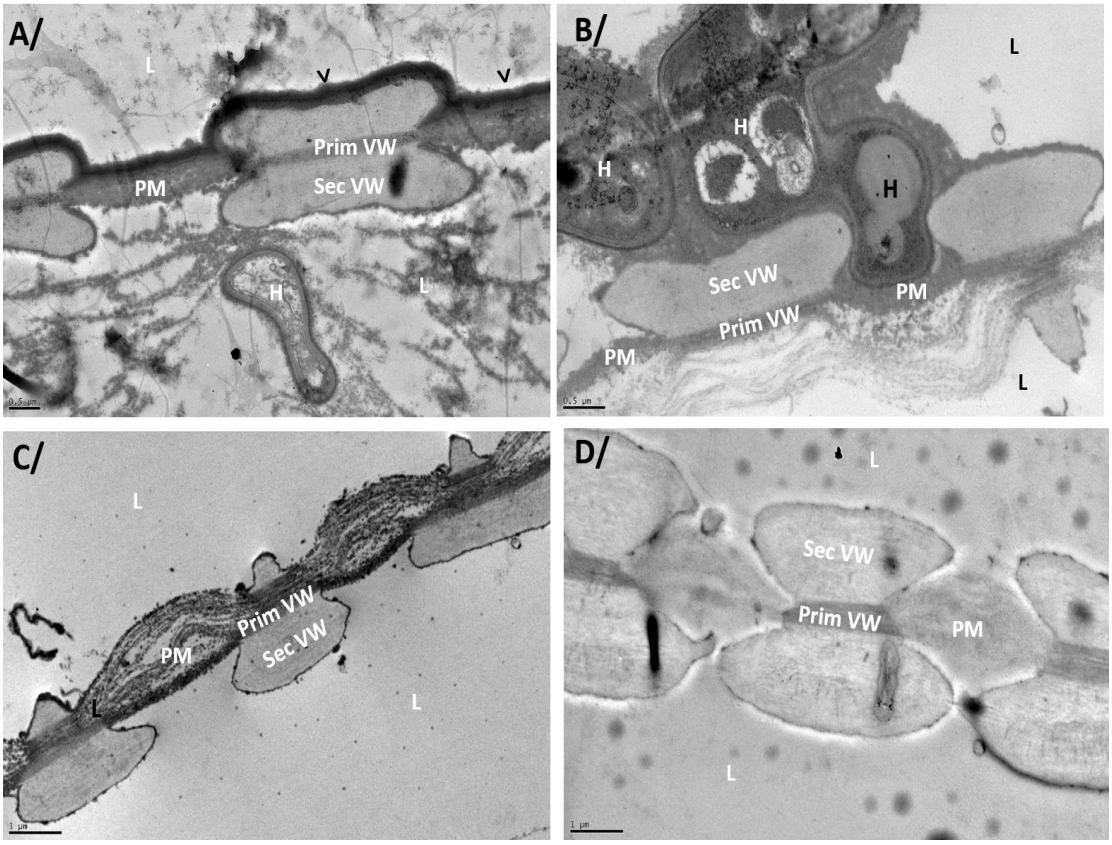

Figure 14. Degradation of pit membranes and secondary walls in the root xylem of oilseed rape seedlings (cultivar Falcon) 28 days after inoculation with *Verticillium longisporum* lineage A1/D2 (isolate A1/D2a) (A) and lineage A1/D1 (isolate VL43) (B, C) in comparison to a mock-inoculated control (D). With fungal infection, pit membranes and secondary vessel walls had a loose and granular appearance and presented an electron-dense coating. Observations were made with transmission electron microscopy on longitudinal sections of stems, fixed with Uranyless and lead citrate. L = vessel lumen, H= hypha, L= vascular lumen, Sec VW= secondary vessel wall, Prim VW= primary vessel wall, PM= pit membrane, arrow heads= electron-dense coating. Bar A= 0.5 µm; B, C= 2 µm.

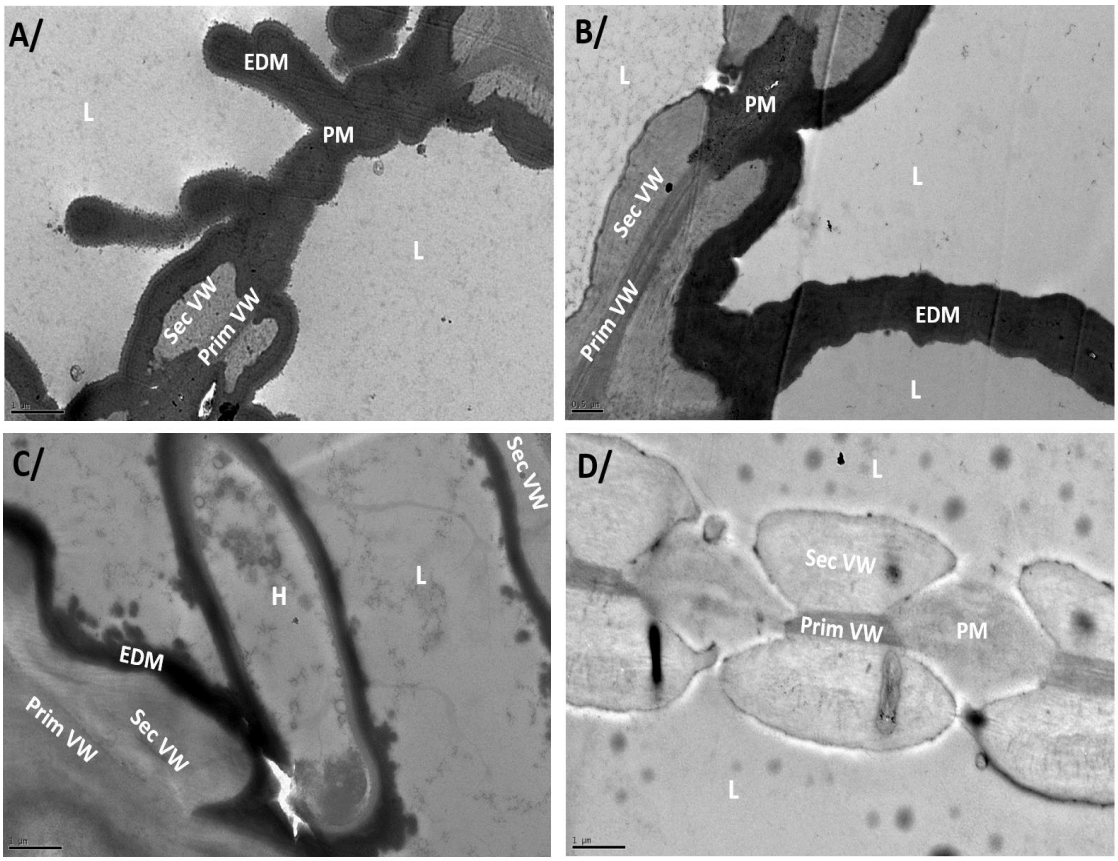

Figure 15. Secondary wall and pit membrane coating in the root xylem of oilseed rape seedlings (cultivar Falcon) 28 days after inoculation with *Verticillium longisporum* lineage A1/D2 (isolate A1/D2a) (A-C) in comparison to a mock-inoculated control (D). Extensive coating of electron-dense material expanded into the vessel lumen (A, B). The coating also covered hyphae that were in direct contact with vessel walls (C). Observations were made with transmission electron microscopy on longitudinal sections of stems, fixed with Uranyless and lead citrate. H= hypha, L= vascular lumen, Sec VW= secondary vessel wall, Prim VW= primary vessel wall, PM= pit membrane, EDM= electron-dense material. Bars A, C, D= 1 µm; B= 0.5 µm.

4. Discussion

4.1. Pathogenicity and systemic colonization assessment

The phenotypic assessment in the greenhouse confirmed the low aggressiveness of lineage A1/D2 in oilseed rape, which had already been reported in previous studies (Depotter et al. 2017a; Novakazi et al. 2015). The three isolates produced very mild leaf symptoms and a slight height reduction (Figure 1). Previous studies reported variability in pathogenicity within lineage A1/D3 (Depotter et al. 2017a; Novakazi et al. 2015). Hence, differences in pathogenicity of lineage A1/D2 might have so far been underestimated due to the low number of available isolates (Inderbitzin et al. 2011b). Despite the mild symptoms caused by A1/D2 isolates, the dry biomass of stems and leaves was not affected (Figure 2). This result is in agreement with Depotter et al. (2017a), who observed minimal root vascular discoloration in oilseed rape seedlings inoculated with the A1/D2c isolate and no significant reduction of aerial plant biomass. However, the present study revealed a

significant reduction in dry root weight caused by A1/D2b and A1/D2c. Vallad and Subbarao (2008) suggested that resistant lettuce cultivars might shed *V. dahliae*-infected lateral roots, which could explain this result.

The assessment of systemic colonization confirmed the relation between the level of fungal vascular colonization of stems and severity of symptoms (Figure 3). This study revealed a minimal systemic colonization by the non-aggressive A1/D2 isolates and a VL43 colonization that was 10 times higher. Similarly, Eynck et al. (2007) showed that fungal DNA in the oilseed rape hypocotyl never exceeded 3 ng/g plant tissue in the non-aggressive interaction of *V. dahliae* with oilseed rape but the DNA amount of VL43 was 20 times higher than *V. dahliae*. For fungal DNA quantification, Eynck et al. (2007) used OLG primers instead of tubulin primers. The study of Knüfer et al. (2017) revealed that the sensitivity of tubulin primers is lower than OLG primers. Thus, the DNA amount of A1/D2 in stems might have been underestimated in the present study. Depotter et al. (2017a) did not report fungal biomass of A1/D2c in stems of oilseed rape seedlings. However, in the same study, samples were harvested at 21 dpi instead of 28 dpi, and a different susceptible cultivar was used. Different levels of fungal systemic colonization in different cultivars with similar mild symptoms had already been reported by Gayoso et al. (2007) for *V. dahliae*.

4.2. External root colonization

The first contact of all tested isolates with oilseed rape roots occurred on root hairs (Figure 4a). This behavior has previously been shown for different *Verticillium* spp. (Eynck et al. 2007; Njoroge et al. 2011; Vallad and Subbarao 2008) and *Fusarium* spp. (Lamo and Takken 2020), regardless of the crop, cultivar, and pathogenicity of the isolate. Although the first contact with the roots was identical for both lineages, VL43 was able to colonize the elongation zone in a net-like pattern (Figure 6a), whereas A1/D2 isolates predominantly grew from root hair to root hair at the differentiation zone (Figure 4c-d). The tight net-like pattern of hyphal growth from VL43 at the elongation zone had already been described by Eynck et al. (2007) for *V. longisporum* in oilseed rape, who suggested that organized hyphal growth of *V. longisporum* in close contact to the root surface is an indication of a compatible interaction with the preferred host.

All isolates showed hyphal growth along the intercellular junctions at the differentiation zone (Figure 6c-d). This growth pattern was already described for other root-colonizing fungi, such as *F. oxysporum* non-pathogenic and pathogenic isolates on roots of tomato seedlings (Bolwerk et al. 2005). The same pattern was observed for *V. dahliae* in different cultivars of cauliflower, broccoli, and lettuce (Njoroge et al. 2011; Vallad and Subbarao 2008), as well as for the beneficial endophyte *Trichoderma harzianum* T-78 in *Arabidopsis thaliana* (Martínez-Medina et al. 2017).

The formation of a hyphal peg for penetration of the root cell wall has already been described for *V. longisporum* in oilseed rape (Eynck et al. 2009a), for *V. dahliae* in cotton and *A. thaliana* (Reusche et al. 2014; Zhao et al. 2016), for *F. oxysporum* in tomato (Lagopodi et al. 2002), and for the root endophyte

Phialocephala fortinii in asparagus (Yu et al. 2001). In this study, in contrast to the report of Reusche et al. (2014), penetration of the aggressive isolate occurred randomly on the root surface independent from the base of root hairs (Figure 8d). Such penetration sites were not observed when plants were inoculated with A1/D2 isolates. Low penetration rates may have prevented the observation of hyphal pegs (Eynck et al. (2007) and explain the low aggressiveness of the A1/D2 lineage. This observation indicates that A1/D2 has a much lower competence to penetrate the root surface. However, the systemic colonization detected by microscopic analysis confirmed the presence of A1/D2 DNA inside the stems (Figure 3). Similarly, Njoroge et al. (2011) reported hyphal growth of *V. dahliae* inside the vascular system of cauliflower and broccoli despite the absence of penetration pegs. Thus, the formation of a penetration peg appears to be specific for individual isolate-host interactions.

Despite differences in hyphal growth patterns on the root, both linages were able to colonize the root quickly, and extensive hyphal growth could already be seen at 3 dpi (Figure 4c-d). Bolwerk et al. (2005) reported that a non-pathogenic *F. oxysporum* isolate colonized the roots more intensely than a pathogenic one. In general, all A1/D2 isolates had more random hyphal growth, which was directed outwards and more prevalent on root hairs than on the root surface (Figure 5). A similar type of fungal root colonization pattern was described in the non-aggressive interaction of *V. dahliae* with oilseed rape (Eynck et al. 2007) and indicates more intense external root colonization of non-aggressive isolates as a response to restricted penetration in a non-preferred host.

Root-colonizing microorganisms have different preferences of root zones for colonization, which might be modulated by distinct root exudates in different root zones (Massalha et al. 2017). This could explain the absence of A1/D2b hyphae on the root cap, although this did not affect colonization and pathogenicity compared to A1/D2b and other A1/D2 isolates. All isolates except A1/D2b produced microsclerotia at 7 dpi on roots (Figure 9d). At 7 dpi, A1/D2b produced thickened hyphae (Figure 9c). Melanized thickened hyphae as resting structures were also described for *V. alfafae* and *V. nonalfalfae* (Inderbitzin et al. 2011a). Nevertheless, these two species do not produce microsclerotia *in vitro*, which A1/D2b does on PDA. Thus, the thickened hyphae may indicate an early phase of microsclerotia formation.

A1/D2a and A1/D2c sporulated at 3dpi in the rhizosphere (Figure 8a). A higher level of sporulation was reported in the non-aggressive interaction of *V. dahliae* with oilseed rape (Eynck et al. 2007), suggesting that early sporulation is triggered by unsuitable conditions due to restricted penetration. Nevertheless, sporulation has been also described at successful infection sites of *V. dahliae* (Klosterman et al. 2009) and *F. oxysporum* (Chen et al. 2019). A1/D2a and A1/D2b produced coiled hyphae on root hairs (Figure 9a-b). These two isolates also showed the most intense superficial hyphal growth. Hyphal coiling on the root surface has previously been described for the endophyte *P. fortinii* in asparagus (Yu et al. 2001). To the best

knowledge of the authors, no coiling of hyphae has so far been described in the literature for vascular pathogens.

4.3. Internal root colonization

The TEM study confirmed that A1/D2a and VL43 isolates colonize the vascular bundle of unwounded roots of *B. napus* seedlings. Penetration through intact tissues was also described for *V. dahliae* in potato roots (Perry and Evert 1982) and for *F. oxysporum* in chickpeas (Jiménez-Fernández et al. 2013). These reports are in contrast with the observations of Bani et al. (2018), who showed that xylem vessels of resistant pea cultivars were only colonized by *F. oxysporum* if roots had been trimmed. The tight contact of hyphae with vessel walls and their arrangement parallel to the vessel axis, as well as the granular and fibrillar material around the hyphae, are common features of vascular pathogens observed inside of xylem vessels (Bishop and Cooper 1983; Eynck et al. 2007). The variety of coating materials around the hyphae (Figure 10a-c) suggests that they are of fungal origin. Since the hyphae could be attached to the vessel walls without the help of these layers (Figure 11a-b), they do not seem to be required for adhesion and their presence or absence might rather be related to different hyphal development and metabolic stages.

Since all root sections used for electron microscopy were longitudinal, it can be assumed that the observed rounded hyphae were conidia (Figure 10a and Figure 12a). This would confirm that *V. longisporum* produces spores to disseminate via the transpiration stream of the plant, as was observed by Eynck et al. (2007). Nevertheless, the high number of elongated hyphae in vessels (Figure 11d) indicates that the role of conidia in longitudinal vascular colonization might be lower than expected, as Bani et al. (2018) already suggested for *F. oxysporum* in pea based on histological observations.

The coating of secondary walls and pit membranes by electron-dense material has been described in potato, pea, and tomato roots infected with different vascular pathogens (Benhamou and Garand 2001; Bishop and Cooper 1983; Perry and Evert 1982). Parham and Kaustinen (1976) related stained phenolic depositions to electron-dense coatings by investigating sections of plant embedded tissues with both light microscopy and TEM. With histological analysis, Eynck et al. (2009a) revealed the role of phenol metabolism in internal defense against *V. longisporum* by reporting a higher accumulation of wall-bound phenolics in the xylem upon VL43 infection in the resistant oilseed rape genotype. Thus, it can be hypothesized that the observed electron-coating depositions on the vessel walls are involved in plant defense associated with phenol metabolism. The vessel coating was more intense in A1/D2a-treated samples than in VL43-treated samples (Figure 15a-b). This indicates that A1/D2a triggers plant defense in Falcon more intensively than VL43, which might explain the lower aggressiveness of A1/D2. Similarly, Benhamou and Garand (2001) reported a more intense vessel coating in pea roots upon infection with a non-aggressive *Fusarium* strain than when plants were inoculated with the pathogenic *F. oxysporum*. Rahman et al. (1999) reported that a resistant cultivar of

pepper produced vascular coating in advance of the invading pathogen *Ralstonia solanacearum*. This would explain why, in this study, some vessels were coated with the electron-dense material despite the absence of A1/D2 hyphae. The absence of A1/D2 hyphae in most samples might simply mean that the colonization level is so low that the hyphae were not found in the examined section. This result is in agreement with the low fungal DNA accumulation observed in the stems (Figure 3). Tyloses which are often described as a typical defense against vascular pathogens (Yadeta and Thomma 2013) were not observed in this study. Similarly, Perry and Evert (1982) were not able to relate tylose formation to intravascular defense mechanisms of potato against *V. dahliae*.

The degradation of pit membranes and secondary walls (Figure 4b-c) occurred in both A1/D2a- and VL43-treated samples without direct hyphal contact, which indicates that degrading enzymes were produced by both the non-aggressive and aggressive isolates and translocated systematically to degrade hemicellulose, cellulose, lignin, and pectin (Bishop and Cooper 1983; Fradin and Thomma 2006; Pouzoulet et al. 2017). In *Verticillium* spp., the production of such enzymes typically correlates with pathogenicity and the level of colonization (Fradin and Thomma 2006), which might explain the higher levels of vessel wall degradation and hyphal colonization in VL43-treated samples in comparison to A1/D2a.

Transversal growth of hyphae through intact pit membranes was described by Bishop and Cooper (1983) and Perry and Evert (1982), but it was not observed in this study. Penetration through root cells towards the xylem vessels could not be described with this analysis, as only the central vascular tissue was selected for ultrathin sections. Transversal growth of VL43 through an opening of secondary wall was observed twice in one sample (Figure 13), but it was not possible to determine whether the transport was between tracheids or between a tracheid and a parenchyma cell. Transversal growth of vascular pathogens between vessels, as well as between a vessel and a xylem parenchyma cell, have been reported by Perry and Evert (1982).

4.4. Conclusions

This study confirmed the non-aggressiveness of lineage A1/D2 in oilseed rape. Additionally, this is the first report describing the root and vascular colonization of lineage A1/D2 in oilseed rape, as well as the first comparative description of the ultrastructural changes produced in the vascular system of oilseed rape by aggressive and non-aggressive isolates of *V. longisporum*. The results indicate that low penetration at the root surface and a poor xylem colonization by A1/D2, as well as a higher intensity of plant defense reactions upon A1/D2 infection in comparison to VL43, might explain the low pathogenicity of the lineage A1/D2.

5. Author contributions

Marta Vega-Marin performed the research. Both authors were involved in the design of the research, discussed collectively the results, and wrote the manuscript.

6. Acknowledgements

This work was financially supported by the German Federal Ministry for Food and Agriculture (BMEL) and the Agency for Renewable Resources (FNR). The authors acknowledge the technical assistance of Isabel Müller in the phenotypic assessment in the greenhouse. Thanks are also extended to Dagmar Tacke for her assistance with DNA extraction and qPCR analysis. Dr. Antonia Wilch, Dima Alnajar, and Alice Eseola provided indispensable guidance in the confocal microscopy assessment. The authors are thankful to Dr. Krzysztof Zienkiewicz for his guidance in sample preparation for electron microscopy. Finally, the authors acknowledge Dr. Ulrike Lipka and Dr. Michael Hoppert for allowing the work on the ultra-microtome and electron microscope at their facilities.

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8. Appendix

Appendix 1. Modified plant nutrient medium composition for *in vitro* cultivation of *oilseed rape* (Johnson et al. 2011)

Component	Amount	Manufacturer
KNO_3	5 mM	Merck Chemicals GmbH, Darmstadt; Germany
$MgSO_4.7H_2O$	2 mM	Carl Roth GmbH, Karlsruhe; Germany
$Ca(NO_3)2$	2 mM	Chemicals GmbH, Darmstadt; Germany
Micronutrient mix:	1 mL/L	
$ZnSO_4.7H_2O$	2 g in 1 L H_2O	Carl Roth GmbH, Karlsruhe; Germany
H_3BO_3	1 g 1 L H_2O	Carl Roth GmbH, Karlsruhe; Germany
$MnSO_4.4H_2O$	0.5 g 1 L H_2O	Carl Roth GmbH, Karlsruhe; Germany
$CoCl_2.5H_2O$	0.16 g 1 L H_2O	Chemicals GmbH, Darmstadt; Germany
$CuSO_4.5H_2O$	0.16 g 1 L H_2O	Carl Roth GmbH, Karlsruhe; Germany
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.11 g 1 L H_2O	Carl Roth GmbH, Karlsruhe; Germany
Fe-EDTA:	2.5 mL/L	
$FeSO_4.7H_2O$	2.6 g in 400 mL H_2O	Carl Roth GmbH, Karlsruhe; Germany
$Na_2EDTA.2H_2O$	3.36 g in 400 mL H_2O	Carl Roth GmbH, Karlsruhe; Germany
Agar	10 g/L	Carl Roth GmbH, Karlsruhe; Germany

Chapter V: Phenotypic evaluation of *Brassica napus* double haploid lines for *Verticillium longisporum* resistance under greenhouse and field conditions

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Abstract

The soil-borne vascular pathogen of *Verticillium longisporum* is one of the most challenging diseases of *Brassica napus*, oilseed rape. Resistance breeding is the most sustainable and effective strategy against vascular pathogens. However, there is limited resistance against *V. longisporum* in the current oilseed rape varieties. In this study, resistance against *V. longisporum* of *B. napus* double haploid lines generated from resynthesized lines was evaluated in the greenhouse and in the field for future QTL mapping and breeding purposes. There was a relatively large variability of responses in the tested *B. napus* lines, both in the field and in the greenhouse. However, the low reproducibility of greenhouse screenings revealed the need of a higher number of replicates for resistance evaluation. For the disease assessment in the field, the systemic fungal colonization in the stems and the microsclerotia development on the stubbles were evaluated. There was a low correlation between greenhouse and field symptoms. The missing correlation might be related to the low number of data points and the low level of symptoms in the field. The differences on microsclerotia development between cropping seasons and locations implied that microsclerotia development might be strongly dependent on moisture and, therefore, might not be a suitable parameter to assess resistance. The results indicate that the disease phenotypes depend on traits that are highly sensitive to the environment, which considerably hinders an accurate disease resistance assessment. Thus, bigger data sets, higher number of replicates, and higher levels of infection in the field through artificial inoculation may be required for accurate disease resistance screening and QTL mapping.

1. Introduction

Rapeseed production has increased more than two-fold since the 2000s; an increase mainly driven by the demand for biofuels (Ismail et al. 2017). Rapeseed is the second most important oilseed crop, accounting for 14% of global vegetable oil production, after oil palm (35%) and soybean (28%) (US Department of Agriculture & USDA Foreign Agricultural Service 2020). The cultivation of rapeseed has also increased due to the benefits it provides when included in the rotation of cereal-based agricultural systems, as it improves soil structure and fertility (Gunstone 2009). *Brassica napus*, oilseed rape, is the dominant rapeseed species in Europe (Gupta 2016). The cultivation expansion of *B. napus* in the 1970s was possible due to the introduction of breeding programs to reduce the amount of erucic acid and glucosinolates to avoid health risks associated to them (Friedt and Snowdon 2010).

To prevent the build-up of diseases, insects, and weeds, oilseed rape should not be grown on the same field more than every three or four year (Hegewald et al. 2018). However, the worldwide demand for biodiesel is driving the oilseed rape production and shortening crop rotations (Ismail et al. 2017). *V. longisporum* is one of the most challenging pathogens of oilseed rape (Depotter et al. 2016) and, so far, has been found in most oilseed rape growing countries, including Canada, the Czech Republic, France, Germany, Sweden, Slovakia, Poland, Russia, and the UK (Bokor et al. 2014; CFIA 2015; Depotter et al. 2016; Gladders et al. 2011; Inderbitzin et al. 2011; Spitzer and Matušinsky 2017; Steventon et al. 2002; Yu et al. 2016).

In oilseed rape, *V. longisporum* does not cause wilting symptoms, unlike in other *Brassica* hosts. Instead, it produces a dark unilateral stem striping during the ripening of the crop. The striping symptoms originate from necrosis, produced after the fungus leaves the xylem and colonizes the parenchyma, which leads to premature ripening of the crop. At plant senescence, microsclerotia start to develop in the stem and roots and are released into the soil after plant decomposition (Dunker et al. 2008). Yield losses by *V. longisporum* have been recorded by Dunker et al. (2008) in individual plants under controlled conditions. In the field, yield losses depend on the cultivar, disease incidence, and weather conditions (Depotter et al. 2019; Zheng et al. 2019). Symptoms caused in oilseed rape by *V. longisporum* in the greenhouse differ from those in field conditions. After root-dip inoculation with a spore suspension at the seedling stage in the greenhouse, plants show chlorosis, vascular discoloration, and stunting (Eynck et al. 2007). The absence of those symptoms in the field is caused by the delay in fungal colonization that plants experience at the beginning of the growing season due to low soil temperatures (Zheng et al. 2019).

The long survival of the fungal resting structures in the soil and the lack of effective curative measures make vascular pathogens very difficult to control. Resistance breeding is the most sustainable and effective strategy against them (Yadeta and Thomma 2013). However, there is limited resistance against

V. longisporum in current oilseed rape varieties, due to past intensive breeding for quality traits and subsequent reduction in the genetic basis of the species (Friedt and Snowdon 2010).

In 2003, *Brassica oleracea* accessions resistant to *V. longisporum* were identified (Happstadius et al. 2003). Thereafter, resynthesized *B. napus* lines with enhanced resistance were developed by interspecific hybridization of *B. oleracea* with *Brassica rapa* (Eynck et al. 2009b; Happstadius et al. 2003; Rygulla et al. 2007). The identification of quantitative trait loci (QTL) for resistance is the starting point for marker-assisted breeding of resistant cultivars. Rygulla et al. (2008) and Obermeier et al. (2013) identified two and three resistance QTLs, respectively, by using segregating double haploid (DH) populations generated from resynthesized *B. napus* lines combined with *B. napus* cultivars. Additional efforts need to be made to map additional QTLs, which can be used to develop genetic markers to localize important genes for stable resistance (Obermeier et al. 2013).

Resistance screening in the greenhouse is a high throughput method that allows the assessment of a high number of genotypes in a short time. Previous studies have revealed the suitability of this method to identify Brassica accessions with enhanced resistance to *V. longisporum,* as well as for mapping resistance QTLs (Eynck et al. 2009b; Obermeier et al. 2013; Rygulla et al. 2007; Rygulla et al. 2008). However, the generated greenhouse data should be validated in the field (Eynck et al. 2009b). Resistance QTLs against powdery and downey mildew in rose and pearl millet, respectively, have successfully been validated in different environmental conditions (Jones et al. 2002; Linde et al. 2006). For the resistance assessment of Brassica accessions against *V. longisporum* in field conditions, two parameters are typically used: the visual assessment of microsclerotia development on stubbles collected after harvest and fungal colonization at the beginning of ripening. In previous studies, microsclerotia assessment has revealed resistance differences between cultivars (Depotter et al. 2019; Dunker et al. 2008; Knüfer et al. 2017). Regarding the assessment of fungal colonization in the stem, Knüfer et al. (2017) reported a strong correlation between greenhouse symptoms and fungal colonization in the field.

In the presented study, *V. longisporum* resistance in *B. napus* double haploid lines (DH) obtained from resynthesized lines was evaluated in the greenhouse and in the field for future QTL mapping and breeding purposes. In addition, the robustness of the different disease parameters, as well as the relationship between them, was assessed.

2. Methods

2.1. Assessment of *B. napus* resistance in greenhouse conditions

2.1.1. *V. longisporum* inoculum

The *V. longisporum* isolate VL43 was obtained from the fungal collection of the Division of Plant Pathology and Crop Protection of the Georg-August University of Göttingen. VL43 was isolated from a diseased oilseed rape plant in Germany in the 1990s (Zeise and Tiedemann 2001 and 2002). The pathogenicity of this isolate is well documented, and it has been extensively used in resistance screenings for identification of less susceptible accessions, as well as for QTL-mapping purposes (Dunker et al. 2008; Gabur et al. 2020; Obermeier et al. 2013; Rygulla et al. 2007).

For the preparation of spore inoculum, 1 mL of spore suspension, which had previously been stored axenically at -80 °C in 25% aqueous glycerol, was added to potato dextrose broth (PDB) in Erlenmeyer flasks amended with 400 ppm streptomycin, 50 ppm chloramphenicol, and 50 ppm rifampicin. PDB flasks were incubated on a shaker (100 rpm) at 22 °C in the dark. After 10 days, the culture was filtered through a sterile sieve. The spore density was determined using a Thoma haemocytometer and diluted to 1 x 10^6 spores / mL. The spore suspension was applied as a 30 min root-dip inoculation. Roots of control seedlings were immersed in water.

2.1.2. Plant material

B. napus cultivars `Falcon´ and `Express´, which are susceptible and moderately resistant to *V. longisporum* (Eynck et al. 2009b), were used as reference cultivars. Two DH-populations were screened for resistance, `R53xEx617´ (n=250) and `SW99-307´ (n=165). R53xEx617 was produced by Saaten Union BioTec GmbH (Leopoldshöhe; Germany) through a cross between the inbred line `Express 617´, derived from the cultivar Express, and `R53´, a resistant resynthesized line. R53 originated from an interspecific hybrid of a cross between *B. oleracea* ssp. *oleracea* and *B. rapa* ssp. *pekinensis* (Obermeier et al. 2013). SW99-307 was produced by Lantmännen SW Seed AB (Svalöv; Sweden) and Deutsche Saatveredelung AG (Lippstadt; Germany) using resynthesized *B. napus* accessions as the resistance donors (Rygulla et al. 2008).

2.1.3. Experimental design

Greenhouse screenings of the two DH populations were conducted in four rounds. The last round consisted of randomly selected lines that had been tested before. Each round had between 70 and 120 lines. Due to problems in germination and seed availability, the complete populations could not be tested (Table 1).

Table 1. Number of *Brassica napus* lines from the double haploid populations R53xEx617 and SW99-307 that were assessed in each screening round in the greenhouse for resistance to *Verticillium longisporum*.

Screening A	94
Screening B	119
Screening C	73
Screening D	82
Total R53xEx617 lines	207
Repeated R53xEx617 lines	94
Total SW99-307 lines	41
Repeated SW99-307 lines	39

Seeds were pre-germinated in quartz sand at 18–24 °C and with a 14 h photoperiod (Horti-Lux HPS-400 Watt). Ten days after sowing, seedlings were removed from the quartz sand and washed thoroughly under running tap water. Root-dip inoculation was carried out as described in section 2.1.1. Seedlings immersed in water were regarded as the control. Due to time limitations, the inoculation of all lines for each screening round was conducted in two consecutive days using the same spore stock solution. Consequently, for each screening round, Falcon and Express seedlings were inoculated twice.

Every treatment (water and VL43) had 12 plants. Treated plants were transplanted into 7 x 7 x 8 cm pots filled with a substrate of sand and standard soil (1:3). Each pot had two plants with the same treatment and pots from different treatments were kept in different trays to avoid cross-contamination. Pots from one treatment were divided in three trays and each tray contained up to 24 pots from twelve different lines. All trays were organized according to a completely randomized design and kept with the same light and temperature conditions as mentioned above.

2.1.4. Disease assessment

Disease assessment was conducted at 7, 14, 21, and 28 days post inoculation (dpi). The assessment of yellowing and death of the leaves was done according to the 9-score assessment key described by Eynck et al. (2009b), and the net area under disease progress curve (AUDPC) was calculated accordingly. At 28 dpi, plant height was measured from the cotyledons to the tip of the longest leaf to calculate the stunting effect caused by the disease. The net AUDPC and plant height were normalized with the values of reference cultivars according to Eynck et al. (2009b) to consider fluctuations between different screening rounds. Because each screening round was inoculated on two consecutive days, the mean value of Falcon and Express from each inoculation day was used to calculate the normalized AUDPC and normalized plant height.

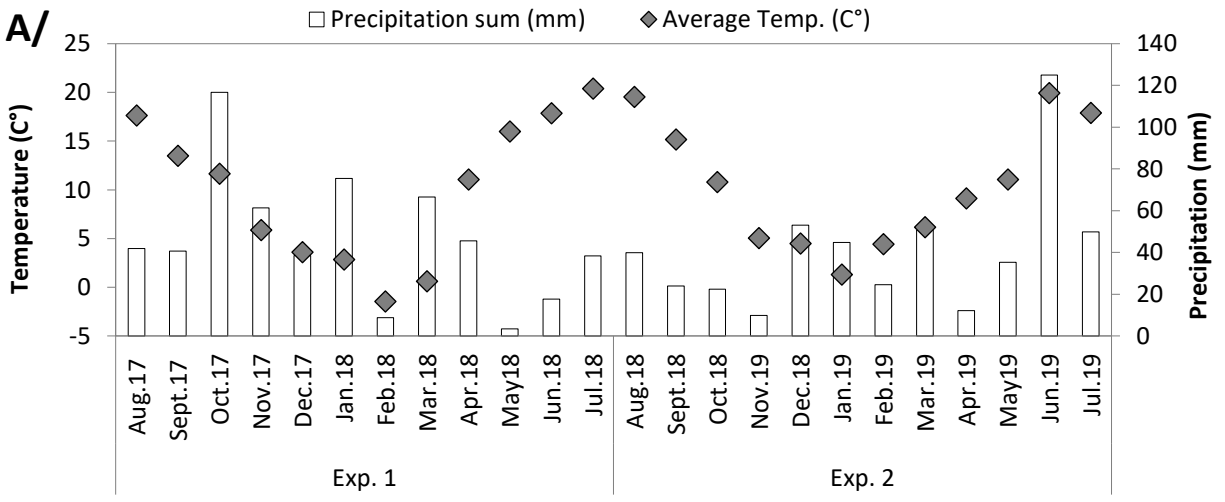
2.2. Assessment of *B. napus* resistance in field conditions

2.2.1. Field locations

Field experiments were conducted to assess the resistance of *B. napus* DH populations under natural conditions. Two geographically distant locations with previous records of *V. longisporum* incidence, Seligenstadt (Bayern) and Nienhagen (Mecklenburg-Vorpommern), were selected for field trials (Table 2). The experiments were conducted in 2017/2018 and 2018/2019 on different plots within the same fields. Field trials located in Seligenstadt were sown and managed by the breeding company KWS Saat SE & Co. KgaA and field trials located in Nienhagen by Quintus GmbH. The monthly mean temperature and precipitation sum during the two growing seasons in both locations are shown in Figure 1. All agronomic measures were performed according to good agricultural practices. No fungicides were applied to either of the two locations and insecticides were not sprayed in Seligenstadt during the cropping season 2017/2018.

Table 2. Geographic and agronomic information of the two selected locations to assess resistance of *Brassia napus* double haploid lines against *Verticillium longisporum.*

Locations	Address	Coordinates 2017/2018	Coordinates 2018/2019	Cultivation *B. napus*	Type of soil
Nienhagen	17166, Groß-Wokern, Mecklenburg-Vorpommern	53.733855, 12.506717	53.733040, 12.509448	Every 3 years	Clay-loam
Seligenstadt	97279, Prosselsheim, Bayern	49.841883, 10.090080	49.504480, 10.063629	Every 4-5 years	Silty-clay



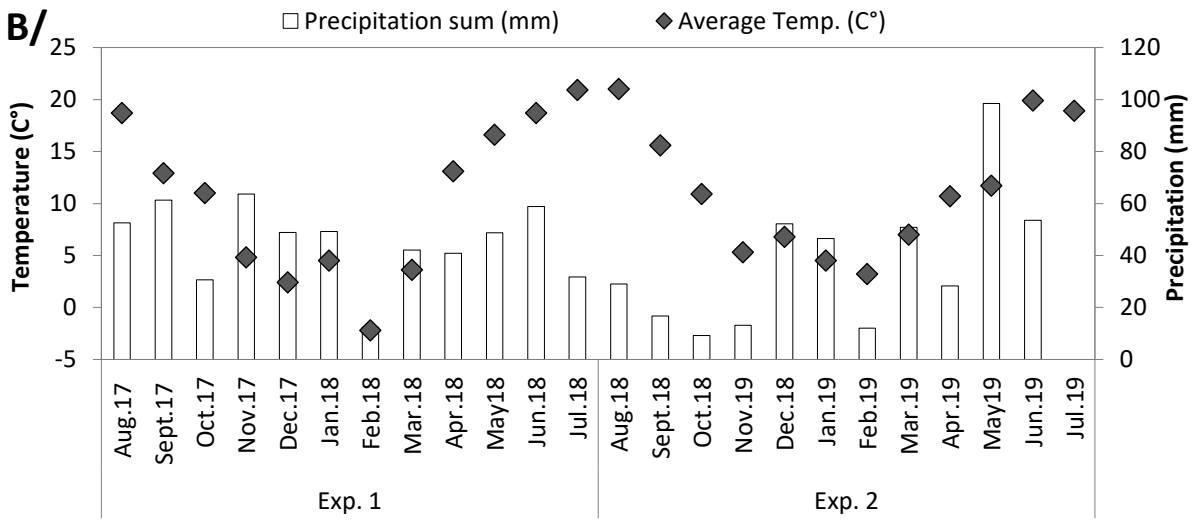


Figure 1. Monthly precipitation sum (mm) and average temperature at Nienhagen (Mecklenburg-Vorpommern) (A) and Seligenstadt (Bayern) (B) during the growing seasons 2017/2018 and 2018/2019. Each season, an experiment (Exp. 1 and Exp. 2) was conducted at each location to test the resistance of *Brassica napus* of double haploid lines to *Verticillium longisporum*.

2.2.2. Experimental design and sampling

The same plant material as in the greenhouse was tested in the field. The experiment had an alpha-lattice design and plot size of 4 x 0.9 m (Figure 2). Between each block there were 2 m paths. For the R53xEx617 population there were eight blocks repeated twice with 36 plots per block. The SW99-307 population was tested on eight blocks with 12 plots each, and each block was repeated twice. Hence, there were 576 plots for the R53xEx617 population and 192 plots for the SW99-307 population. Due to germination and establishment problems, as well as limited seed availability, the complete populations could not be tested. An exemplifying sowing plan is shown in Appendix.

Figure 2. Drone image (April 2018) of the alpha-lattice design of the field experiment for resistance assessment of different *Brassica napus* double haploid lines against *Verticillium longisporum*

2.2.3. Disease assessment

Disease resistance in the field was evaluated by assessing microsclerotia development on the stubble at senescence, as well as the fungal DNA colonization at the beginning of ripening (Table 3). For fungal DNA assessment in Seligenstadt, preliminary qPCR analysis of 2017/2018 showed no detection of DNA in the reference cultivars. In 2018/2019, the amount of DNA detected in samples from Seligenstadt was lower than the detection signal. In both growing seasons, there were no microsclerotia detected in samples from Seligenstadt. Thus, for both field assessment parameters, only Nienhagen was evaluated. In the cropping season 2017/2018, only one block repetition could be evaluated due to contamination of volunteer oilseed rape in one block. Due to time limitations, microsclerotia visual assessment was only performed in the R53xEx617 population in both seasons.

Table 3. Sowing, harvesting, and sampling dates of field trials in Nienhagen during two growing seasons to assess the resistance of *Brassica napus* lines against *Verticillium longisporum*. The first sampling was done for fungal quantification in stems; the second for the assessment of microsclerotia development in the stubbles.

Growing seasons	Sowing	Sampling 1	Harvest	Sampling 2
2017/2018	01.09.2017	13.-15.06.2018	unknown	25.07.2018
2018/2019	26.08.2018	12./13.06.2019	31.07.2019	14.08.2019

For qPCR assessment, 10 stubbles were randomly selected from each plot. From each stubble, a 10 cm fragment was harvested 5 cm above the hypocotyl. Stubble fragments from one plot were pooled as one sample and collected in breathable bags. First, bags were air-dried for four weeks. Then, samples were dried in a lyophilizer for 48 hours and then crushed with a hammer. In 2018/2019, three crushed subsamples filled in 30 mL tubes were considered three biological replicates. In 2017/2018, only one subsample was assessed. Each subsample was homogenized with a ball mill at a frequency of 30/90 s using TissueLyser II (Qiagen, Hilden; Germany) and 15–20 steel balls (5mm Ø). DNA from 30 mg homogenized subsample was extracted in a 96-well microplate with 1.2 mL Biosprint96 tubes (Qiagen, Hilden; Germany). Then, samples were vortexed for 30 s with RTL buffer (Qiagen, Hilden; Germany) and centrifuged for 20 min (6000 x g) at room temperature. The 200 µl supernatant was used for DNA extraction according to the protocol of BioSprint 96 DNA Plant Kit (Qiagen, Hilden; Germany). After dilution of DNA in 65µl Milli-Q water, 45–50 µl were transferred to a new 96-well microplate. DNA was quantified with Qubit dsDNA BR Assay Kit (Qubit 2.0 Fluorometer, Invitrogen; USA) and dissolved to 5 ng/µl with Milli-Q H_2O water.

The ViiA7 Real-Time PCR System (Applied Biosystems; USA) with 384-well Framestar PCR plate (4titude, Wotton; UK) was used for amplification and quantification of fungal DNA with SYBR Green Master mix (Roche, Basel; Switzerland) using OLG forward (CAGCGAAACGCGATATGTAG) and reverse (GGCTTGTAGGGGGTTTAGA) primers (Eynck et al. 2007).The amplification reaction had a total volume of 10

µL, which consisted of 10 µM of each primer and 3 µl of template DNA (5ng/µl). The PCR program consisted of a 2 min initial denaturation step at 94 °C, 36 cycles of 20 s at 95 °C, 30 s at 59 °C, and 40 s at 72 °C, followed by a final extension of 5 min at 72 °C. For qPCR analysis, three technical replications were carried out for each biological replicate.

For the visual assessment of microsclerotia development, stubbles were left on the field for approximately two weeks after harvest (Table 4). The assessment of the level of microsclerotia formation on the stem epidermis and pith, as well as on the roots, was done using a key with four qualitative scores according to Eynck et al. (2009b). Disease severity based on microsclerotia formation was calculated both for the stem (mean value from the epidermis and pith) and roots. For each plot, 10 randomly selected stubbles were assessed.

2.3. Statistical analyses

Statistical analyses were carried out with R (version 4.0.2.). Frequency distributions of symptoms from reference cultivars in different greenhouse screening rounds were represented in boxplots for comparison. Disease severity of the reference cultivars was assessed with a two-way ANOVA with a previous box-cox transformation, if necessary, based on variance homogeneity. For post hoc analysis, a Tukey HSD test at a significance level of 0.05 was performed. Data from different years, greenhouse screening rounds, and parameters was correlated with a Pearson correlation

Table 4. Assessment key for *Verticillium longisporum* disease severity on *Brassica napus* stubbles based on microsclerotia development according to Eynck et al. (2009b).

Score	Description
1	Healthy tissue: no microsclerotia visible in tissue
2	Slight infection: just a few single microsclerotia visible
3	Advanced infection: black patches of microsclerotia
4	Severe infection: long black patches covering most of the stem, epidermis is peeling off

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3. Results

3.1. Assessment of *B. napus* resistance to *V. longisporum* in greenhouse conditions

When screening a population for disease resistance, differences between screening rounds should be minimal. To assess this, net AUDPC and plant height of the reference cultivars in each screening round were compared (Figure 3). Except for net AUDPC from screening A, there were no significant differences between Falcon and Express. Despite the lack of significant differences between cultivars, the net AUDPC median value of Falcon was higher than the median of Express in all screening rounds. Likewise, the median relative height of Falcon was always lower than Express. Screening C was the screening round that led to the largest differences of symptoms.

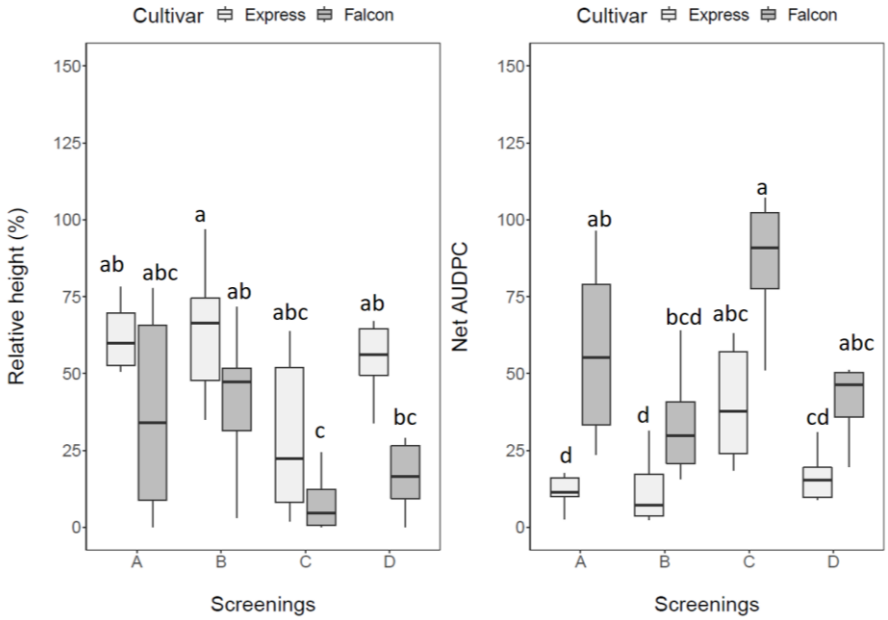

Figure 3. Frequency distribution of net area under the disease progress curve (AUDPC) and height relative to the control (%) of *Brassica napus* moderately resistant and susceptible cultivars Express and Falcon caused by root-dip inoculation with *Verticillium longisporum* (1.10^6 spores / mL) in four different greenhouse screening rounds (A-D). Each data point shows a boxplot with the median of six biological replicates, each consisting of merged data from four plants. Borders of boxes represent 25% and 75% quartiles. Error bars refer to standard deviation. Different letters indicate significant differences of the mean (Tukey-test, $P \leq 0.05$).

In general, there was a relatively wide range of variation in the response of the double haploid lines to root-dip inoculation with *V. longisporum* (Figure 4–7). For simplification and better visualization, only normalized height is shown, and each screening round is plotted separately. The normalized net AUDPC of each screening round is presented in Appendices Appendix–Appendix. Each screening round had DH lines that were more diseased than Falcon and less diseased than Express. Parents of the R53xEx617 population were screened in round B and showed less symptoms than Express, with R53 having the lowest symptoms. In all screening rounds, the normalized net AUDPC and relative height values were between zero and two, except for

screening round C, where the normalized height values went up to five. Within the disease distribution, a clear grouping of the two populations was not observed. However, in screening C, in contrast to the R53xEx617 population, most of the SW99-307 lines showed disease severity values lower than Express.

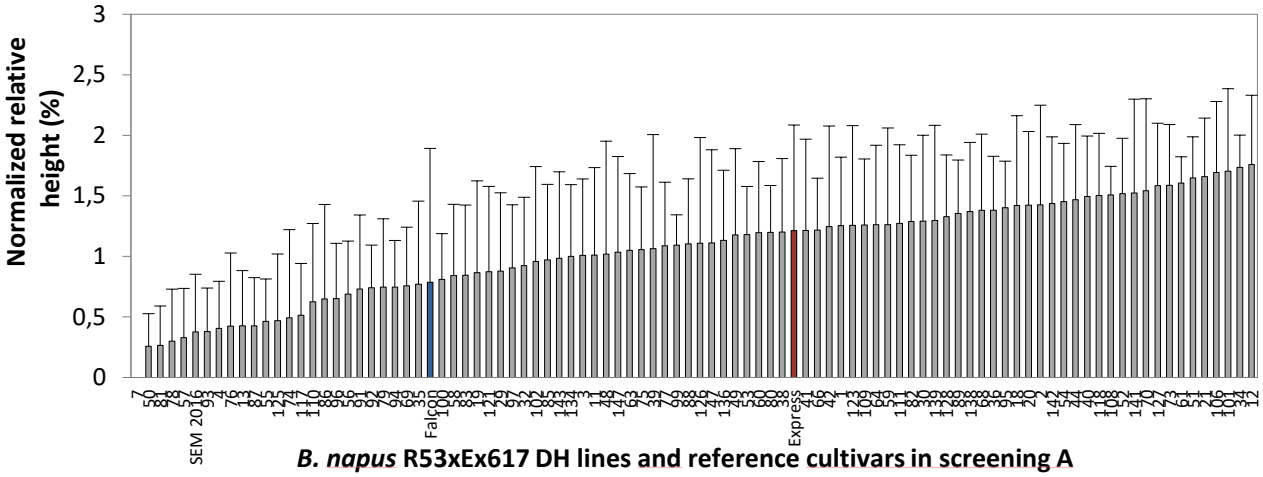

Figure 4. Response of *Brassica napus* seedlings of double haploid lines from the R53xEx617 population to *Verticillium longisporum* root-dip inoculation (1.10^6 spores / mL) under greenhouse conditions in screening round A. Normalized relative height is shown and each data point corresponds to the mean of 12 plants. Responses from moderately resistant and susceptible cultivars Express and Falcon are shown in red and blue, respectively. Error bars indicate the standard deviation.

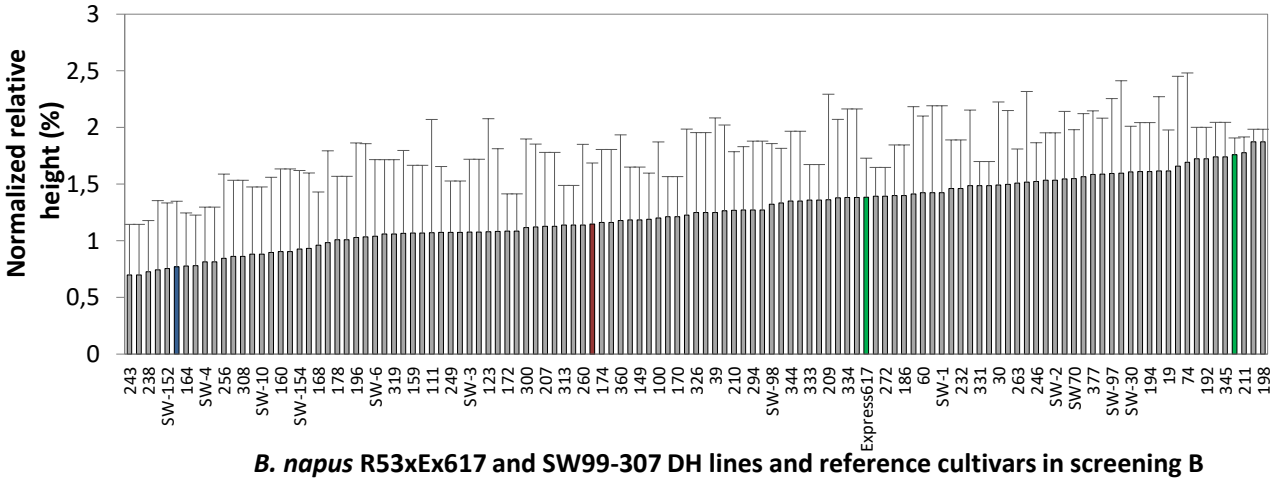

Figure 5. Response of *Brassica napus* seedlings of double haploid lines from populations R53xEx617 and SW99-307 to *Verticillium longisporum* root-dip inoculation (1.10^6 spores / mL) under greenhouse conditions in screening round B. Normalized relative height is shown and each data point corresponds to the mean of 12 plants. Responses from moderately resistant and susceptible cultivars Express and Falcon are shown in red and blue, respectively. Parents of the R53xEx617 population are shown in green. Error bars indicate the standard deviation.

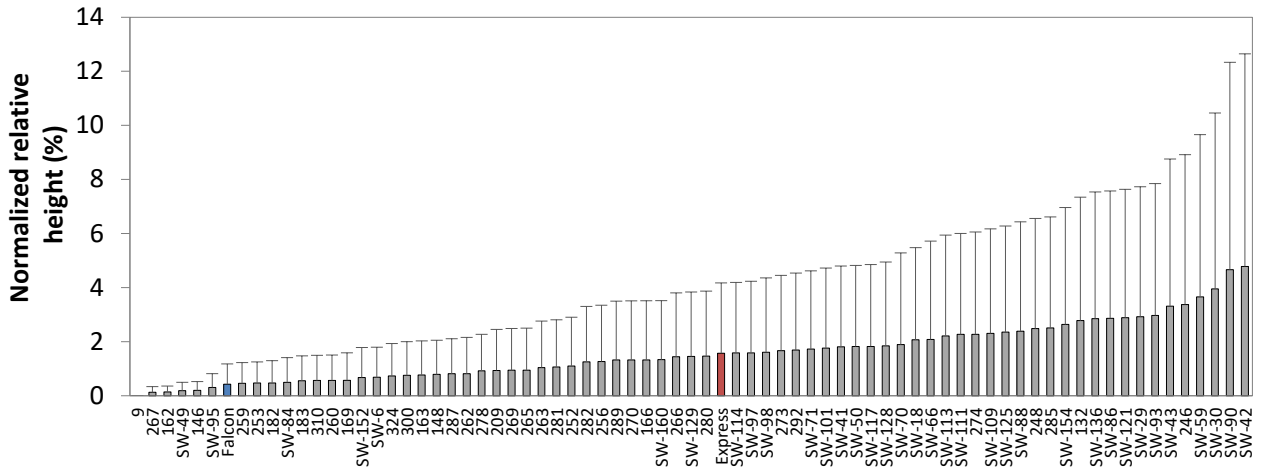

Figure 6. Response of *Brassica napus* seedlings of double haploid lines from the populations R53xEx617 and SW99-307 to *Verticillium longisporum* root-dip inoculation (1.10^6 spores / mL) under greenhouse conditions in screening round C. Normalized relative height is shown and each data point corresponds to the mean of 12 plants. Responses from moderate resistant cultivar Express and susceptible cultivar Falcon are shown in red and blue, respectively. Error bars indicate the standard deviation.

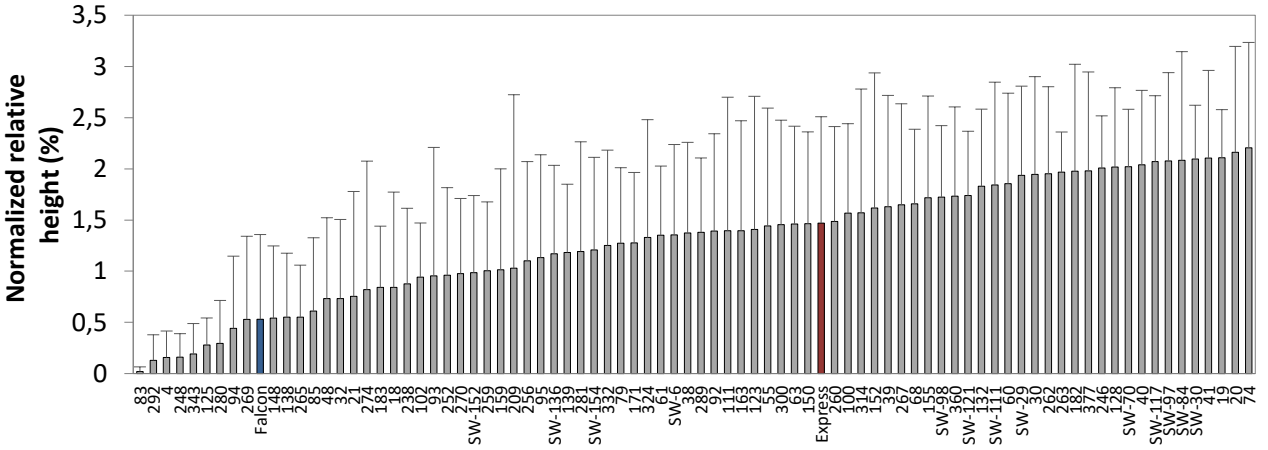

Figure 7. Response of *Brassica napus* seedlings of double haploid lines from the populations R53xEx617 and SW99-307 to *Verticillium longisporum* root-dip inoculation (1.106 spores / mL) under greenhouse conditions in screening round D. Normalized relative height is shown and each data point corresponds to the mean of 12 plants. Responses from moderate resistant cultivar Express and susceptible cultivar Falcon are shown in red and blue, respectively. Error bars indicate the standard deviation.

Correlation of the two parameters assessed in the greenhouse varied between screening rounds (Figure 8). Screening A had the weakest correlation value between the normalized AUDPC and the normalized relative height (r=0.12, $P \leq 0.05$), whereas screening D had the strongest correlation between disease parameters (r=0.78, $P \leq 0.05$).

The last screening (D) was made with a random selection of lines that had been tested in screening rounds A and C. The correlation of symptoms of lines tested in different screening rounds was assessed, but this evaluation showed no significant correlation between the symptoms from different screening rounds (Figure 9).

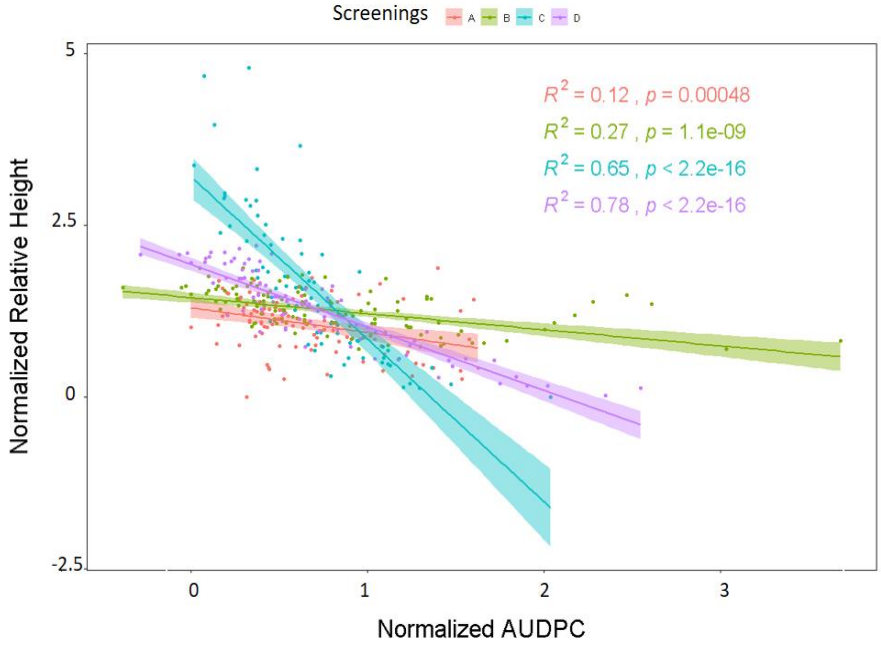

Figure 8. Correlation of mean normalized net area under the disease progress curve (AUDPC) and normalized relative height of *Brassica napus* seedlings caused by root-dip inoculation with *Verticillium longisporum* (1.10^6 spores / mL) under greenhouse conditions in four different screening rounds (A-D). *Brassica napus* lines from double haploid populations R53xEx617 and SW99-307, as well as susceptible and moderately resistant cultivars Falcon and Express, were tested. The four Pearson correlations (N_A=94, r_A=0.12; N_B=119, r_B=0.27; N_C=73, r_C= 0.65; N_D=73, r_D= 0.78) were significant ($P \leq 0.05$).

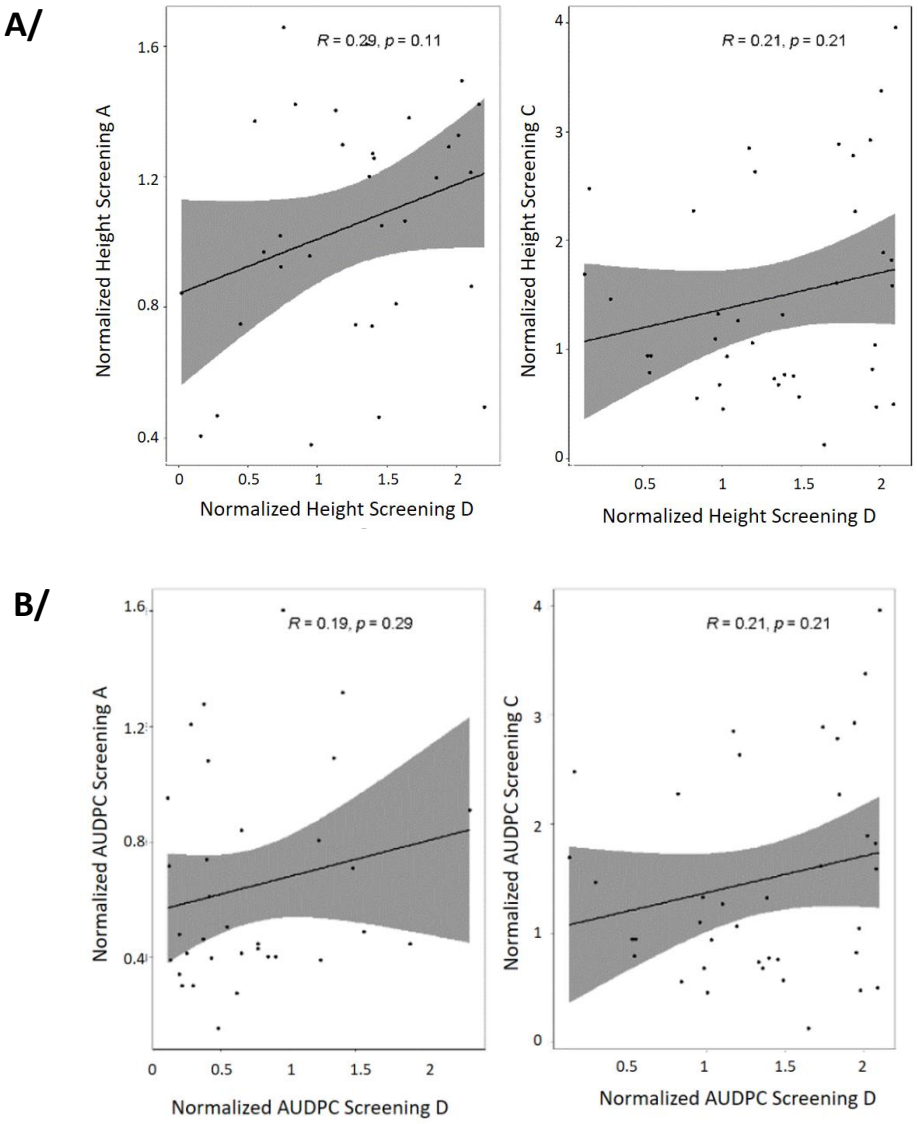

Figure 9. Correlation of normalized height relative to the control (A) and normalized area under the disease progress curve (AUDPC) (B) between greenhouse screenings A and D (N=33) and screenings C and D (N=38) caused by root-dip inoculation with *Verticillium longisporum* (1.10^6 spores / mL) in *Brassica napus* seedlings from the double haploid populations R53xEx617 and SW99-307, as well as on susceptible and moderate resistant cultivars Falcon and Express. The Pearson correlations were not statistically significant ($P > 0.05$).

3.2. Visual assessment of *B. napus* resistance to *V. longisporum* in the field

Low precipitation was a limiting factor for crop establishment, which led to missing samples from some plots. Additionally, seed availability limited the number of lines that could be sown. Thus, there were 221 and 219 lines assessed each growing season, respectively, for microsclerotia development. In Nienhagen, one block repetition could not be assessed in 2017/2018 due to volunteer oilseed rape. Thus, 1682 and 5358 stubbles from the R53xEx617 population and reference cultivar, respectively, were assessed in the first and second growing period. The disease severity of the first year was very low (1.6), in contrast to the second growing season, which had moderate disease severity (2.25) (Table 5). Disease severity was higher on roots than on

stems, and this tendency could also be seen in the reference cultivars (Figure 10). Disease severity was significantly higher in the susceptible cultivar Falcon than in Express.

Table 5. *Verticillium longisporum* disease severity on stubbles from *Brassica napus* double haploid lines of the R53xEx617 population, as well as from the susceptible and moderately resistant cultivars Falcon and Express, based on microsclerotia development on stems and roots according to a qualitative scale from Eynck et al. (2009b) (1= no microsclerotia, 4= severe infection). Stubbles were collected from Nienhagen (Mecklenburg-Vorpommern) during the growing seasons 2017/2018 (N=1682) and 2018/2019 (N=5358). The standard deviation is indicated in brackets.

Location	Stubble section	Disease score 2017/2018	Disease score 2018/2019
Nienhagen	Stem	1.5 (0.7)	2 (0.8)
	Root	1.7 (0.6)	2.5 (0.9)

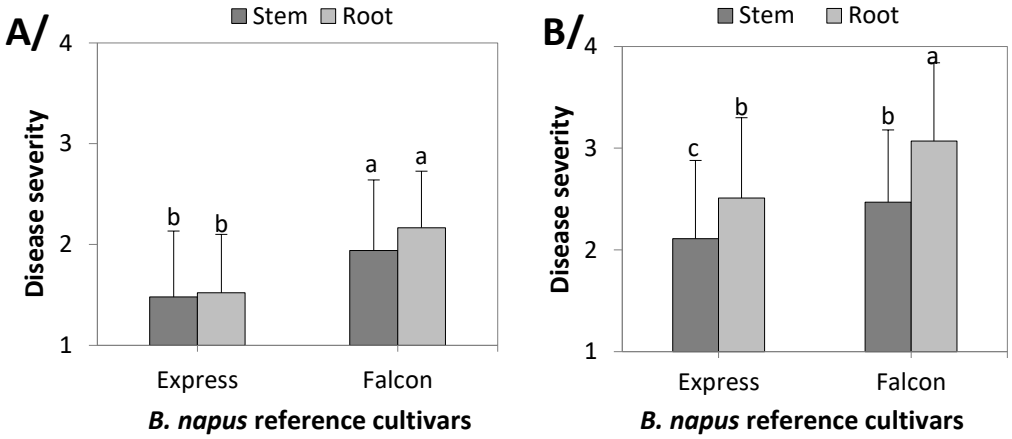

Figure 3. *Verticillium longisporum* disease severity of the moderately resistant and susceptible *Brassica napus* cultivars Express and Falcon based on microsclerotia development on stubbles according to a qualitative scale from Eynck et al. (2009b) (1= no microsclerotia, 4= severe infection) in field trials in Nienhagen (Mecklenburg-Vorpommern) during growing seasons 2017/2018 (A) and 2018/2019 (B). Each data point corresponds to the mean of 20 (A) or 36 (B) biological replicates, each consisting of merged data from 10 stubbles. Error bars refer to the standard deviation. Different letters indicate significant differences (Tukey test, $P \leq 0.05$).

Despite the lack of severe symptoms, there was a broad distribution of disease expression in both growing seasons (Figure 11 and 12). There was a mild significant correlation (r=0.29, $P \leq 0.05$) of the stem average disease severity from both years (Figure 13).

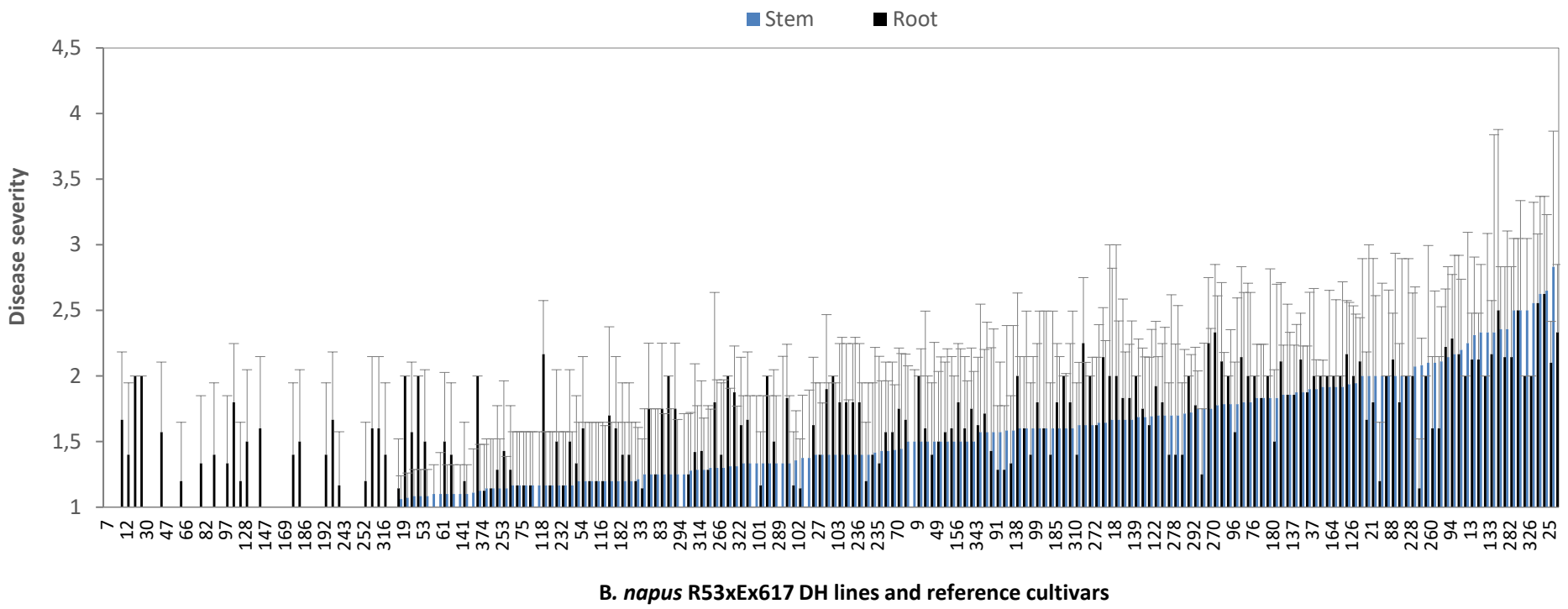

Figure 11. *Verticillium longisporum* disease severity from 221 *Brassica napus* double haploid lines (DH) lines of the R53xEx617 population based on microsclerotia development on stubbles according to a qualitative scale from Eynck et al. (2009b) (1= no microsclerotia, 4= severe infection) at field trials in Nienhagen (Mecklenburg-Vorpommern) during the 2017/2018 growing season. Each data point corresponds to the mean disease severity of 10 stubbles in one plot. Error bars show the standard deviation.

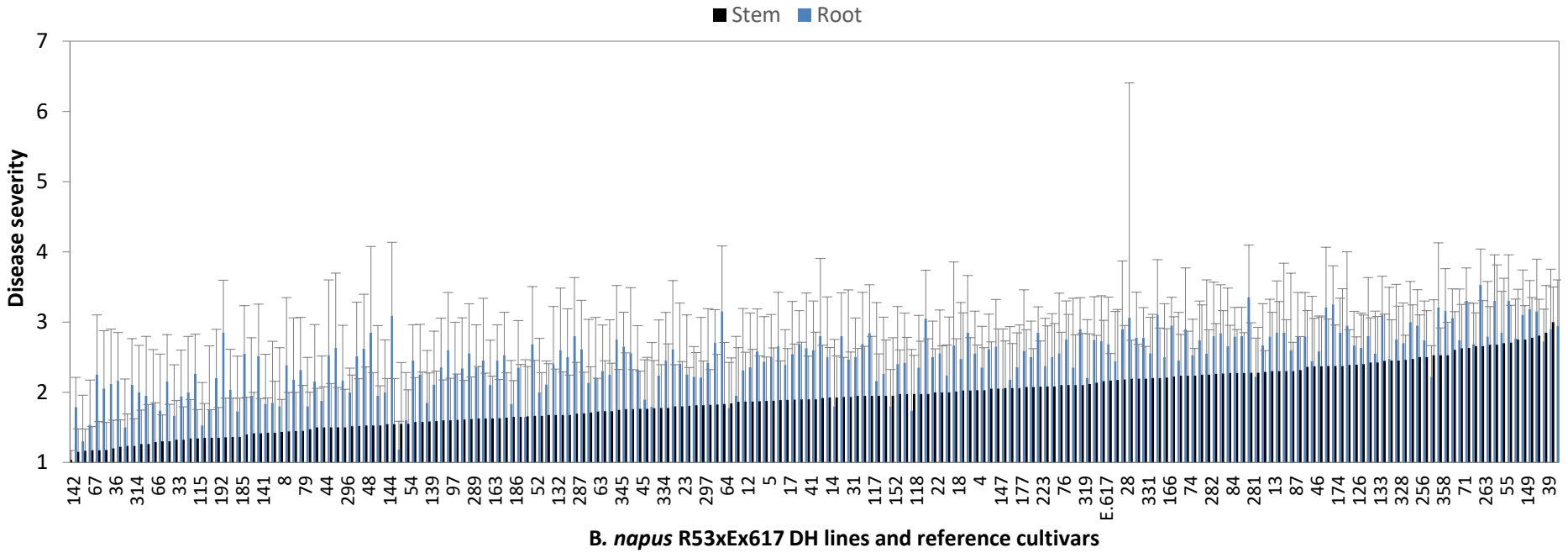

Figure 12. *Verticillium longisporum* disease severity of 212 *Brassica napus* double haploid lines (DH) lines of the R53xEx617 population based on microsclerotia development on stubbles according to a qualitative scale from Eynck et al. (2009b) (1= no microsclerotia, 4= severe infection) at field trials in Nienhagen (Mecklenburg-Vorpommern) during the 2018/2019 growing season. Each data point shows the mean disease severity of two samples, each corresponding to 10 stubbles from one plot. Error bars depict the standard deviation

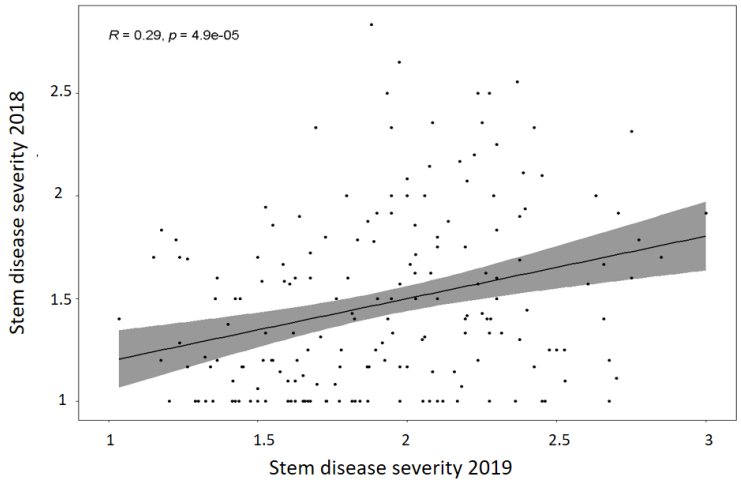

Figure 13. Correlation of *Verticillium longisporum* disease severity on *Brassica napus* double haploid lines of the R53xEx617 population based on microsclerotia development on stems according to a qualitative scale from Eynck et al. (2009b) (1= no microsclerotia, 4= severe infection) in Nienhagen (Mecklenburg-Vorpommern) between seasons 2017/2018 and 2018/2019. Pearson correlation revealed a low but significant correlation (N=185, r=0.3, $P \leq 0.05$).

The relationship between microsclerotia development in the field and the two parameters used in the greenhouse was assessed. Due to the higher disease severity in that growing season, field data from 2018/2019 were used for correlation. However, there was no significant correlation between the level of microsclerotia development on the stems and normalized AUDPC or height (Figure 14).

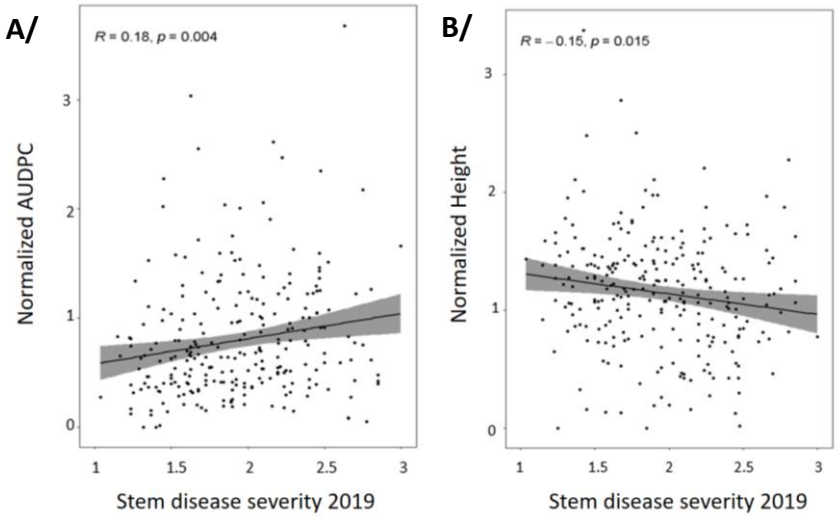

Figure 14. Correlation between *Verticillium longisporum* field disease severity and greenhouse symptoms (A= normalized net AUDPC, B= normalized relative height) of *Brassica napus* double haploid lines from the R53xEx617 population, as well as reference cultivars Falcon and Express, in Nienhagen (Mecklenburg-Vorpommern) during the 2018/2019 growing season. Disease severity was based on microsclerotia development on stems according to a qualitative scale from Eynck et al. (2009b) (1= no microsclerotia, 4= severe infection). Pearson correlation revealed a low but significant correlation (N=259, r=0.18, r= 0.15, $P \leq 0.05$).

3.3. Assessment of fungal systemic colonization in the field

For fungal DNA quantification in the DH lines harvested from Nienhagen in the 2017/2018 growing season, there were 246 and 81 lines assessed from the R53xEx617 and SW99-307, respectively. In 2018/2019, there were 249 and 86 lines from the R53xEx617 and SW99-307 populations, respectively. In 2017/2018, the average total detected DNA was 0.03 ng per 5 ng of plant DNA, whereas, in the samples from 2018/2019, the average detected DNA was 0.016 ng per 5 ng of plant DNA (Table 6). In both seasons, the quantified amount of fungal DNA in Express was lower than in Falcon.

Table 6. Systemic colonization of *Verticillium longisporum* in the *Brassica napus* moderately resistant and susceptible cultivars Express and Falcon and the double haploid populations R53xEx617 and SW99-307, as well as in the parental lines of the R53xEx617 population, in Nienhagen (Mecklenburg-Vorpommern) during the seasons 2017/2018 and 2018/2019. Systemic colonization was calculated as fungal DNA per 5 ng plant DNA. For season 2018/2019, each sample corresponds to milled powder of 10 stubble fragments from one plot. For season 2018/2019, each data point is the mean value of three biological replicates from two samples, each consisting of milled powder of 10 stubbles. Values in brackets represent the standard deviation. The line R53 was not sown in the season 2018/2019.

Populations, cultivars, and parental lines	2017/2019		2018/2019	
	Fungal DNA/5 ng of plant DNA	Number of samples	Fungal DNA/5 ng of plant DNA	Number of samples
R53xEx617 population	0.025 (0.043)	246	0.016 (0.015)	249
SW99-307 population	0.020 (0.026)	81	0.011 (0.01)	86
Express	0.022 (0.044)	25	0.3 (0.22)	2
Falcon	0.074 (0.067)	32	0.17 (0.13)	2
Express617	0.043	1	0.037 (0.022)	2
R53	0.021	1		

The correlation of fungal DNA between seasons was significant but low (r_p=0.19, $P \leq 0.05$) (Figure 15). To assess the correlation between the two parameters used in the field for disease assessment, only data from the 2018/2019 season was used due to low microsclerotia development in 2017/2018 (Figure 16). The correlation between the two parameters was significant, but low (0.2). When assessing the correlation between the greenhouse and fungal colonization in the field, the fungal DNA from each cropping season significantly correlated with AUDPC symptoms, but the correlation coefficients were below 0.2 (Figure 17). There was no correlation between plant height in the greenhouse and fungal DNA in field stubbles.

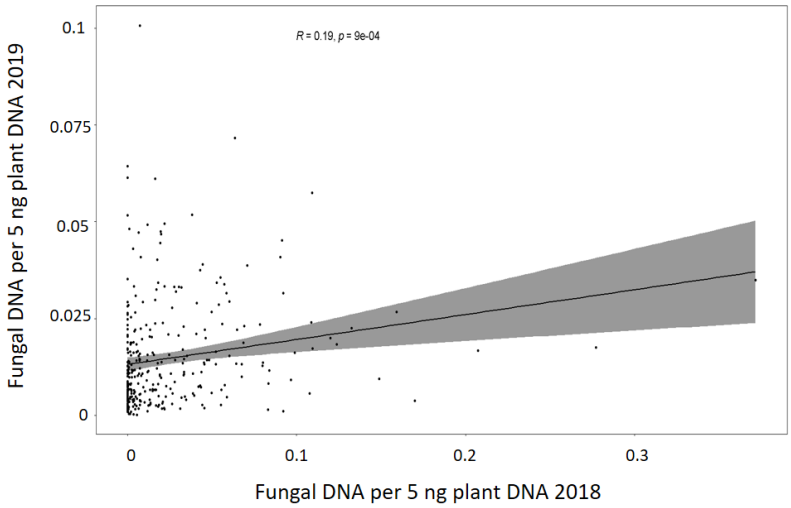

Figure 15. Correlation of *Verticillium longisporum* systemic colonization on stems of *Brassica napus* double haploid lines of the R53xEx617 and SW99-307 populations in Nienhagen (Mecklenburg-Vorpommern) during the 2017/2018 and 2018/2019 growing seasons. Systemic colonization was calculated as fungal DNA per 5 ng of plant DNA. Pearson correlation was used for the analysis, which revealed a low but significant correlation (N=318, r=0.19, $P \leq 0.05$).

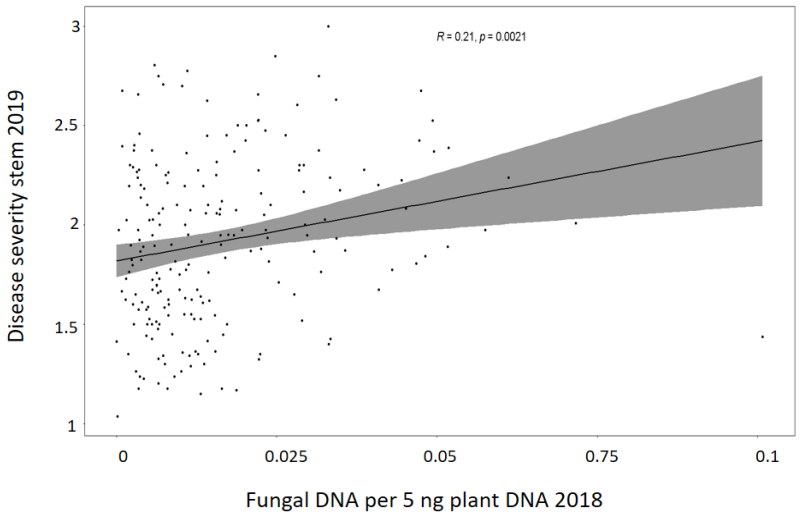

Figure 16. Correlation of *Verticillium longisporum* systemic colonization and disease severity on stems of *Brassica napus* double haploid lines of the R53xEx617population in Nienhagen (Mecklenburg-Vorpommern) during the 2018/2019 growing season. Disease severity was based on microsclerotia development according to a qualitative scale from Eynck et al. (2009b) (1= no microsclerotia, 4= severe infection). Systemic colonization was assessed by fungal DNA per 5 ng. Pearson correlation was used for the analysis, which revealed a low but significant correlation (N=210, r=0.19, $P \leq 0.05$).

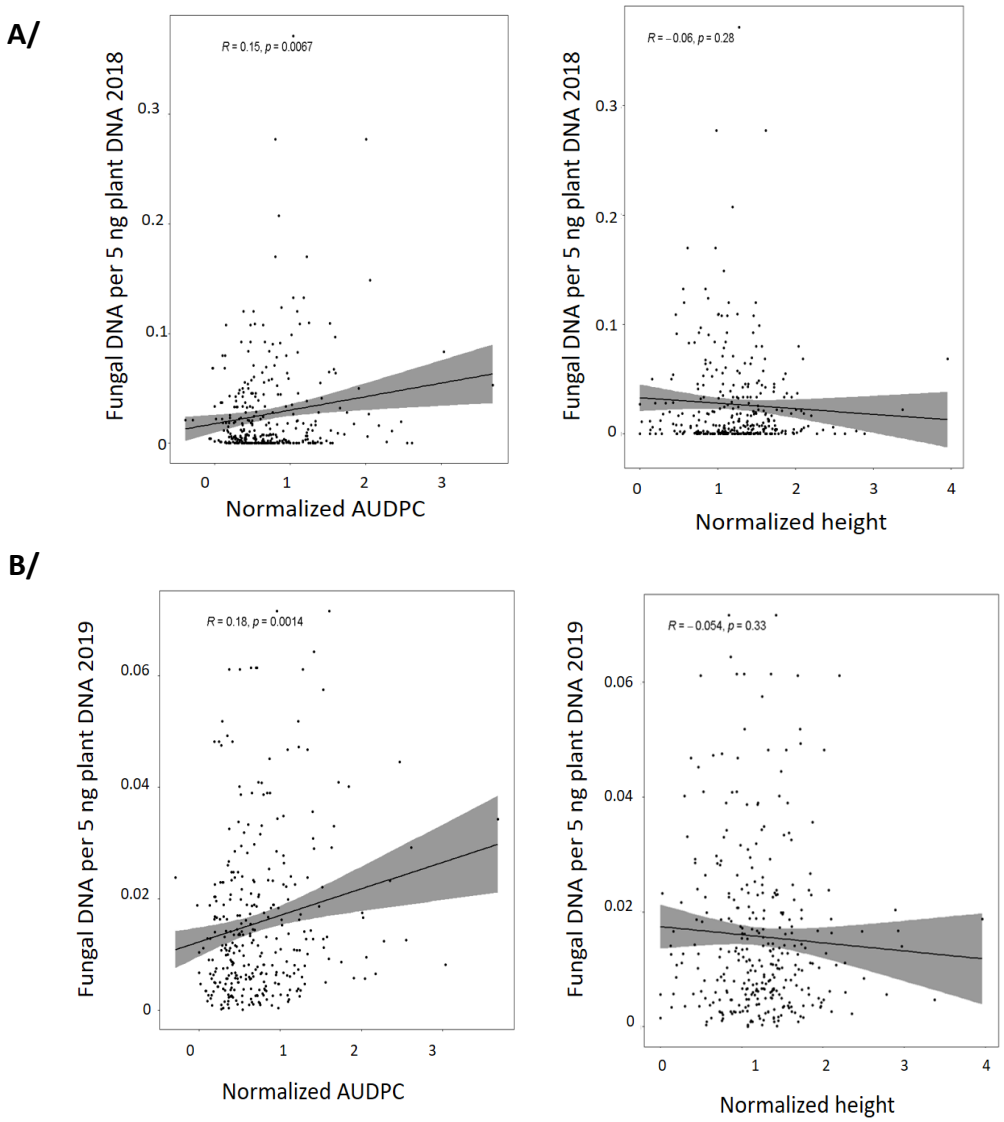

Figure 17. Correlation between the *Verticillium longisporum* systemic colonization in the field and greenhouse symptoms (A= normalized net AUDPC, B= normalized relative height) of *Brassica napus* double haploid lines of the R53xEx617 and SW99-307 populations in Nienhagen (Mecklenburg-Vorpommern) during the 2017/2018 (A, N=331) and 2018/2019 (B, N=331) growing seasons. Systemic colonization was assessed by fungal DNA per 5 ng. Pearson correlation was used for the analysis, which revealed, for both seasons, a significant low correlation between fungal DNA and normalized AUDPC (r_A=0.15, r_B=0.18, P<0.005), but no correlation between fungal DNA and normalized height (r_A=-0.06, r_B=-0.054, P>0.05).

4. Discussion

Root-dip inoculation of seedlings is a well-established method for the screening of resistance in *B. napus* to *V. longisporum* that allows screening a large number of lines in a short time (Eynck et al. 2009b). The greenhouse screenings from the present study showed a relatively high variability in the development of symptoms in DH lines (Figure 4-7). Screenings A and B revealed a low correlation between the two disease parameters in comparison to screening rounds C and D (Figure 8). This might indicate that, for some lines, a height reduction may not be accompanied by yellowing symptoms or vice-versa, which suggests a variety of

potential underlying resistance mechanisms. Due to the low number of lines tested from the SW99-307 population, it was not possible to determine if the two populations had different correlation trends of the two greenhouse disease parameters.

Analysis of the disease distribution of Falcon and Express in the greenhouse showed that there was variability in the development of symptoms in each screening round (Figure 3). The analysis also revealed that there was a lack of correlation of disease severity in lines tested in different screening rounds (Figure 9). The missing correlation might be influenced by the low number of data points and it indicates that a higher number of replicates are needed to ensure reproducibility (Eynck et al. 2009b). The observed variability associated with each screening might impede the detection of minor differences between genotypes. Statistical assessment of the symptoms from Falcon and Express showed no significant differences between the two cultivars (Figure 3), which may affect the normalization of disease parameters. The moderately resistant cultivar SEM might be able to provide a consistent differential response to Falcon, as it has been showed in previous studies (Eynck et al. 2009b; Zheng et al. 2019).

The field assessment revealed that, despite the low levels of disease severity in both cropping seasons, disease levels varied among the tested lines. In the case of the assessment based on microsclerotia development, the disease severity on roots was, in general, higher than on stems (Table 5). This trend has already been described by Zheng et al. (2019) and is in tune with previous findings that showed that the hypocotyl is an important barrier for the systemic colonization, hindering the progression of infection from roots to shoots (Eynck et al. 2009a).

This study found a minimal correlation between greenhouse symptoms and the level of microsclerotia development on stubbles in the field (Figure 14). The low level of disease incidence in the field, as well as the relatively low number of replicates in both experimental set ups, could be factors influencing the low correlation. At the ripening stage, the fungus leaves the xylem and colonizes the parenchyma, where it forms necrotic lesions. At plant senescence stages, the fungal saprophytic phase takes over and microsclerotia develop (Depotter et al. 2016). It is still unknown how resistance mechanisms that take place before senescence, as well as environmental factors, influence microsclerotia development in dead tissue. In Seligenstadt and in the first field trial in Nienhagen, the rainfall sum in June was below 60 mm. In contrast, the precipitation in Nienhagen in June 2019 was 120 mm. Microsclerotia development on stubbles was the highest at this location during this growing period. This suggests that moisture on stubbles might be required for fungal survival right before senescence for subsequent microsclerotia formation. Consequently, microsclerotia development might be strongly dependent on precipitation and, therefore, might not be suitable to assess fine differences in resistance between genotypes, as Knüfer et al. (2017) already suggested.

For both cropping seasons, the two DH populations revealed a lower fungal DNA content than the reference cultivars (Table 6), which indicates the potential to locate QTL markers in these populations. Knüfer et al. (2017) reported a strong correlation between greenhouse symptoms and fungal colonization in the field at the beginning of ripening. However, the present study revealed a low significant correlation between normalized AUDPC in the greenhouse and systemic colonization in the field (Figure 17). Moreover, plant height did not correlate with fungal colonization in the field. This absence of correlation with plant height might be related to the differential correlation between greenhouse symptoms that different lines presented (Figure 8). The low fungal colonization detected in the field might have contributed to the weak correlation between AUDPC and fungal DNA in the field. Due to the low sensibility of *V. longisporum*-specific tubulin primers, OLG primers amplifying the internal transcribed spacer region (ITS) region were selected. However, due to the low specificity of these primers, false positives in plant samples contaminated with *Alternaria* spp. or *Botrytis* spp., although unlikely, cannot be ruled out (Knüfer et al. 2017).

For the field screenings carried out in this study, natural infection was used instead of artificial inoculation, in contrast to Dunker et al. (2008), Eynck et al. (2009b), and Knüfer et al. (2017). With phylogenetic analysis based on five protein coding genes and the ITS region, Inderbitzin et al. (2011b) concluded that *V. longisporum* consists of three genetic lineages that originated from three independent hybridization events involving four haploid *Verticillium* parents. Two of these parents, D2 and D3, correspond to *V. dahliae*. Parents A1 and D1 are unknown *Verticillium* species. While isolates from the lineage A1/D1 and A1/D3 have a wider geographic distribution and host range, A1/D2 has so far only been found in horseradish in Illinois and Ontario (Inderbitzin et al. 2011; Yu et al. 2016). Pathogenicity tests in the greenhouse revealed that A1/D1 was the most aggressive lineage on oilseed rape, while A1/D2 was the least aggressive on all tested *Brassica* and non-*Brassica* crops (Depotter et al. 2017; Novakazi et al. 2015). However, extensive lineage monitoring and pathogenicity characterization of *V. longisporum* in oilseed rape is missing. Caranta et al. (1997) and Geffroy et al. (2000) reported isolate-specific QTLs for *Colletotrichum lindemuthianum* in bean and potyviruses in pepper, respectively. Different *V. longisporum* pathotypes and/or lineages on different fields could trigger different disease reactions, which might explain the lack of correlation between field symptoms and disease severity in the greenhouse.

Other factors besides the presence of different pathotypes could explain the weak correlation between field and greenhouse symptoms, as well as between cropping seasons. For example, it has been shown that differences on temperature modulate the severity of *V. longisporum* infection (Siebold and Tiedemann 2013), and Onaga et al. (2017) reported that elevated temperature increases the expression of pathogenicity related genes in *Magnaporthe oryzae*. Chen et al. (2018) described that the wheat microbiome can affect the pathogenicity of *Fusarium graminearum*. Trivedi et al. (2017) showed that the abundance of Actinobacteria predicted soil suppressiveness against *F. oxysporum*. In addition, the effect of soil chemical properties on the

effectiveness of soil suppressiveness have already been reported (Tenuta and Lazarovits 2004). Moreover, the differences in spatial distribution in the soil of microsclerotia could have also play a role in the differences of symptoms recorded between locations and plots (Xiao et al. 1997).

In conclusion, this study reported a relatively large variability of responses in different *B. napus* lines to infection with *V. longisporum*. The results evidence that disease expression depends on traits that are highly sensitive to the environment, which considerably hinder and accurate assessment (Young 1996). Thus, bigger data sets, a higher number of replicates, and higher levels of infestation in the field through artificial inoculation might be required for disease resistance screening for QTL mapping purposes.

5. Author contributions

Marta Vega-Marín analyzed data from the greenhouse screenings and microsclerotia assessment, Harmeet Singh and Christian Obermeier analyzed the qPCR data. All authors were involved in the design of the research and discussed collectively the results. Marta Vega-Marín and Andreas von Tiedemann wrote the manuscript.

6. Acknowledgements

This work was financially supported by the German Federal Ministry for Food and Agriculture (BMEL) and the Agency for Renewable Resources (FNR). The research was carried out within the framework of a cooperation project with the Gemeinschaft zur Förderung von Pflanzeninnovation e. V. (GFPi). The authors acknowledge Martin Wissen and his team from KWS Saat SE & Co. KGaA, as well as Quintus GmbH, for conducting the field experiments and sampling the stubbles for microsclerotia assessment. The authors thank the technical assistance of Isabel Müller for conducting and evaluating the greenhouse trials, as well as the disease assessment of the stubbles and field sampling of stems for qPCR assessment. Thanks are also extended to Chelsea Schreiber, Dagmar Tacke, Muhammad Khan, and Bálint Bakondi for their help with the microsclerotia visual assessment. We acknowledge the sampling of stems for the qPCR assessment by Natalia Sierra, Hubertus Reintke, Vinay Kumar, Reneé Heise, David Gualotuña, Fruzsi Ficze, and Maik Knobel.

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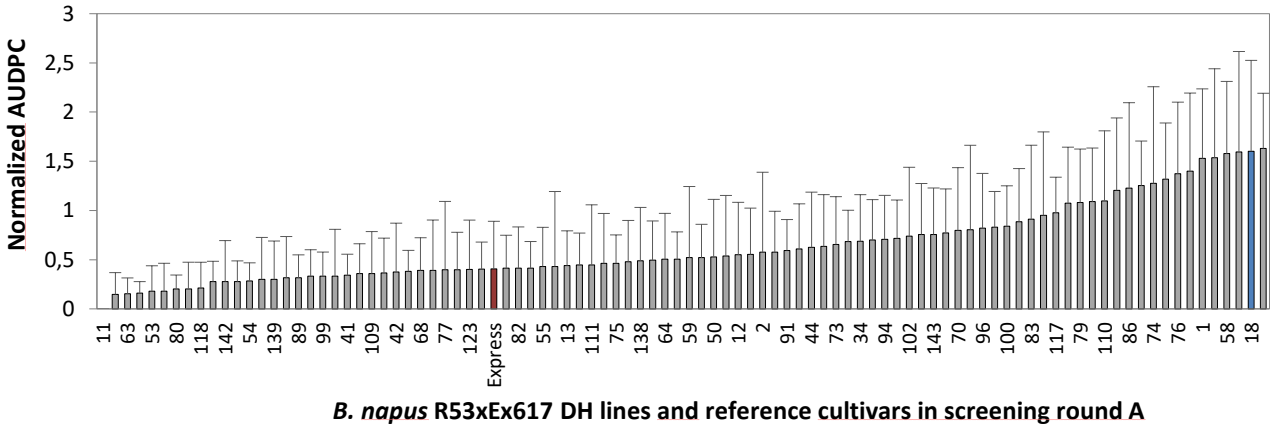
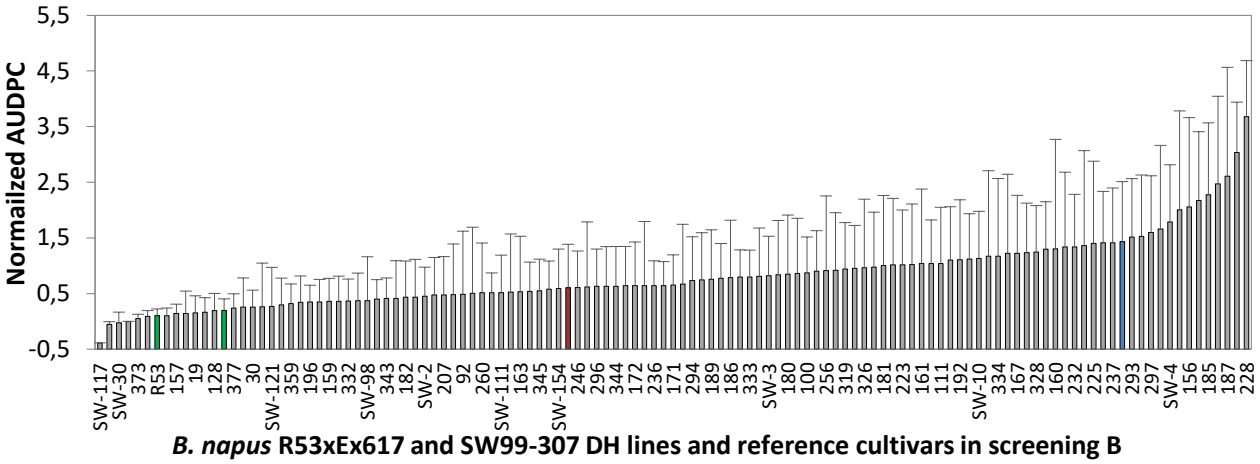
8. Appendix

Appendix 1. Exemplifying sowing plan

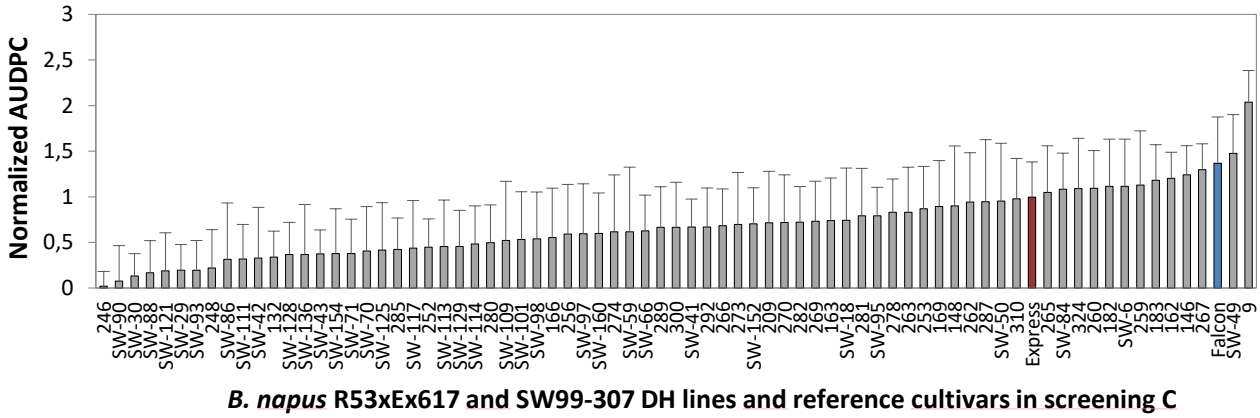


***B. napus* R53xEx617 DH lines and reference cultivars in screening round A**

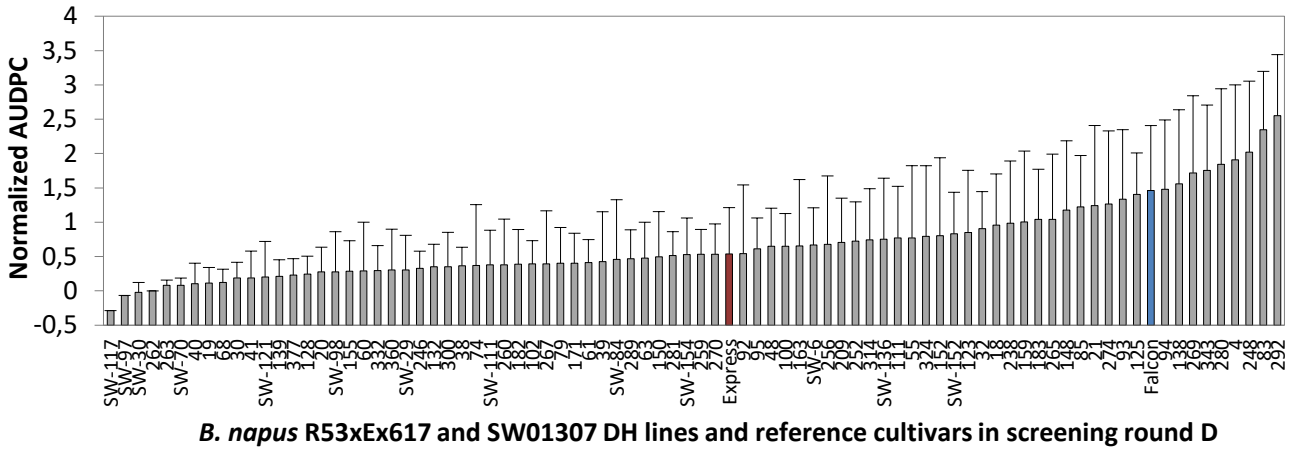
Appendix 2. Response of *Brassica napus* seedlings of double haploid lines from the R53xEx617 population to *Verticillium longisporum* root-dip inoculation (1.10^6 spores / mL) under greenhouse conditions in the screening round A. Normalized net area under the disease progress curve (AUDPC) is shown and each data point corresponds to the average of 12 plants. The responses from the moderately resistant cultivar Express and the susceptible cultivar Falcon are shown in red and blue, respectively. Error bars indicate the standard deviation.



Appendix 3. Response of *Brassica napus* seedlings of double haploid (DH) lines from the R53xEx617 and SW99-307 populations to *Verticillium longisporum* root-dip inoculation (1.10^6 spores / mL) under greenhouse conditions in the screening round B. Normalized net AUDPC is shown and each data point corresponds to the average of 12 plants. The responses from the moderately resistant cultivar Express and the susceptible cultivar Falcon are shown in red and blue, respectively. The parents of the of the R53xEx617 DH population are colored green. Error bars indicate the standard deviation.



Appendix 4. Response of *Brassica napus* seedlings of double haploid (DH) lines from the populations R53xEx617 to *Verticillium longisporum* root-dip inoculation under greenhouse conditions in the screening round C. Normalized net AUDPC is shown and each data point corresponds to the average of 12 plants. The responses from the moderately resistant cultivar Express and the susceptible cultivar Falcon are shown red and blue, respectively. Error bars indicate the standard deviation.



***B. napus* R53xEx617 and SW01307 DH lines and reference cultivars in screening round D**

Appendix 5. Response of *Brassica napus* seedlings of double haploid (DH) lines from the populations R53xEx617 to *Verticillium longisporum* root-dip inoculation (1.10^6 spores / mL) under greenhouse conditions in the screening round D. Normalized net AUDPC is shown and each data point corresponds to the average of 12 plants. The responses from the moderately resistant cultivar Express and the susceptible cultivar Falcon are shown red and blue, respectively. Error bars indicate the standard deviation.

Chapter VI: General Discussion

The phylogenetic analysis by Inderbitzin et al. (2011), based on five protein-coding genes and the nuclear ribosomal internal transcribed spacer region (ITS), revealed that *V. longisporum* consists of three lineages (A1/D1, A1/D2, and A1/D3) that originated from three independent hybridization events of four haploid *Verticillium* parents. This discovery added a new layer of complexity to the control of this soil-borne vascular pathogen. Since then, different studies have genetically characterized the *V. longisporum* lineages, as well as their pathogenicity and distribution (Banno et al. 2015; Depotter et al. 2017b; Novakazi et al. 2015; van Tran et al. 2013; Zou et al. 2020). *V. longisporum* typically infects *Brassica* crops, of which cabbage, cauliflower, and oilseed rape are the economically most important (Depotter et al. 2016; Inderbitzin et al. 2011). The worldwide demand for biodiesel is driving oilseed rape production and shortening crop rotations (Ismail et al. 2017), which considerably contributes to the increase of biotic stresses (Hegewald et al. 2018). The current threat that *V. longisporum* poses on oilseed rape production, as well as the knowledge gaps in the biology of this pathogen, have motivated the performance of this study, which aimed to further characterize the genetic and pathogenicity diversity of *V. longisporum*. Additionally, due to limited resistance against *V. longisporum* in the current oilseed rape varieties (Friedt and Snowdon 2010), oilseed rape double haploid lines (DH) were evaluated for future quantitative trait locus (QTL) mapping and breeding purposes.

The genetic diversity and population structure of *V. longisporum* within and across lineages from different geographic origins, as well as its relevance for pathogenicity, has not been well studied in oilseed rape. Thus, the first objective of this study (chapter II) was the performance of an extensive lineage monitoring in the main oilseed rape growing regions in Europe and Canada. The lineage monitoring, which was carried out with a multiplex-PCR according to Inderbitzin et al. (2013), revealed that A1/D1 was the dominant lineage in oilseed rape (Figure 2). A phylogenetic analysis of A1/D1 isolates based on genotyping by sequencing (GBS) was conducted to determine whether genetic subgroups exist within the A1/D1 lineage (Figure 3). The A1/D1 genetic clusters were, in general, independent of geographic region or field. The pathogenicity characterization in the greenhouse of A1/D1 isolates revealed pathogenicity diversity within the lineage, also within one genetic cluster and among isolates from the same field.

The long survival of fungal resting structures in the soil and lack of effective curative measures make vascular pathogens the most difficult plant pathogens to control. The limited availability of control measures calls for the investigation of new alternatives, such as the use of microorganisms as biocontrol agents (Deketelaere et al. 2017; Yadeta and Thomma 2013). One biocontrol approach against vascular pathogens is using non-pathogenic or non-aggressive isolates from the same species or from other vascular pathogens (Deketelaere et al. 2017). Thus, in chapter III, the biocontrol ability of the non-aggressive A1/D2 lineage was investigated in oilseed rape. The study revealed a biocontrol effect of A1/D2 against a *V. longisporum* aggressive A1/D1 isolate with root-dip inoculation (Figure 3). The induction of salicylic acid (SA), a signal in basal and cultivar-

related resistance against *V. longisporum* (Zheng et al. 2019a), was not involved in the biocontrol mechanism (Figure 5). Applying a biocontrol agent as a seed coat is the most practical and economically feasible application method in fields of arable crops (Alabouvette et al. 2009; Rocha et al. 2019). However, the biocontrol effect of A1/D2 was lost with this application technique (Figure 6-7). Confocal microscopy analyses revealed that seed coating resulted in less A1/D2 hyphal establishment in the roots than the root-dip inoculation (Figure 9), which might explain the lack of A1/D2 biocontrol effect with a seed coat.

In chapter IV, the non-aggressive interaction of *V. longisporum* lineage A1/D2 with oilseed rape was compared to the aggressive interaction of an A1/D1 isolate. Phenotypic assessments in the greenhouse confirmed the non-aggressiveness of A1/D2 on oilseed rape. The external root colonization assessment by confocal microscopy revealed sporulation, hyphal coiling, and unorganized outward hyphal growth by A1/D2 at the rhizosphere (Figure 5, 8 and 9), in contrast to the organized net-like hyphal growth of A1/D1 close to the root surface (Figure 6). Electron microscopy and qPCR assays revealed minimal vascular colonization by A1/D2 in the roots and stems (Figure 3 and 10), respectively. Additionally, electron microscopy observations showed a more intense xylem vessel coating in A1/D2-treated samples than in A1/D1-treated samples (Figure 15), as well as stronger secondary vessel wall degradation by A1/D1 than by A1/D2 (Figure 14). The results indicate that low penetration at the root surface, a low vascular colonization by A1/D2, and a higher intensity of plant defense reactions upon A1/D2 infection in comparison to A1/D1 might explain the low pathogenicity of the lineage A1/D2.

Intensive breeding for quality traits in the past decades and the subsequent reduction of the genetic basis of the species has led to limited resistance against *V. longisporum* in the current oilseed rape varieties (Friedt and Snowdon 2010). Thus, the final goal of this study (chapter V) consisted of the evaluation of oilseed rape double haploid lines (DH) for future QTL mapping and breeding purposes. The greenhouse and field assessments revealed relatively high variability within the DH populations in their response to *V. longisporum* (Figure 4-7, 11 and12). There was a low correlation between greenhouse and field symptoms (Figure 14), which evidences the difficulty of performing disease screenings in field conditions.

In the sections that follow, the most important findings of the four previously mentioned chapters of this study are discussed. Besides examining the different individual findings independently, the relationship between them, and their overall agronomic relevance are also analyzed.

I. Phylogenetic analysis of *V. longisporum* A1/D1 did not reveal clustering based on geographic region or field of origin, which might indicate gene flow between populations.

Population genetics is a useful tool to gain insight into the evolution history of an organism (Sætre and Ravinet 2019). In plant pathology, specifically, it can provide information on disease origin and dispersal pathways (Depotter et al. 2017b; Dutech et al. 2012). The phylogenetic study of 81 A1/D1 isolates revealed

genetic clustering within this lineage. However, the clusters were, in general, independent of geographic region and field. It is unclear how asexual fungal pathogens, like *V. longisporum*, adapt to environmental changes. A lack of sexual reproduction is putatively considered to negatively affect the ability to generate genetic diversity (Jonge et al. 2013). Nevertheless, the observed genetic groups within lineage A1/D1 indicate that the role of non-sexual recombination in shaping population diversity of *V. longisporum* might have previously been underestimated, as it has already been proposed for *V. dahliae* (Faino et al. 2016; Jonge et al. 2013). The absence of clear clustering based on origin of isolates evidences that spatial population structure is not as relevant for asexual organisms as it is when there is sexual recombination (Sætre and Ravinet 2019). In addition, it indicates gene flow between locations or introductions from a common source (Wheeler et al. 2019).

Seed transmission has been proposed as a mechanism for long distance dispersal of vascular pathogens. Zheng et al. (2019b) reported minimal seed transmission of *V. longisporum* in natural conditions, which could contribute to dispersal and genetic reshaping of populations. Other hosts could also play a role in the dispersal of the pathogen. This is illustrated by seed transmission of *V. dahliae*, which has been reported in several hosts, such as cotton, spinach, and olive trees (Du Toit et al. 2005; Göre et al. 2011; Karajeh 2006). *V. longisporum* typically infects *Brassica* hosts (Depotter et al. 2016), but it has been isolated from the Amaranthaceae crop sugar beet (Jackson and Heale 1985), which suggests that the host range of *V. longisporum* might be broader than expected. Besides crops, weeds could also play a role in the dispersal of the disease. It was reported that *V. longisporum* developed microsclerotia in scentless mayweed after root-dip inoculation (Johansson et al. 2006). Similarly, the potential role of weeds as disease reservoirs was reported for *V. dahliae* in lettuce fields (Vallad et al. 2005) and for wheat-infective *Fusarium graminearum* strains in North America (Lofgren et al. 2018). In addition, the coexistence within a *Verticillium* species of aggressive and asymptomatic lifestyles has previously been reported (Gurung et al. 2015; Inderbitzin and Subbarao 2014; Malcolm et al. 2013). A cryptic asymptomatic lifestyle could contribute to the maintenance of inoculum, as well as to the evolution and dispersal of the pathogen. Thus, a better understanding of the host range of *V. longisporum* would provide information on the dispersal and evolution of the pathogen, as well as the basis for improved reduction of microsclerotia in the soil by adjustment of crop rotations and weed management (Malcolm et al. 2013).

II. Phenotypic assessments revealed variability in aggressiveness within A1/D1 lineage, which did not correlate with the genetic clusters from the phylogenetic analysis.

Previous reports have shown differential geographic distribution and host preferences for the different *V. longisporum* lineages (Banno et al. 2015; Depotter et al. 2017b; Inderbitzin et al. 2011; Yu et al. 2016). This was confirmed with the lineage monitoring in this study, which revealed that A1/D1 is the dominant lineage in oilseed rape, as previous studies have also reported (Depotter et al. 2017b; Inderbitzin et al. 2011;

Zou et al. 2020). The reported differential host preference and geographic occurrence between different *V. longisporum* lineages putatively led to differential evolutionary paths between the lineages. This is illustrated by the work of Novakazi et al. (2015) and Depotter et al. (2017a), who reported that A1/D1 was the most aggressive lineage in oilseed rape with root-dip inoculation. However, the pathogenicity assessment of A1/D1 isolates in chapter II revealed a higher level of pathogenicity diversity within this lineage than previously reported, as well as A1/D1 isolates that were non-aggressive. This indicates that the mechanisms that control pathogenicity in this pathogen are not lineage-dependent.

The phylogenetic analysis did not reveal clustering based on pathogenicity. Similarly, a genotypic characterization based on microsatellites did not reveal differences in genetic diversity, genotypic richness, or diversity between pathogenic and endophytic *V. dahliae* isolates from different sympatric hosts (Wheeler et al. 2019). Likewise, phylogenetic studies based on RAPD (Random Amplification of Polymorphic DNA) banding patters and GBS did not show any correlation between genetic clustering and pathogenicity of *V. dahliae* (Bhat and Subbarao 1999; Dung et al. 2019).

Plant-pathogen interactions imply the constant coevolution of the plant and pathogen to overcome the deployed weaponry for both pathogen infection and plant defense (Depotter et al. 2019). So far, no research has been carried out to genetically characterize the pathogenicity of *V. longisporum*. In contrast, different studies have attempted to unveil the underlying mechanisms that modulate pathogenicity in *V. dahliae*. The locus *Ave1* of *V. dahliae* race 1 encodes a protein that activates *Ve1*-mediated resistance in tomato. *Ave1* confers pathogenicity to *V. dahliae* when infecting plants lacking *Ve1,* but the *Ave1* deletion does not lead to a complete loss of pathogenicity. Homologous Ave1 proteins have been described in different fungal pathogens, as well as bacteria (Jonge et al. 2012), but its presence has never been assessed in *V. longisporum*. During plant infection, effectors are delivered into the plant by the fungus and putatively act as pathogenicity enhancers (Kachroo et al. 2017). Klosterman et al. (2011) reported that approximately 120 genes of *V. dahliae* and *V. albo-atrum* could potentially encode effectors involved in pathogenicity. Non-aggressive Fusarium strains present a lower effector profile than pathogenic isolates (Lamo and Takken 2020). Likewise, a different effector profile between *V. longisporum* isolates with different aggressiveness should be expected. Other studies have revealed that chromosomal rearrangements determine pathogenicity differences within *V. dahliae*. These chromosomal rearrangements among genetically similar *V. dahliae* isolates lead to repeat rich regions surrounded by transposable elements, which drive pathogenicity differences between isolates (Faino et al. 2016; Jonge et al. 2013). If similar chromosomal rearrangements are responsible of *V. longisporum* pathogenicity, they could have not been detected by GBS.

III. Variable aggressiveness within the lineage A1/D1 complicates resistance screenings in field conditions.

For the genetic and pathological characterization of lineage A1/D1 in chapter II, *V. longisporum* isolates were obtained from oilseed rape stubbles from different locations in Europe and Canada. The isolation method required high levels of microsclerotia infestation on the epidermis of stubbles. Despite the inevitable selection effect of this isolation method, the isolates obtained with this method presented a broad range of pathogenicity diversity on root-dip inoculated oilseed rape seedlings (chapter II). Microsclerotia development on stubbles is a parameter often used to assess disease severity in field conditions (Dunker et al. 2008; Knüfer et al. 2017; Zheng et al. 2019c). However, the reported pathogenicity diversity of A1/D1 in chapter II indicates that saprophytic growth on senescent tissue neither depends on the pathogenicity of the isolate nor on plant defense mechanisms. This indicates that it is not a suitable parameter to evaluate disease severity, as Knüfer et al. (2017) already reported. This is supported by the results from the resistance field screenings of two DH oilseed rape populations in chapter V, where microsclerotia development on stubbles was assessed to determine disease severity in DH lines under field conditions. Microsclerotia incidence was highest at the location and year that had the highest precipitation in June. This result indicates that moisture on the stubbles, rather than host resistance or fungal aggressiveness, is the main factor determining the level of microsclerotia development, as it allows fungal survival before saprophytic growth at plant senescence.

For the field disease assessment of DH lines in chapter V, fungal DNA colonization at the beginning of ripening was evaluated in addition to the assessment of microsclerotia development. Knüfer et al. (2017) reported a direct correlation between greenhouse symptoms and the level of fungal DNA colonization in the field at the beginning of ripening, making this parameter more suitable than the assessment of microsclerotia to detect fine resistance differences between oilseed rape genotypes in field conditions. However, in the present study, both parameters for disease assessment in field conditions led to a low correlation with greenhouse symptoms (r_p<0.2). The results provide evidence that disease reactions depend on traits that are highly sensitive to climatic conditions, which considerably hinders an accurate disease resistance assessment (Young 1996). However, since field screenings were conducted with natural infection of *V. longisporum*, pathogenicity differences among the isolates that infected the DH lines within a field might have also contributed to the missing correlation. This is in agreement with Caranta et al. (1997) and Geffroy et al. (2000), who reported isolate-specific plant defense reactions for *Colletotrichum lindemuthianum* in bean and potyviruses in pepper, respectively. In addition to interfering with field disease resistance screenings, the pathogenicity diversity of *V. longisporum* within a field hinders prediction of disease incidence based on soil inoculum. This is in agreement with Johansson et al. (2006), who reported no significant correlation between inoculum density in soil and disease incidence in oilseed rape in Swedish fields.

IV. Microscopic assessments indicated differences in plant defense activation between aggressive and non-aggressive isolates in oilseed rape.

In chapter IV, the interactions of non-aggressive A1/D2 isolates and the aggressive isolate VL43 with the root surface and root vascular system of oilseed rape cultivar Falcon were investigated by confocal and transmission electron microscopy (TEM), respectively. The investigations of A1/D2 external root colonization patterns through confocal microscopy in chapter IV revealed a lack of fungal penetration structures at the root surface. In contrast, VL43 started forming penetration structures three days after inoculation. Despite the absence of penetration structures of A1/D2, TEM and qPCR analyses confirmed the vascular system colonization of A1/D2. Eynck et al. (2007) suggested that low penetration rates might impede the observation of hyphal pegs with confocal microscopy. Cole and Diener (2013) showed that root-expressed QTL RFO3 in Arabidopsis restricted the penetration of *F. oxysporum* f.sp. *matthiol* but not of crucifer-infecting *F. oxysporum*. The QTL RFO3 encodes receptor-like kinases, which may detect an extracellular Fusarium-derived signal that is only present in the *F. oxysporum* f.sp. *matthiol* infection. A similar detection of A1/D2 isolates on the root surface might be responsible of their low penetration and aggressiveness in oilseed rape.

The TEM assessment revealed that both isolates degraded the secondary vessel wall, although A1/D2a to a lesser extent than VL43. This observation indicated a higher production of wall-degrading enzymes by the aggressive isolate, as Benhamou and Garand (2001) hypothesized. The role of such enzymes in shaping the aggressiveness of *V. dahliae* was reported by Tzima et al. (2011) through fluorescence microscopic assessments. The analysis revealed that the disruption of a gene (VdSNF1) involved in the expression of hydrolytic cell wall-degrading enzymes led to a reduced root penetration and xylem vessel colonization in tomato.

TEM analysis in chapter IV also revealed that A1/D2a-treated samples presented a more intense coating of the secondary vessel walls than VL43-treated samples. Likewise, Benhamou and Garand (2001) reported that inoculation with a non-aggressive *Fusarium* strain induced more intense cell wall coating than the pathogenic *F. oxysporum.* It is generally accepted that electron-dense coating is produced by the plant to inhibit lateral and vertical spreading of vascular pathogens in the xylem (Yadeta and Thomma 2013). This is ilustrated by the work of Rahman et al. (1999), who reported no vessel coating in the susceptible pepper cultivar upon infection of *Ralstonia solanacearum* and extensive spread of the bacteria in the xylem vessels, in contrast to the resistant cultivar.

Parham and Kaustinen (1976) related stained phenolic depositions to electron-dense coatings by investigating sections of embedded plant tissues with both light microscopy and TEM. Similarly, Street et al. (1986) confirmed the phenolic nature of electron-dense depositions on tomato xylem walls upon infection of *Verticillium albo-atrum* by inhibiting the deposition with the application of L-α-aminooxy-

β-phenylpropionate, which is a specific inhibitor of phenylpropanoid synthesis**.** Eynck et al. (2009) revealed with histological analysis the role of phenol metabolism in internal defense against *V. longisporum* by reporting a higher accumulation of wall-bound phenolics in the hypocotyl xylem upon VL43 infection in the resistant oilseed rape genotype. Likewise, the role of phenylpropanoid metabolism in plant defense has also been reported for other vascular pathogens, such as *Fusarium* spp. in flax (Kostyn et al. 2012) and *V. dahliae* in pepper (Novo et al. 2017). Thus, it can be hypothesized that the observed electron-coating depositions on the vessel walls are involved in plant defense associated with the phenylpropanoid metabolism. The study of Zheng et al. (2019a) revealed that SA is a defense signal molecule involved in basal resistance against *V. longisporum* in the oilseed rape cultivar Express. In chapter III, the induction of SA by A1/D1 and A1/D2 was evaluated in cultivar Falcon. However, this assessment revealed that SA is not involved in plant defense induction upon *V. longisporum* infection in Falcon. Similarly, the induction of resistance against *Fusarium oxyporum* in tomato does also not required the involvement of SA (Lamo and Takken 2020). It remains to be elucidated which signal molecules in the plant activate vessel coating upon infection of vascular pathogens (Yadeta and Thomma 2013).

In previous studies, the hypocotyl has been presented as the section where plant defense is activated against aggressive *V. longisporum* isolates, which hinders the progress of infection from roots to shoots in resistant oilseed rape cultivars (Eynck et al. 2009; Zheng et al. 2019a). This is illustrated by the microsclerotia assessment of stubbles from DH lines in chapter V, where the disease severity on roots was in general higher than on stems. In the TEM assessment in chapter IV, intense plant defense reactions were already observed at the root level upon infection with the non-aggressive A1/D2. Similarly, Benhamou and Garand (2001) reported a more intense vessel coating in pea roots upon infection with a non-aggressive *Fusarium* strain than when plants were inoculated with the pathogenic *F. oxysporum*. This indicates non-aggressive isolate cannot overcome the plant defense mechanisms activated at the root level, whereas the mechanisms activated in the hypocotyl might be involved in deterring the systemic colonization of aggressive isolates.

V. Microscopic assessments of the interaction of A1/D2 with oilseed rape provided evidence on the potential biocontrol mechanisms of A1/D2.

In chapter III, the biocontrol effect of the non-aggressive A1/D2 lineage against the aggressive isolate VL43 was investigated. When A1/D2 isolates were root-dip inoculated simultaneously or a week before VL43, a significant reduction in VL43 symptoms was observed. Such a biocontrol effect is typically due to competition for space on the roots and/or induction of plant defense (Deketelaere et al. 2017). Although these hypotheses could not be confirmed by mere phenotypic assessment of the biocontrol effect, they were supported by the microscopic assessments in chapter IV, where the interaction between non-aggressive A1/D2 isolates and roots of oilseed rape was investigated and compared to the aggressive interaction of isolate VL43. Confocal microscopy analysis revealed that A1/D2 strongly colonized the root hairs. This

indicates that A1/D2 non-aggressive isolates are good competitors for space at root hairs, which are common infection sites of vascular pathogens (Eynck et al. 2007; Lamo and Takken 2020; Njoroge et al. 2011; Vallad and Subbarao 2008). In addition, electron microscopy observations revealed that A1/D2 induced intense plant vessel coating, which indicates that A1/D2 provides protection against VL43 by inducing plant defense. These microscopic observations indicate that A1/D2 confers protection against VL43 by directing activating plant defense rather than by priming. In contrast, Benhamou et al. (1996) detected a priming effect on the induction of electron-dense depositions upon *F. oxysporum* f. sp. *pisi* penetration in pea roots that had been pre-treated with B*acillus pumilu*. The deposition was absent in roots that had only been treated with the bacteria.

A post-inoculation treatment with A1/D2a a week after VL43 led to significant worsening of leaf symptoms in comparison to plants only treated with VL43. These observations evidenced different levels of non-aggressiveness within the A1/D2 lineage. Analysis of internal root colonization by means of electron microscopy in chapter IV revealed that A1/D2a-treated samples presented some secondary wall degradation, which might be related to the worsening of VL43 symptoms with an A1/D2a post-inoculation. An electron microscopy assessment of the infection of other non-aggressive isolates could reveal if differences in their ability to degrade xylem walls exist, which might explain the differences of their biocontrol effect with the post-inoculation approach.

The assessment of the biocontrol potential of A1/D2 as a seed coat in chapter III did not result in a significant reduction of VL43 symptoms. A confocal microscopic assessment showed that A1/D2b root colonization was lower when applied as a seed treatment than when applied using root-dip inoculation. With lower hyphal colonization of roots, a lower competition ability and induction of plant defense by the biocontrol agent is expected. These results demonstrate the difficulty of an effective biocontrol against vascular pathogens in natural conditions. It remains unclear whether higher application rates would have led to a biocontrol effect. The colonization patterns of A1/D2 in oilseed rape with microsclerotia inoculation were not investigated in this study. However, the isolation of A1/D1 non-aggressive strains from severely infected stubbles in chapter II revealed that non-aggressive *V. longisporum* isolates can systemically colonize oilseed rape plants in the field and produce inoculum. Lievens et al. (2003) reported simultaneous colonization of different vascular pathogens in tomato plants. Thus, simultaneous infections of non-aggressive and aggressive *V. longisporum* strains within a plant can be expected, which might impact host-pathogen interactions and the development of the disease.

Outlook

This study revealed high genetic and pathogenicity variability within *V. longisporum* populations in oilseed rape growing regions. Further phylogenetic and pathogenicity analyses including strains isolated from the

soil and from different plant developmental stages and *Brassica* hosts could provide a better picture of the overall *V. longisporum* diversity. Since seed transmission might be a factor contributing to gene flow between different geographic regions, seed transmission of *V. longisporum* in other *Brassica* crops (including cover crops) should be assessed in future studies. A better understanding of the complete host range and potential cryptic lifestyle of the pathogen could provide more information about its dispersal, evolution, and pathogenicity, which would ultimately allow better management of the disease.

The biocontrol and microscopic assessments provided information on the underlying interactions in this pathosystem. Additional microscopic assessments of more isolates, as well as comparative, genomics, transcriptomic, or proteomic analysis, could reveal the underlying mechanisms that shape the differences in pathogenicity and plant responses upon *V. longisporum* infection. The biocontrol results opened the door to investigate the potential use of non-aggressive *V. longisporum* isolates as biocontrol agents. However, further research on the concentration and application of the biocontrol agent is still needed.

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Summary: Occurrence and genetic diversity of *Verticillium longisporum* lineage in Europe and their interaction with oilseed rape*, Brassica napus* L.

The phylogenetic analysis by Inderbitzin et al. (2011), based on five protein-coding genes and the nuclear ribosomal internal transcribed spacer region (ITS), revealed that *V. longisporum* consists of three lineages (A1/D1, A1/D2, and A1/D3) that originated from three independent hybridization events of four haploid *Verticillium* parents. This discovery added a new layer of complexity to the control of this soil-borne vascular pathogen. Since then, different studies have genetically characterized the *V. longisporum* lineages, as well as their pathogenicity and distribution (Banno et al. 2015; Depotter et al. 2017b; Novakazi et al. 2015; van Tran et al. 2013; Zou et al. 2020). *V. longisporum* typically infects *Brassica* crops, of which cabbage, cauliflower, and oilseed rape are the economically most important (Depotter et al. 2016; Inderbitzin et al. 2011). The worldwide demand for biodiesel is driving oilseed rape production and shortening crop rotations (Ismail et al. 2017), which considerably contributes to the increase of biotic stresses (Hegewald et al. 2018). The current threat that *V. longisporum* exerts on oilseed rape production, as well as the knowledge gaps in the biology of this pathogen, have motivated the performance of this study, which aimed to further characterize the genetic and pathogenicity diversity of *V. longisporum*. Additionally, due to limited resistance against *V. longisporum* in the current oilseed rape varieties (Friedt and Snowdon 2010), oilseed rape double haploid lines (DH) were evaluated for future quantitative trait locus (QTL) mapping and breeding purposes.

The genetic diversity and population structure of *V. longisporum* within and across lineages from different geographic origins, as well as its relevance for pathogenicity, has not been well studied in oilseed rape. Thus, the first objective of this study (chapter II) was the performance of an extensive lineage monitoring in the main oilseed rape growing regions in Europe and Canada. The lineage monitoring, which was carried out with a multiplex-PCR according to Inderbitzin et al. (2013), revealed that A1/D1 was the dominant lineage in oilseed rape. A phylogenetic analysis of A1/D1 isolates based on genotyping by sequencing (GBS) was conducted to determine if genetic subgroups exist within the A1/D1 lineage. The A1/D1 genetic clusters were, in general, independent of geographic region or field. The pathogenicity characterization in the greenhouse of A1/D1 isolates revealed pathogenicity diversity within the lineage, also within one genetic cluster and among isolates from the same field.

The long survival of fungal resting structures in the soil and lack of effective curative measures make vascular pathogens some of the most difficult plant pathogens to control. The limited availability of control measures calls for the investigation of new alternatives, such as the use of microorganisms as biocontrol agents (Deketelaere et al. 2017; Yadeta and Thomma 2013). One biocontrol approach against vascular pathogens is

using non-pathogenic or non-aggressive isolates from the same species or from other vascular pathogens (Deketelaere et al. 2017). Thus, in chapter III, the biocontrol ability of the non-aggressive A1/D2 lineage was investigated in oilseed rape. The study revealed a biocontrol effect of A1/D2 against a *V. longisporum* aggressive A1/D1 isolate with root-dip inoculation. The induction of salicylic acid (SA), a signal in basal and cultivar-related resistance against *V. longisporum* (Zheng et al. 2019a), was not involved in the biocontrol mechanism. Applying a biocontrol agent as a seed coat is the most practical and economically feasible application method in fields of arable crops (Alabouvette et al. 2009; Rocha et al. 2019). However, the biocontrol effect of A1/D2 was lost with this application technique. Confocal microscopy analyses revealed that seed coating resulted in less A1/D2 hyphal establishment in the roots than the root-dip inoculation, which might explain the lack of A1/D2 biocontrol effect with a seed coat.

In chapter IV, the non-aggressive interaction of *V. longisporum* lineage A1/D2 with oilseed rape was compared to the aggressive interaction of an A1/D1 isolate. Phenotypic assessments in the greenhouse confirmed the non-aggressiveness of A1/D2 on oilseed rape. The external root colonization assessment by confocal microscopy revealed sporulation, hyphal coiling, and unorganized outward hyphal growth by A1/D2 at the rhizosphere, in contrast to the organized net-like hyphal growth of A1/D1 close to the root surface. Electron microscopy and qPCR assays revealed minimal vascular colonization by A1/D2 in the roots and stems, respectively. Additionally, electron microscopy observations showed a more intense xylem vessel coating in A1/D2-treated samples than in A1/D1-treated samples, as well as stronger secondary vessel wall degradation by A1/D1 than by A1/D2. The results indicate that low penetration at the root surface, a low vascular colonization by A1/D2, and a higher intensity of plant defense reactions upon A1/D2 infection in comparison to A1/D1 might explain the low pathogenicity of the lineage A1/D2.

Intensive breeding for quality traits in the past decades and the subsequent reduction of the genetic basis of the species has led to limited resistance against *V. longisporum* in the current oilseed rape varieties (Friedt and Snowdon 2010). Thus, the final goal of this study (chapter V) consisted of the evaluation of oilseed rape double haploid lines (DH) for future QTL mapping and breeding purposes. The greenhouse and field assessments revealed relatively high variability within the DH populations in their response to *V. longisporum*. There was a low correlation between greenhouse and field symptoms, which evidences the difficulty of performing disease screenings in field conditions.

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Marta Vega Marín, Leonard Sundermann, and Andreas von Tiedemann (2019). Biocontrol potential of the non-aggressive lineage A1/D2 of *Verticillium longisporum*. Poster at the International Rapeseed Conference, Berlin.

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Marta Vega Marín, Leonard Sundermann, and Andreas von Tiedemann (2018). Biocontrol potential by the apathogenic A1/D2 lineage of *V. longisporum* against aggressive strains of *V. longisporum* and their interaction with roots of oilseed rape. Meeting of the working group Integrated Control in Oilseed Crops from the International organization for Biological Control, Zagreb (Croatia).

Marta Vega Marín and Andreas von Tiedemann (2016, 2017, and 2018). Gefährdung des Rapsanbaus durch neue Pathotypen der Krankhaften Abreife- Untersuchungen zu Pathogenitätsunterschieden bei *Verticillium longisporum* und Verbesserung der Resistenz von Winterraps gegen ein erweitertes Pathotypenspektrum. Gemeinschaft zur Förderung von Pflanzeninnovation e. V. Jahrestagungen, Berlin (2016 and 2017) and Bonn (2018).

Acknowledgements

The submission of this doctoral thesis would have not been possible with the countless education opportunities my parents have financed through my academic path. There are no words to express my gratitude for their absolute dedication, enormous sacrifices, and unconditional support.

My deepest gratitude goes to my supervisor Prof. Dr. Andreas von Tiedemann for giving the opportunity to carry out my PhD under his supervision. His passion for teaching, his love for phytopathology as well as his leadership skills have truly been inspirational. His problem-solving abilities and his useful suggestions have been indispensable for tackling all the obstacles that came on the way. I am also thankful for his kindness and guidance on a personal level. I am greatly indebted to Prof. Dr. Petr Karlovsky for being the co-referee of my thesis. Great gratitude is expressed to Prof. Dr. Volker Lipka for accepting to be a member of my exam committee. I also would like to express my gratitude to Dr. Birger Koopmann for being a member of my thesis committee and his supportive role in the cooperation breeding project.

I was lucky enough to have a wonderful team of my people by my side, without which the completion of this work would have not been possible. To the cooperation partners from the GFPi and to Dr. Christian Obermeier and Dr. Harmeet Sighn from the Department of Plant Breeding of the Justus-Liebig-University Giessen, I am very thankful for the collective work we have carried out these years. I gratefully acknowledge Dr. Christian Obermeier and Dr. Zehng Xiaoarong for their scientific expertise and their valuable contributions to the thesis. I would like to thank Isabel Müller and Dagmar Tacke, the two technical assistants assigned to my research project. Their absolute tireless commitment was indispensable. Special thanks go to all the students and technical assistants that helped in the field and lab work. My sincere thanks go to Hubertus Reintke and Maik Knobel for their work in the greenhouse and in the field experiments. I am also thankful to Martin Wissen and his team from KWS, as well as to Qintus GmbH for conducting and sampling the field experiments. I also would like to thank all the collaborators that provided oilseed rape material for the lineage monitoring. Dr. Antonia Wilch provided indispensable guidance for the confocal microscopy. I also gratefully acknowledge to Dr. Krzysztof Zienkiewicz, Dr. Ulrike Lipka and Dr. Michael Hoppert for the support with the electron microscopy. I would like to show my gratitude to Dr. Antonio Nogueira and Dr. Anke Sirrenberg for proofreading the thesis.

To all staff members in our division, I would like to express my gratitude for the wonderful working environment. In particularly, my greatly thanks to Martina Bode for her efficient help with burocratic issues. I want to thank the other students and friends of the division -Yao, Annette, Barbara, Sebastian, Dima, Musrat, Sarenqimuge, Christian, and Cheryl- for their help, suggestions, encouragement, and kindness, as well as for the fun memories. Special thanks goes to Alice Eseola for guiding me in the lab, for her contagious laughter, and her resilience.

The guidance and encouragement I have received from different teachers throughout the years have undoubtedly played a significant role in inspiring me to take over such an academic challenge. I am deeply thankful to Dr. Susanne Weigand for accepting me into the Crop Protection master program, which ultimately opened me the doors to agronomic and phytopathological research. I am also thankful to my teachers during my exchange program at the University of Iowa, who that imprinted in me the love for science. Special thanks go to Dr. Lori Adams and Dr. Linh Bui Raborn, with whom I first ever discussed the possibility to pursue a PhD. Their passion for plant science as well as their determination as female scientists still inspires me today. To Dr. Antonio Segura, for introducing me to the field of phytopathology. Also, to all my German teachers and my data analysis teacher Dr. Mehmet Gültas, whose teachings have been very valuable for this thesis.

I would like to mention all my Göttingen friends out of the division for their unconditional support and all the memories we have gathered. First, to my roommates Piyal and Bhakti, as well as Christian and Virginia, with whom I built two wonderful homes. To my “Sobremensa” group, for becoming my second family in Göttingen. To “Cadencia do Mar”, Johan, and my salsa group, for letting me rediscover the healing ability of music. To Elena, Natalia G., Jon, Cristina, Tomos, and Roberta, for their support and fun moments. To Jonas, for his inexhaustible help during my first years in Germany and with the PhD application process. To Anupa and her family, for their unmeasurable kindness. To Marcel, for wiping my tears, for his helping hand, for never giving up. To my Crop Protection friends, for our parties and their academic help. Natalia S. and Piyal also provided wonderful scientific advice. I also have to mention my friends from Spain. Marta, Stephy, and Laura, for always showing me distance can never tear us apart. My university girlfriends, for inspiring me with their integrity and tenacity. Special thanks go to Rosana, for her constant daily encouragement. Also to Marta S., Daniel, and Kate, for always trying to meet when I was back home.

This project was partly supported by funding of German Ministry of Agriculture and consortium of German plant breeders GFPi, to which I am highly indebted.

Statutory declaration

I hereby declare that this dissertation was undertaken independently and without any unaccredited aid.

Göttingen, 7 December 2020

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