Verticillium longisporum on oilseed rape (*Brassica napus* L.) – Differential roles of salicylic acid, seed transmission and plant colonization in greenhouse and field conditions

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Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less.

- Marie Curie, Physicist

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The contents of chapter 2, 3 and 5 of this thesis have been published:

1. Zheng X, Pfordt A, Khatri L, Eseola AB, Wilch A, Koopmann B, von Tiedemann A (2019). **Contrasting patterns of colonization with *Verticillium longisporum* in winter and spring type oilseed rape (*Brassica napus* L.) in the field and greenhouse and the role of soil temperature**. Plant Disease 103 (8): 2090-2099. DOI: doi.org/10.1094/PDIS-01-19-0236-RE.
2. Zheng X, Lopisso DT, Eseola AB, Koopmann B, von Tiedemann A (2019). **Potential for seed transmission of *Verticillium longisporum* in oilseed rape (*Brassica napus* L.)**. Plant Disease 103 (8): 1843-1849. DOI: doi.org/10.1094/PDIS-11-18-2024-RE.
3. Zheng X, Koopmann B, von Tiedemann A (2019). **Role of salicylic acid and components of the phenylpropanoid pathway in basal and cultivar-related resistance of oilseed rape (*Brassica napus*) to *Verticillium longisporum*.** Plants 2019, 8, 491. DOI: doi.org/ 10.3390/plants8110491.

The published paper 3 has been used in chapter 5 of this thesis.

Chapter 1: General introduction

1.1 Oilseed rape (*Brassica napus* L.)

Oilseed rape (*B. napus*) is the second most important oilseed crop (Fig. 1.1), which belongs to the mustard family (Brassicaceae) that consists of 338 genera with more than 3,709 species distributed worldwide, especially in temperate and alpine regions (Al-Shehbaz et al. 2006; Warwick et al. 2010). *Brassica napus* is a relatively young crop species (AACC genome, 2n=38), which is derived from a spontaneous hybridization between *B. rapa* (AA genome, 2n=20) and *B. oleracea* (CC genome, 2n=18) (Koh et al. 2017). Europe, China, Canada and India are the major growing regions for oilseed rape production contributing 86.4% (60.7 million metric tons) of the total rapeseed production worldwide in the season 2016/17 (USDA 2017). The exact location and time of its origin is not known but it is estimated to be grown in Europe for vegetable oil already since 400 years, starting in the early 17th century (Prakash et al. 2012).

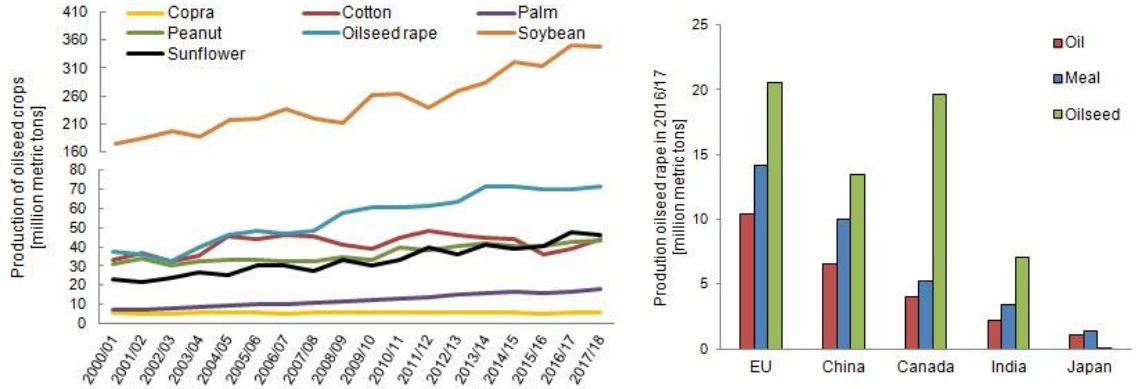

Figure 1.1 World production of oilseed crop from 2000 to 2017 (left) and production of rapeseed oil, meal and seeds in the five top producing countries in 2016/17 (right). (Source: USDA)

Oilseed rape is a winter or spring type annual crop. In contrast to the spring type, which is normally sown in early spring, the winter type is usually sown in late summer or in early autumn to benefit from the cold stimulation during winter, which is needed for flowering. Winter oilseed rape is the most cultivated type in Europe, while spring oilseed rape is more popular in Canada, northern Europe and Australia (Prakash et al. 2012). Beside these two types, a semi-winter type of oilseed rape was produced by crossing winter type *B. napus* with Chinese *B. rapa* for adaptation to local environments. A short period of low temperatures is essential for the full development of semi-winter type (Chen et al. 2008; Wu et al. 2014). Seeds of old time oilseed rape varieties contained more than 40% of erucic acid and glucosinolates, which are undesirable components in rapeseed for food production (Friedt and Snowdon 2009). High content of erucic acid in oil production

causes bitter taste and may cause heart disease (Knutsen et al. 2016). High levels of glucosinolates can release by-products in meal for animal feeding causing liver and kidney damage in monogastric animals (Vogtmann et al. 1975; Tripathi and Mishra 2007). To reduce negative effects on oil and meal of oilseed rape to animals and humans, a new oilseed rape variety, named canola was developed by Stefansson and Downey through conventional plant breeding in Canada in the 1970s (Booth and Gustone 2009). The seeds of this variety contain less than 2% erucic acid and less than 25 μmol/g glucosinolates (Dimov and Möllers 2010). Nowadays, canola oil has become one of the healthiest oils for cooking on the market containing about 60% oleic acid, 20% linoleic acid and 10% linolenic acid. In contrast to canola, a high erucic acid rapeseed (HEAR) containing up to 50-55% erucic acid in the total oil production was developed to meet the demands of the non-food sector used in the oleochemical industry (Knutsen et al. 2016).

Thanks to the intensive breeding progress, oilseed rape provides various benefits. Canola oil was primarily consumed as plant-based culinary oil. In recent years, rapeseed oil was also used as a renewable energy source leading to significant greenhouse-gas cuts ranging from 25.0% to 39.7% compared with conventional fuels (Pehnelt and Vietze 2012; Gilbert 2012). Among the oilseed crops, oilseed rape is one of the most productive species, producing a three to four fold higher yield of oil compared to soybeans. In Europe, 77% of biodiesel production is made up by rapeseed oil (Zentková and Cvengrošová 2013), and oilseed rape has become the major oilseed crop, because of large demands of rapeseed oil for the biodiesel industry (Carré and Pouzet 2014). Further, rapeseed meal left after the oil extraction is a good source of protein in animal feeding containing 38% to 42% protein. Due to these advantages production of oilseed rape increased rapidly in the last decade and reached a higher production than cotton in 2005/06 (Fig. 1.1).

The increasing acreage grown to oilseed rape resulted in less diverse and shorter crop rotations fostering biotic stresses to oilseed rape production. Besides, oilseed rape production is as well threatened by abiotic stresses. Cold stress is one of the important abiotic stresses causing lower density of plants after winter and less production of seeds on plant (Lardon and Triboi-Blondel 1995). On the other hand, extreme high temperature can also cause negative effects on the oilseed rape, such as sterility of flowers and abnormal growth of seeds (Polowick and Sawhney 1988). Cabbage stem flea beetle, pollen beetle, cabbage seed weevil, cabbage stem weevil, rape stem weevil, cabbage root fly and brassica pod midge are the seven major pests occurring in oilseed rape growing areas in Europe, which can lead to serious yield losses (Williams 2010). When high densities of pests are present, plants are not able to compensate damaged or lost plant

organs, which then are likely to have a greater impact on yield (Ferguson et al. 2003). Additionally, oilseed rape can be infested by several fungal diseases, such as blackleg (*Leptosphaeria maculans* and *L. biglobosa*), light leaf spot (*Pyrenopeziza brassicae*), Sclerotinia stem rot (*Sclerotinia sclerotiorum*), clubroot (*Plasmodiophora brassicae*), Verticillium stem striping (*Verticillium longisporum*), Alternaria blight (*Alternaria brassicae*) and grey mold (*Botrytis cinerea*), which can lead to yield losses from 10% to 60% (Dunker et al. 2008; Clarkson et al. 2014; Strehlow et al. 2015; Cai et al. 2017) and are the economically most important production constraints in many oilseed rape growing areas worldwide. Among these diseases, *V. longisporum* has gained an increasing importance in major oilseed rape growing regions in recent years.

1.2 *Verticillium longisporum*

The genus *Verticillium* belongs to the fungal phylum Ascomycota, to which ten species have been currently assigned. Although it is a small group, it has an agricultural importance causing vascular wilt diseases resulting in significant economic losses in numerous vegetable, ornamental, forest and industrial crops in many parts of the world (Inderbitzin et al. 2013). Among these species, *V. dahliae* is the most widespread and economically important, which can infect more than 200 host species (Pegg and Brady 2002; Inderbitzin et al. 2011b), while *V. longisporum, V. albo-atrum*, *V. alfalfa* and *V. nonalfalfae* have a more narrow host range (Fradin and Thomma 2006; Johansson et al. 2006a; Inderbitzin et al. 2011b; Depotter et al. 2016). *Verticillium longisporum*, which is considered as an amphidiploid hybrid, has three different lineages deriving from as unknown species A1 as a common parent hybridized either with unknown species D1, *V. dahliae* D2 or *V. dahliae* D3 (Inderbitzin et al. 2011a; Inderbitzin and Subbarao 2014). These three lineages showed different pathogenicity and virulence to Brassicaceae species. Compared to *V. dahliae*, *V. longisporum*, especially the lineage A1/D1, is more aggressive to Brassicaceae (Eynck et al. 2007). The first report of *V. longisporum* in *Brassica* spp. was recorded in Hamburg in 1961 (Stark 1961). Today, *V. longisporum* is widely spread and established in mostly all oilseed rape production areas in Europe (Germany, France, Belgium, Czech Republic, Netherlands, Poland, southern Russia, Sweden and United Kingdom (England and Wales)) (Depotter et al. 2016). In 2014, *V. longisporum* was recantly detected in canola in Canada, and since then, a nationwide survey has taken place and confirmed that *V. longisporum* is widely distributed in most canola production areas in Canada (Manitoba, Alberta, Saskatchewan, British Columbia, Ontario and Quebec) (CFIA 2017). Although *V. longisporum* was only found in cabbage, cauliflower and horseradish in United States (California and Illinois), the lineages present in these regions are A1/D1 and A1/D2, which were suggested to have a wider potential

host range than currently assumed (Novakazi et al. 2015). On cabbage fields in Japan, the lineages A1/D1 and A1/D3 were also identified (Banno et al. 2015). In 2015, *V. longisporum* was first recorded in cabbage fields in China (Yu et al. 2015). According to recent research, lineage A1/D1, which is the most virulent and predominant lineage in oilseed rape, has two sub-clusters, A1/D1 West and A1/D1 East, which are correlated with the geographic distribution of the isolates. Compared to A1/D1 East, the A1/D1 West population has more genetic diversity (Depotter et al. 2017).

Verticillium longisporum has a monocyclic disease development on oilseed rape. During the non-host periods, *V. longisporum* survives in the form of microsclerotia in soil. Hyphae germinated from microsclerotia grow on and along root hairs closely attached to the surface of the main and lateral roots and cover the root surface with a hyphal net, which grows along the grooves of the junctions of the epidermal cells. After that, hyphae are able to penetrate the epidermal cells by forming a slight hyphal swelling. Subsequently, hyphae grow intracellularly and intercellularly through the root cortex and reach the vascular system (Eynck et al. 2007). Disease symptoms caused by *V. longisporum* on oilseed rape are different to *Verticillium* spp. infesting other crops: normally, no wilting symptom can be observed (Depotter et al. 2016). Under greenhouse conditions, infected plants show discoloration of leaves and a reduction in plant height (Zeise and von Tiedemann 2001; Eynck et al. 2009b). Under field conditions, disease symptoms are not visible before the ripening stage of the plants, when dark and unilateral striping indicating the necrosis of cortical tissue can be observed (Heale and Karapapa 1999; Knüfer et al. 2017). Therefore, the disease caused by *V. longisporum* on oilseed rape under field conditions was recently re-named as ‘Verticillium stem striping’ (Depotter et al. 2016). Shortly before harvest, black microsclerotia are formed by the pathogen in the root and under the stem epidermis and in the stem pith. Microsclerotia of the pathogen will remain in the plant debris and be released into the soil during plant decomposition. Microsclerotia are the resting bodies of *V. longisporum*, which may remain dormant and viable in the soil for more than ten years (Wilhelm 1955; Depotter et al. 2016). Furthermore, the pathogen might be spread in non-symptomatic plant parts such as seeds and thus transferred to *V. longisporum* free growing regions by long distance transports. However, no clear evidence for seed transmission has been provided so far.

Yield losses caused by *V. longisporum* were observed in individual plants under controlled conditions (Dunker et al. 2008), while yield losses in the field have not yet been experimentally verified. As described by Dunker et al. (2008), no significant reduction of thousand seed weight and oil content was found after artificial inoculation of oilseed rape with *V. longisporum* in the field. Conversely, Gladders et al. (2013) estimated a reduction

in thousand seed weight of 12% to 24% in four commercial crops severely affected by *V. longisporum*. Due to systemic infection, *V. longisporum* grows and spreads inside the xylem vessels of oilseed rape, current disease control strategies, such as fungicide application, do not provide protection against this pathogen. Thus, the control of this disease is still a big challenge. An integrated disease management strategy needs to be developed for the successful control of this disease. Soil treatment, such as fumigation and heating, is a successful way to reduce the fungal population in the soil, however, it has a potential risk to the environment and is very energy consuming (Powelson and Carter 1973; Pullman 1981). Therefore, this control method is not allowed in many countries. Crop rotation may also be effective to control this disease, but the persistence of microsclerotia and the presence of non-brassicaceous plants as potential alternative hosts might reduce the decreasing effect of a wide crop rotation (Novakazi et al. 2015). Use of biological control agents (e.g. *V. isaacii* isolate Vt305, *Serratia plymuthica* HRO-C48) and organic soil amendments have been reported to suppress the symptom development on oilseed rape caused by *V. longisporum* (Subbarao et al. 1999; Müller and Berg 2008; Tyvaert et al. 2014). Breeding for resistant cultivars is the most feasible mean to control 'Verticillium stem striping' in oilseed rape. Due to the narrow genetic basis of the currently available cultivars on the market, resistance sources from *B. oleracea*, *B. rapa* and even outside the Brassicaceae family may be used for breeding resistant *B. napus* culivars.

1.3 Response of *B. napus* to infection with *V. longisporum*

In *Arabidopsis thaliana*, several resistance mechanisms against *V. longisporum* were described. Genes in *A. thaliana* related to cell wall synthesis, proteolysis and plant defense were up-regulated during early stages of infection with *V. longisporum* (Tischner et al. 2010). Regarding plant hormones, jasmonic acid and ethylene were suggested to be important for the regulation of *V. longisporum* resistance mechanisms in *A. thaliana*, however, plants displaying a misfunction of the salicylic acid pathway due to mutation, such as *Nah*G, *eds1-1, pad4-1*, did not express increased susceptibility to *V. longisporum* (Johansson et al. 2006b). Soluble phenylpropanoids were involved in defense response (König et al. 2014). Similar to the salicylic acid pathway in *A. thaliana*, genes of the phenylpropanoid pathway were reported to be candidates for *V. longisporum* resistance in *B. napus* (Obermeier et al. 2013). Furthermore, several phenolic acids, intermediates in the phenylpropanoid pathway, especially in lignin biosynthesis, were accumulated in *B. napus* after infection with *V. longisporum* and considered to play a role in resistance (Eynck et al. 2009a). An enhancement of levels of salicylic acid and its glucoside was found in the xylem sap of *B. napus* plants infected with *V. longisporum*, which were

positively correlated with disease severity (Ratzinger et al. 2009). Strong increases of the salicylic acid and salicylic acid mediated resistant marker *PR1* and *PR2* were also found in stems and hypocotyls after infection, but it seems that there is a lack of relation to resistance of *B. napus* to *V. longisporum* (Ratzinger et al. 2009; Kamble and Bhargava 2007). However, the exact role of salicylic acid in the interaction of *B. napus* and *V. longisporum* has not been investigated thoroughly yet.

1.4 Aims and objectives of the thesis

Although the disease cycle of *V. longisporum* has been well described in previous studies, seed transmission has not been thoroughly investigated so far. Furthermore, the natural infection process and plant colonization of *V. longisporum* under field conditions is still unclear. While no gene to gene resistance has been identified, quantitative resistance seems to be important for its control and opens the potential for breeding of resistant cultivars in the future. As described above, the phenylpropanoid pathway has a crucial but complicated role in the interaction of *B. napus* with *V. longisporum*, which demands more efforts to elucidate the potential causal relationships. Thus, based on this background, the present thesis focused on the natural development of *V. longisporum* and functional analysis of *V. longisporum* resistance on *B. napus*. The specific objectives of the respective chapters are briefly described as follows:

1) A qPCR assay was conducted to monitor the presence of *V. longisporum* in different plant parts of field-grown winter and spring oilseed rape. The major objective of this part (Chapter 2) was to reveal the natural development of *V. longisporum* in *B. napus* and its effect on yield and seed quality parameters.

2) Seed transmission of *V. longisporum* is an important issue with regard to epidemiology and to the international seed trade market of oilseed rape. To verify the potential of seed transmission, several experiments were conducted under controlled and field conditions. The general objective of this study (Chapter 3) was to explore whether *V. longisporum* is capable to be transmitted by seeds of *B. napus*, and whether a dissemination of the disease is possible

3) Since phenylpropanoids have been found to be important for resistance of oilseed rape to *V. longisporum*, a study was conducted to analyze the role of secondary metabolites, enzyme activities and gene expressions involved in the phenylpropanoid pathway of *B. napus* during *V. longisporum* infection. Since salicylic acid and lignin are two important components among phenylpropanoids and potentially competitive during plant defense response because of their common precursors, the objectives of these studies were to investigate the effect

of salicylic acid on *V. longisporum* and *B. napus* (Chapter 4) and the role of salicylic acid in resistance responses of *B. napus* to *V. longisporum* including its relationship to lignin synthesis (Chapter 5).

4) Potential sources of resistance to infection with *V. longisporum* are of large interest for phytopathologists and breeders. Therefore, the objectives of these studies were to identify plant tissues (Chapter 6) or plant genotypes (Chapter 7) with enhanced disease resistance to *V. longisporum*.

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Chapter 2: Differential pattern of colonization of field-grown winter and spring oilseed rape (*Brassica napus* L.) with *Verticillium longisporum*

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Abstract: Oilseed rape is the second most important source of vegetable plant oil, however, production is threatened by *Verticillium longisporum*, a soil-borne vascular pathogen so far occurring in Europe and Canada, which causes premature ripening potentially resulting in significant economic losses. In spite of intensive research on *V. longisporum* in the last decades, some basic knowledge is still lacking about the time course of infection in the field and disease development in susceptible and resistant cultivars. In this study, field experiments with microsclerotia inoculated plots were conducted. The colonization of spring and winter oilseed rape with *V. longisporum* from the seedling to the mature plant stage was monitored by qPCR. Cultivars contrasting in resistance to *V. longisporum* were used. Endogenous SA, a typical response of oilseed rape to *V. longisporum* infection, was followed as well with the development of *V. longisporum*. *Verticillium longisporum* was able to colonize roots of winter oilseed rape already before winter at BBCH 14. Possibly due to the increased temperature and lacking of SA, biomass of *V. longisporum* increased rapidly at flowering stage. In contrast to susceptible winter oilseed rape, in which *V. longisporum* easily spreads into the entire plants, especially accumulating in the stem, resistant cultivar can restrict the growth of *V. longisporum* in the hypocotyl and prevent *V. longisporum* from further developing into the stem.

Keywords: oilseed rape, *Verticillium longisporum*, resistant, susceptible, qPCR, salicylic acid, seed quality

2.1 Introduction

Oilseed rape (*Brassica napus* L.) is the second most important crop for oil production in

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the world, after soybean (Depotter et al. 2016). Due to the multiple uses as edible vegetable oil, animal feed and biofuel, a strong rise in the production of oilseed rapeworldwide have been witnessed in the last 40 years. The European Union, China and Canada are the main oilseed rape growing regions contributing 76.3% (53.6 million metric tons) of rapeseed production in the season 2016/17 (USDA 2017). Oil with high contents of erucic acid tastes bitter and might cause heart disease when large quantities are consumed. Oilseed rape meal with high levels of glucosinolates may contain by-products causing liver and kidney damage in monogastric animals (Vogtmann et al. 1975; Tripathi and Mishra 2007). Therefore, oilseed rape cultivars, which have 'double low' ('00') quality, i.e. <2% erucic acid in the oil and <25µmol/g glucosinolates in the seeds, are dominant in oilseed rape production in Europe (Dimov and Möllers 2010; Piazza and Foglia 2001).

Since the 1980s, due to the increasing growing area of oilseed rape and the relatively intense crop rotation, *Verticillium longisporum* (VL), a soil-borne vascular fungal pathogen, has become an increasing threat to oilseed rape production in Europe and North America, causing 'Verticillium stem striping' (Karapapa et al. 1997; Zeise and von Tiedemann 2002; Depotter et al. 2016). Its melanized microsclerotia can be dormant and viable in the soil for several years and cause substantial yield losses by induction of premature ripening (Dunker et al. 2008; Gladders et al. 2011). *Verticillium longisporum* is supposed to have a hemibiotrophic life cycle in oilseed rape (Depotter et al. 2016). The fungal hyphae germinated from the microsclerotia colonize along the surface of the root hairs for the initial biotrophic stage and directly penetrate into the root cortex and spread further into the vascular system. In greenhouse experiments with *B. napus* inoculated with *V. longisporum* by root dipping in conidia suspension, the first penetration of *V. longisporum* into the roots was observed at 60 hpi without showing any visible discolouration or necrotic symptom. By three weeks after inoculation, *V. longisporum* started to colonize the xylem vessels of the shoot without occupation of the entire vascular system. However, the hyphae of the fungus were capable to penetrate into adjacent vessels after being trapped at vessel end walls (Zhou et al. 2006; Eynck et al. 2007). Low level of *V. longisporum* was detectable in pooled plant samples at 7 dpi. Clear symptoms were observed at 21 dpi (Eynck et al. 2007). In contrast, under field conditions, no visible symptoms of *V. longisporum* were observed before the ripening stage of the plants. The necrotrophic life stage starts at the onset of plant maturity leading the fungus to escape from the vessels and colonize the stem parenchyma where it forms melanized microsclerotia under the stem epidermis and in the stem pith (Knüfer et al. 2017). The microsclerotia are transferred with stubbles into the soil after harvest, and have been estimated to be able to remain in soil for more than ten years (Heale and Karapapa 1999;

Depotter et al. 2016). Until today, the lack of effective fungicides has limited any direct control measures. Preventive non-chemical management of this disease thus includes crop rotation with non-host cultivar, delayed sowing date, application of biological control agents and the use of resistant cultivars (Rygulla et al. 2008; Tyvaert et al. 2014).

Observation of dark unilateral stem striping symptoms and visual assessment of the quantity of microsclerotia on harvested stubbles are common approaches to detect and assess 'Verticillium stem striping' disease in the field. In addition, a qPCR based method using *ß*-tubulin primers, which showed a high specificity to *V. longisporum* isolates, was developed to detect and quantify *V. longisporum* in symptomless plants growing in the field. This assay can clearly differentiate levels of quantitative resistance to *V. longisporum* in oilseed rape genotypes under field conditions (Knüfer et al. 2017). Previous studies followed the colonization of *B. napus* with *V. longisporum* in a controlled environment (Zhou et al. 2006), however, studies are lacking which investigated the temporal pattern of entry and colonization in plants from early to mature growing stages in the field. According to assessments of yield losses in single plants, the overall yield losses potential due to *V. longisporum* has been estimated to range between 10% and 50% (Dunker et al. 2008). However, this figure has not yet been experimentally verified with contemporary commercial cultivars. Besides, effects on seed weight and oil content after infection with *V. longisporum* are insufficiently investigated (Dunker et al. 2008).

Salicylic acid (SA) is an important phytohormone occurring in plants in a free and conjugated form, and possessing a high metabolic and physiological activity, such as regulatory functions in plant development and responses to biotic and abiotic stresses (Abreu and Munné-Bosch 2008; Vlot et al. 2009; Wang et al. 2012). Salicylic acid is involved in the regulation of senescence indicated by yellowing of leaves due to chlorophyll degradation (Vogelmann et al. 2012). Furthermore, SA is known as a signal molecule playing a role in yield formation in tomato and wheat (Yıldırım and Dursun 2009; Sharafizad et al. 2012). Previous studies with *B. napus* implied that SA accumulation in stem tissue and xylem sap was associated with *V. longisporum* infection and appeared to be correlated with susceptibility of plants to the disease (Ratzinger et al. 2009). Although a functional role of SA in the interaction of oilseed rape and *V. longisporum* awaits clarification, SA could thus serve as a biochemical marker of plant responses to infection and disease severity.

Based on qRCR detection, visual observations and SA analyses from seedling to maturity stages, the present study elucidates the temporal patterns of colonization and development of *V. longisporum* in field-grown winter and spring oilseed rape. The use of the two seasonal types of oilseed rape in separate field experiments was expected to

provide deeper insights into the impact of climate condition and plant ontogensis on the course of entry and colonization. In addition, resistant and susceptible cultivars were selected for the study in order to determine the behavior of *V. longisporum* in oilseed rape genotypes differing in quantitative resistance.

2.2 Materials and methods

2.2.1 Plant material

Two winter oilseed rape cultivars, *B. napus* L. cv. Falcon (Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, NPZ, Hohenlieth, Germany) and SEM 05-500256 (Syngenta, Germany), and two spring oilseed rape cultivars, *B. napus* L. cv. Visum and OP-DLE 7 (NPZ, Germany) were used. Cultivar Falcon and Visum are German commercial oilseed rape, which are susceptible to *V. longisporum*. SEM 05-500256 and OP-DLE 7 are breeding lines resistant to *V. longisporum*. The seeds were pretreated with tetramethylthiuram disulfide (TMTD) (5.71 ml/kg seeds) as seed coating. Plant growth stages were assessed following the BBCH identification key described by Weber and Bleiholder (1990) and Lancashire et al. (1991) in Appendix 1.

2.2.2 Conditions and design of field trials

Table 2.1 Weather data recorded at the weather station Göttingen in 2015/16 (WSG 2015, 2016).

	Temperature [°C]	Precipitation [mm]	Sum of sun [h]	Wind speed [km/h]
Aug.2015	19.7	91.4	250.9	4.6
Sep. 2015	12.8	54.6	132.3	5.7
Oct. 2015	8.6	37.0	87.5	2.8
Nov. 2015	8.1	124.6	66.0	11.5
Dec. 2015	7.0	35.0	51.5	9.7
Jan. 2016	1.3	63.4	51.4	0.0
Feb. 2016	3.6	73.2	62.5	7.2
Mar. 2016	4.5	28.2	81.8	8.2
Apr. 2016	8.3	29.0	176.2	7.4
May 2016	14.3	17.6	238.0	6.5
Jun. 2016	17.6	98.2	229.8	4.8
Jul. 2016	18.9	19.0	234.1	6.5
Aug. 2016	17.9	36.8	238.9	6.2

Two field experiments were conducted in the northwest of Göttingen, Lower Saxony, Germany (51°33’56.9’’ N, 9°55’09.0’’ E) in the season 2015/16. This site was selected due to its virgin cropping history as regards oilseed rape cultivation. The annual mean temperature and precipitation were 8.7°C and 644.9 mm (WSG, Wetterstation Göttingen), respectively. Temperature and precipitation data recorded during the experimental season are shown in table 2.1.

The plots arranged in completely randomized block design with four replications were conducted in both winter and spring oilseed rape experimental fields. There were 16 plots, each 3 m by 16 m in size. Each plot consisted of two sub-plots for sampling and yield analysis. Sowing rate was 60 seeds per m^2 for winter oilseed rape and 100 seeds per m^2 for spring oilseed rape. For winter oilseed rape, fertilization (205.3 kg/ha nitrogen, 40 kg/ha sulfate), pesticides against weeds (2.5 l/ha Butisam® Gold), insects (0.2 l/ha Trebon®) and snails (4 kg/ha Patrol® Meta Pads®) and growth regulator (0.7 l/ha Caramba®) were conducted according to good and local agricultural practice. Fertilization (172.9 kg/ha nitrogen, 40 kg/ha sulfate), pesticides against weeds (2.5 l/ha Butisam® Top), insects (0.75 l/ha Karate® Zeon, 0.2 l/ha Trebon®, 0.17 l/ha Avaunt®, 0.3 l/ha Biscaya®), pathogens (0.5 l/ha Cantus® Gold) and snails (4 kg/ha Arinex®, 4 kg/ha Mollustop®) and growth regulator (0.7 l/ha Moddus®) were applied in spring oilseed rape field. All agronomic measures were performed according to good agricultural practice.

2.2.3 Fungal inoculum and field inoculation

Stubbles of oilseed rape heavily infested with *V. longisporum* microsclerotia were collected in autumn 2015 from an experimental field of KWS in Fehmarn, northern Germany. The milled dry powder of stubbles (Kompost-Meister 2400, Germany) was homogenously spread on the soil surface by hand at a rate of 15 g per m^2 shortly before sowing (27th August 2015 for winter oilseed rape, 7th April 2016 for spring oilseed rape) and incorporated into the upper soil layer using a rotary harrow (Kreiselegge, Germany).

2.2.4 Measurement of seed quantity and quality

Clean seed yield was determined in dt per ha at a standard water content of 9%. Random samples taken from each plot were used for quality analysis. Protein, oil and glucosinalates were determined by Dr. Christian Möller in the Plant Breeding Division, Georg August University Göttingen using near-infrared reflectance spectroscopy (NIRS). Because of the strong damage caused by pollen beetle (*Meligethes aeneus*) during the flowering period and extreme thundery weather leading to lodging, no valid yield analysis was possible on spring oilseed rape.

2.2.5 Disease assessment of stubbles after harvest

From each plot, 50 stubbles of winter oilseed rape and 25 stubbles of spring oilseed rape were randomly collected 3 weeks after harvest for the phenotypic disease assessment. Disease incidence was assessed as the proportion of diseased plants to the total number of assessed plants. Disease severity was scored by visual and microscopic observation of the presence and intensity of microsclerotia on subepidermal and in pith tissue of stems as well as in the roots using an assessment key (Knüfer et al. 2017) with four severity

classes as Table 2.2.

Table 2.2 Assessment key for scoring disease severity of *Verticillium longisporum* on oilseed rape stubbles. (Knüfer et al. 2017)

Score	Description
1	Healthy tissue: no microsclerotia visible in tissue
2	Slight infection: low abundance of microsclerotia
3	Advanced infection: high abundance of microsclerotia
4	Severe infection: tissue heavily infested with microsclerotia, epidermis is peeling off from the stubble

2.2.6 Quantification of DNA of *V. longisporum* in different plant tissues

Five plants per plot were harvested at six time points and separated into three parts, root, hypocotyl and stem as illustrated in Fig. 2.1.

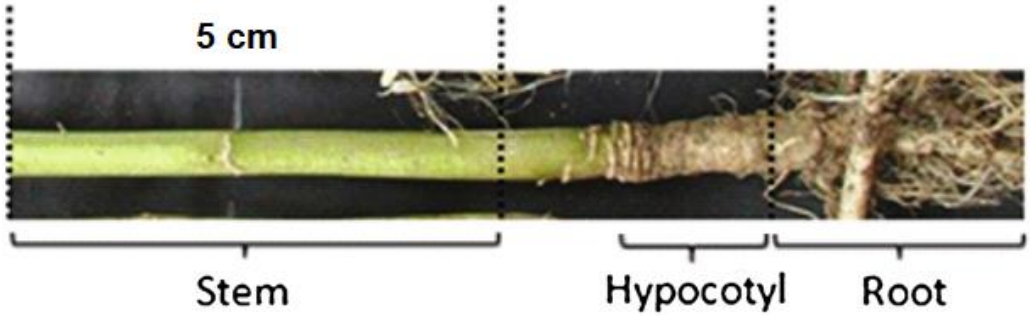

Figure 2.1 Section of oilseed rape sample for detection and quantification of *Verticillium longisporum* by qPCR and analysis of salicylic acid by HPLC (modified from Knüfer et al. 2016).

Samples were lyophilized and ground into fine powder. Total DNA was extracted from about 50 mg dry, ground sample using the cetyltrimethylammonium bromide (CTAB) method (Brandfass and Karlovsky 2008). The samples were homogenized in 1 ml CTAB with 2 µl ß-mercaptoethnol and 1 µl 1x proteinase K. The mixture was sonicated for 5 s and incubated for 10 min at 42°C, and subsequently for 10 min at 65°C. For degradation of RNA, 1.5 µl RNAse was added and incubated for 15 min at 37°C. For isolation of DNA from the mixture, 800 µl chloroform: isoamyl alchol (24:1) was added and incubated on ice for 10 min. After centrifugation at 12,000 rpm for 10 min, the supernatant was mixed in 193.6 µl 30% PEG and 100 µl 5 M NaCl for precipitation. The pellet after centrifugation for 5 min was washed with 70% ethanol. The washed pellet was dried in a centrifugal concentrator for 10 min at 30°C and dissolved overnight in 200 µl TE buffer at 4°C.

The extracted total DNA stained with ethidium bromide (EB) was qualified with 1% agarose gel electrophoresis. A CFX384 real-time PCR detection system (Bio-Rad laboratories, Inc.) was used for the amplification and quantification of *V. longisporum* DNA using ß-tubulin primers forward (*5'-GCAAAACCCTACCGGGTTATG-3'*) and reverse (*5'-*

AGATATCCATCGGACTGTTCGTA-3') (Debode et al. 2011). The amplification mix consisted of 5 µl premix (qPCRBIO SyGreen Mix Lo-Rox, Nippon Genetics Europe GmbH), 0.4 µM of each primer and 1 µl of template DNA and filled up to a total volume of 10 µl with ddH_2O. The PCR-program considered a 3 min initial denaturation step, followed by 40 cycles with a denaturation step (5 s at 95°C), annealing (15 s at 68°C) and subsequent extension (15 s at 72°C). Fluorescence was detected after each elongation step and the program was completed with a final elongation step of 2 min at 72°C. Melting curve analysis was performed with 95°C for 10 s, followed by a 55°C step for 10 s and subsequent measurements within a range of 65°C to 95°C with every 5 s in 0.5°C temperature increments. PCR for all treatment samples were performed with four biological and three technical replicates and data were analyzed using CFX Manager Software (Bio-Rad laboratories, Inc.).

2.2.7 Quantification of salicylic acid in different plant tissues

2.2.7.1 Extraction of salicylic acid

Salicylic acid was extracted from plant tissues according to a modified method described by Enyedi et al. (1992). The samples used for SA analysis were the same as used for DNA extraction. About 50 mg samples were homogenized in 1.5 ml acetone, shaken vigorously and centrifuged at 5,500 rpm at 4°C for 45 min.

Free salicylic acid. The supernatant was transferred to a fresh tube 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 added subsequently. The upper phase from this mixture was transferred to a fresh tube and evaporated to dryness at 35°C. The residue was dissolved again in 200 µl of HPLC grade methanol.

Conjugated salicylic acid. The acetone pellet was dried in a water bath at 65°C for 60 min and hydrolyzed in 0.8 ml of 2 M NaOH. The mixture was heated in a water bath to 80°C for 2.5 h. For acidification of the mixture, 0.64 ml concentrated HCl (37%) was added and the mixture was incubated at 80°C in a water bath for 60 min. To extract SA, 1.6 ml ethyl acetate was added and the mixture was transferred to a fresh tube with subsequent evaporation to dryness by speed vacuum centrifugation at 35°C. The residue was dissolved in 200 µl of HPLC grade methanol. Both free and conjugated SA was stored at -20°C till measurement.

2.2.7.2 Standards and sample preparation

Salicylic acid was dissolved in HPLC grade methanol to a concentration of 20 mM. Dilutions with concentration of 100 nM, 250 nM, 500 nM, 700 nM, 1 µM, 2.5 µM, 5 µM,

7.5 µM, 10 µM, 20 µM were used as internal standards. Peak of SA was identified by comparing retention times of samples and standards, and confirmed by addition of standard SA to the samples. Before loading into a HPLC vial, all the samples or standards were centrifuged at 500 rpm for 5 min to precipitate unsolvable particles and to prevent injection problems.

2.2.7.3 Determination of salicylic acid by HPLC

The HPLC-fluorescence system consisted of a Varian 410 automatic injector, two Varian 210 pumps with 10 W SS head, a LiChrospher RP-18 column (250 x 4 mm, 5 µm) protected by a Security Guard™ Carbo-H precolumn (4 x 3 mm, 5 µm) kept in a column oven at 30°C and a Varian 363 fluorescence detector with excitation wavelength at 315 nm and emission wavelength at 405 nm. Each sample was analyzed for 33 min under a bi-mobile phase of (A) 20 mM sodium acetate, pH 5.0 and (B) methanol with a flow rate of 1 ml/min with the following biphasic protocol: initial 10% B for 2 min, linear gradient to 38% B in 13 min, increased to 98% B in 30 s and held for 9 min, equilibrated to initial condition in 30 s and hold for 8 min. The injection volume was 10 µl.

2.2.8 Statistical analysis

The experimental field data was analyzed as completely randomized design with four replications using STATISTICA 13.2. Before testing for statistical significance, a probability plot with Shapiro-Wilk test was used to test for normal distribution. The data were analyzed with factorial ANOVA. A multiple comparison was analyzed by Fisher LSD test. The experimental results are presented as means ± standard error at 5% significance level.

2.3 Results

2.3.1 Colonization and development of *V. longisporum* on oilseed rape

2.3.1.1 Development of *V. longisporum* in different plant tissues of winter oilseed rape

Samples collected at BBCH 14, 18, 30-32, 55, 61-65, 79-80 were used for detection of colonization and development of *V. longisporum* in susceptible cultivar Falcon and resistant cultivar SEM. No visible symptom (stem striping) was observed on these collected samples. Before winter at BBCH 14, *V. longisporum* DNA was already detectable in the root tissue of both cultivars (Fig. 2.2). However, a 100-fold higher amount of *V. longisporum* DNA was recorded in the susceptible (0.322 ng/g DW) than in the resistant cultivar (0.003 ng/g DW).

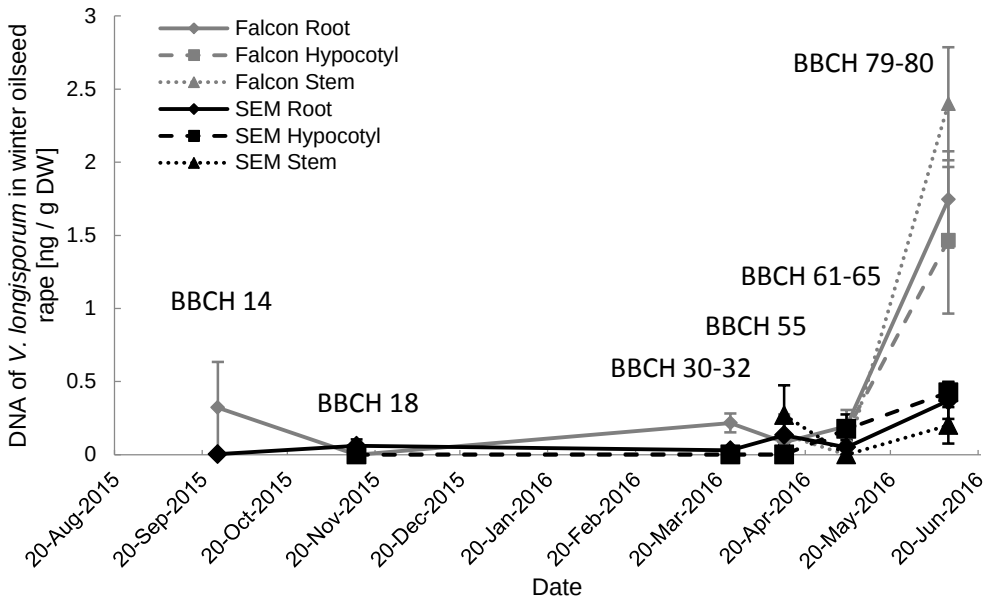

Figure 2.2 Course of colonization of roots, hypocotyls and stem tissues of winter oilseed rape (*Brassica napus*) with *Verticillium longisporum* from seedling to maturity growth stages in the field. Samples from inoculated plots were presented. Bars indicate standard errors.

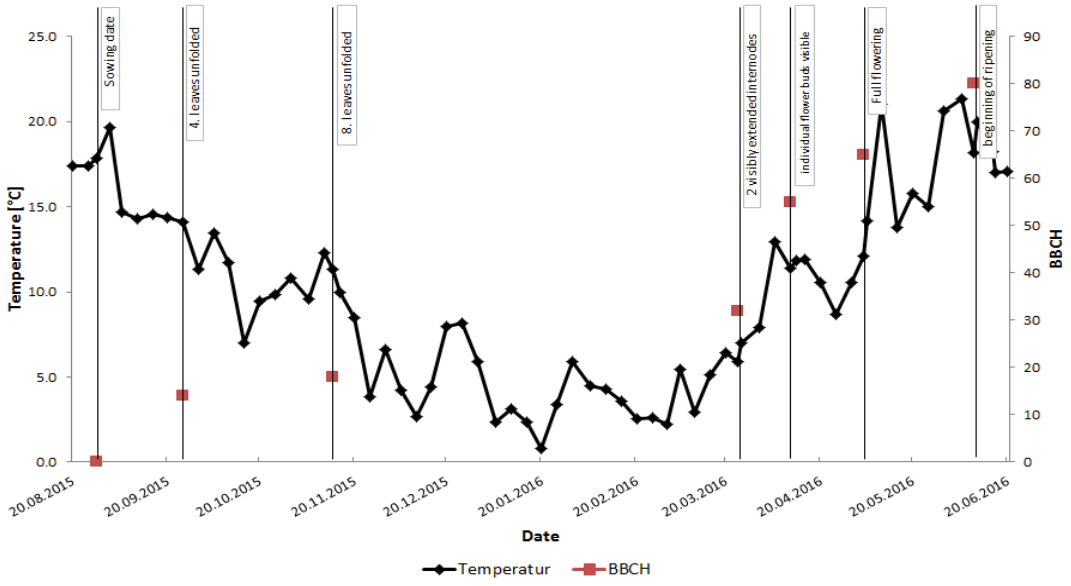

Figure 2.3 Soil temperatures in 5 cm depth in the growing season of winter oilseed rape in 2015/16 in Göttingen. Red dots indicate the growing stages of *Brassica napus* at which samples were collected.

Soil temperatures at 5 cm depth during winter were below 10°C but above 0°C (Fig. 2.3). The accumulation of *V. longisporum* was observed during this phase. At BBCH 30-32, shortly after winter, *V. longisporum* colonized the hypocotyls of the susceptible cultivar, while no fungal growth occurred in the resistant cultivar. The soil temperature increased again in the end of April (Fig. 2.3). With the increase of soil temperature, a more than 2-fold increase of *V. longisporum* DNA was observed at BBCH 61-65 in all plant tissues in both cultivars. Close to harvest, *V. longisporum* growth drastically better developed in the

susceptible compared to the resistant cultivar (P<0.05), and significantly higher amounts of *V. longisporum* DNA were found in the stems compared with the roots and hypocotyls. In contrast, less colonization of *V. longisporum* was found in the stems of the resistant cultivar, compared with the roots and hypocotyls.

2.3.1.2 Salicylic acid in different plant tissues of winter oilseed rape

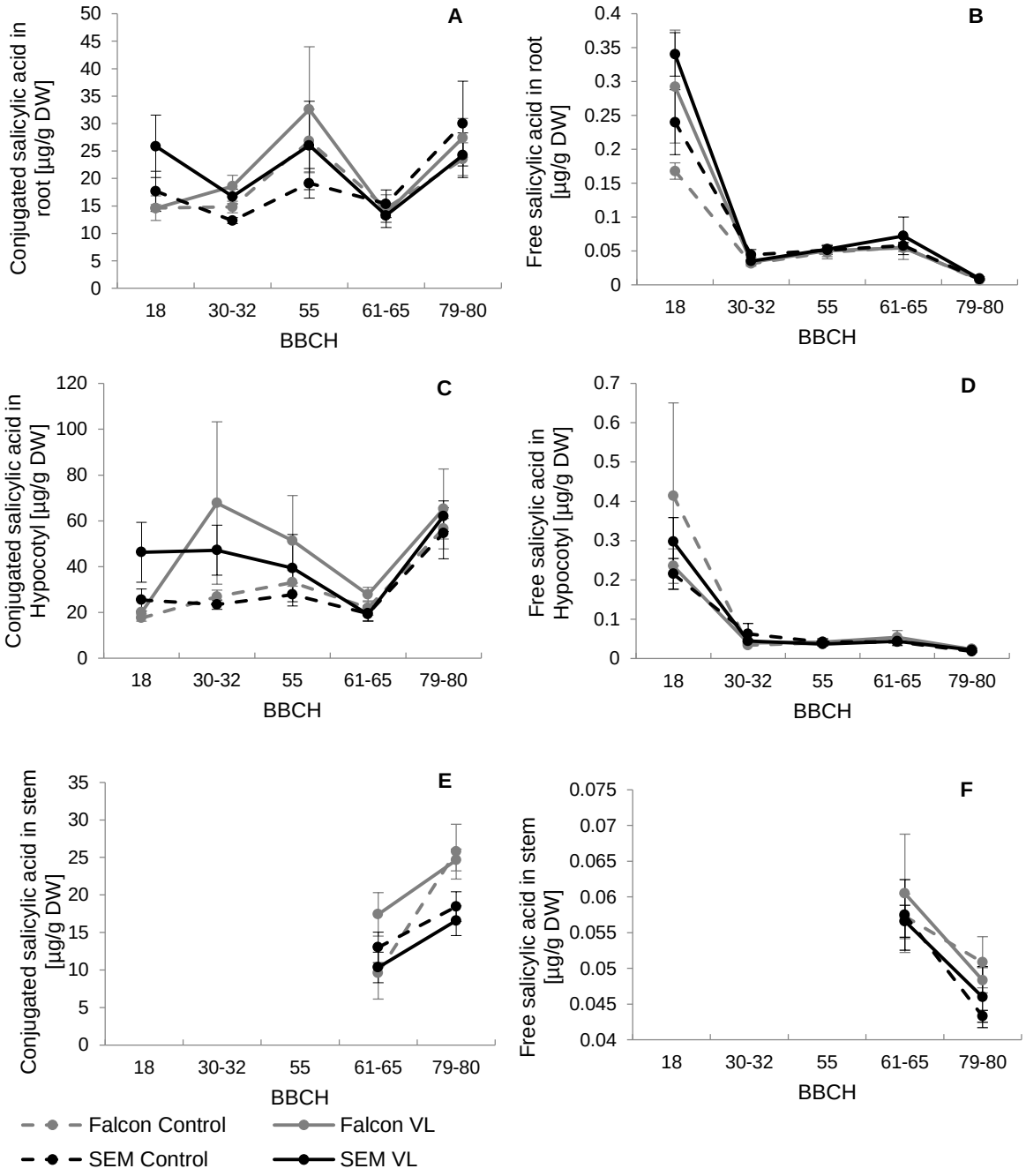

Figure 2.4 Conjugated (A, C, E) and free (B, D, F) salicylic acid content in root (A, B), hypocotyl (C, D) and stem (E, F) of winter oilseed rape (*Brassica napus*) at different growing stage. Bars indicate standard errors.

Compared to free SA, levels of conjugated SA were relatively higher and constant during the period of sampling. Free SA was clearly reduced after winter. Besides, higher levels of

conjugated SA were found in the hypocotyls compared with the roots and stems (Fig. 2.4C). A slight induction of conjugated SA by *V. longisporum* was found in both susceptible and resistant cultivars until flowering. A faster increase of conjugated and free SA was recorded in the roots of the *V. longisporum* inoculated resistant cultivar (Fig. 2.4A, B). In contrast to BBCH 18, relatively higher levels of conjugated SA were observed after winter in the susceptible cultivar inoculated with *V. longisporum* comparing with the resistant cultivar (Fig. 2.4A, C). In the flowering stage (BBCH 61-65), levels of conjugated SA were reduced in the roots and hypocotyls. After flowering, conjugated SA increased again in all plant parts, while free SA decreased. However, at this stage (BBCH 79-80), induction of conjugated SA by *V. longisporum* was no more observed.

2.3.1.3 Development of *V. longisporum* in different plant tissues of spring oilseed rape

The samples collected at BBCH 14, 55, 60, 65, 80 were used for detection of colonization and development of *V. longisporum* in susceptible cultivar Visum and resistant cultivar OP-DLE 7. In general, soil temperature during the vegetation stages was above 10°C (Fig. 2.6). In the greenhouse phenotyping, Visum was clearly more susceptible to *V. longisporum*, showing more severe yellowing of leaves and stunting of plant height, compared with OP-DLE 7 (not shown). However, in the field, no visible symptoms, i.e. stem striping, were observed on both cultivars.

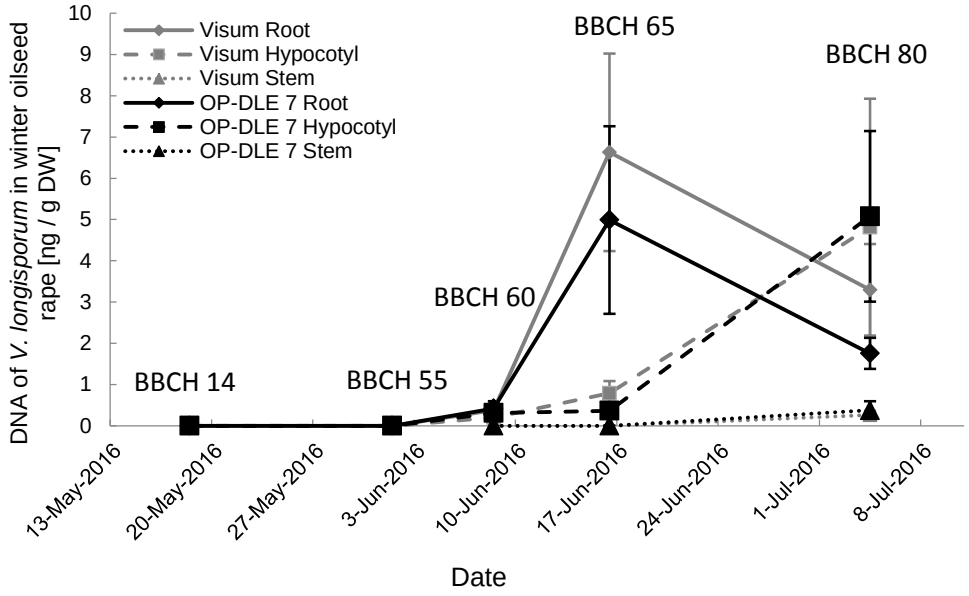

Figure 2.5 Biomass of *Verticillium longisporum* represented by the amount of fungal DNA in root, hypocotyl and stem tissue of field grown spring oilseed rape (*Brassica napus*) at different growth stage. Samples were taken from inoculated plots. Bars indicate standard errors.

No *V. longisporum* was detectable in any tissue of both cultivars up to BBCH 60 (Fig. 2.5).

Similar to winter oilseed rape, levels of *V. longisporum* DNA at BBCH 60 in both genotypes were equivalent. One week later, a more than 10-fold increase of *V. longisporum* DNA was found at full flowering (BBCH 65) in the root of both cultivars, compared with BBCH 60. Levels of *V. longisporum* DNA in the roots at this stage were clearly higher than in winter oilseed rape. However, only a slightly higher *V. longisporum* DNA was found in the roots and hypocotyls of the susceptible cultivar. Stem tissues of both cultivars were free of *V. longisporum* until the end of flowering, and were only colonized at the onset of ripening (BBCH 80). In this growth stage, better development of *V. longisporum* was found in the hypocotyls, while the amounts of *V. longisporum* DNA decreased in the roots.

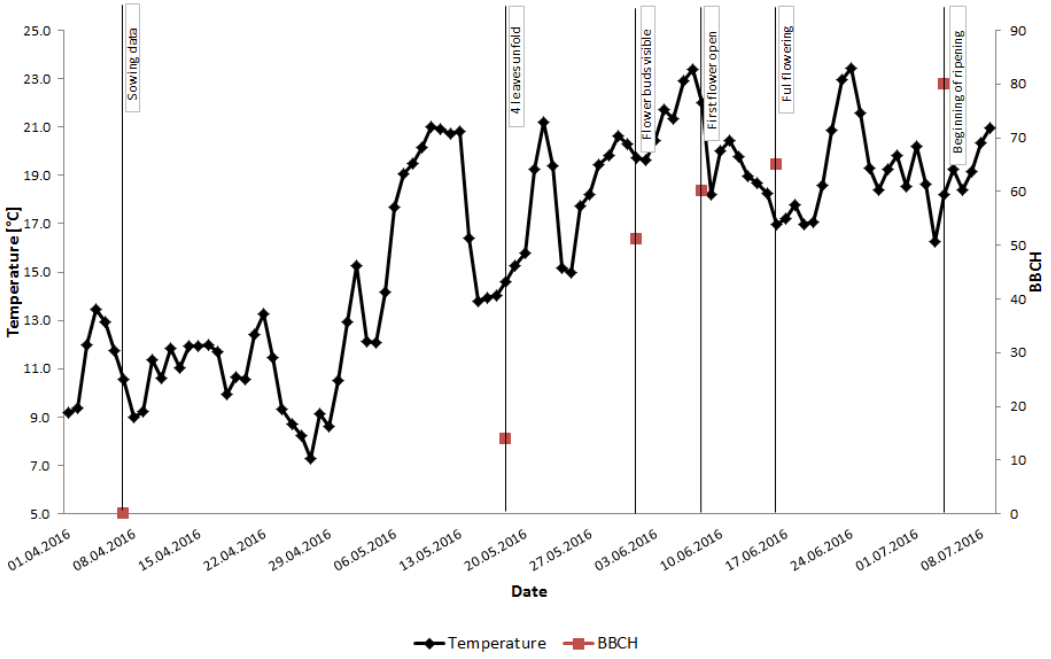

Figure 2.6 Soil temperatures in 5 cm depth in the growing season of spring oilseed rape in 2016 in Göttingen. Red dots indicate the growth stages of *Brassica napus* and the date of sample collection.

2.3.1.4 Salicylic acid in different plant tissues of spring oilseed rape

In contrast to winter oilseed rape, no strong induction of SA was observed after inoculation of *V. longisporum*. Both conjugated and free SA were slightly increased with growth of plants. At BBCH 55, a slight induction of conjugated SA was observed in the root and hypocotyl of the resistant cultivar, while in the susceptible cultivar, induction was only observed in the hypocotyl (Fig. 2.7A, C). No difference between control and *V. longisporum* inoculated plants was observed at BBCH 60 in all plant parts of both cultivars. At BBCH 80, a general increase in conjugated SA was found in the susceptible but not in the resistant cultivar (Fig. 2.7A, E). Free SA did not show any interesting increase or decrease in the plants inoculated with *V. longisporum*.

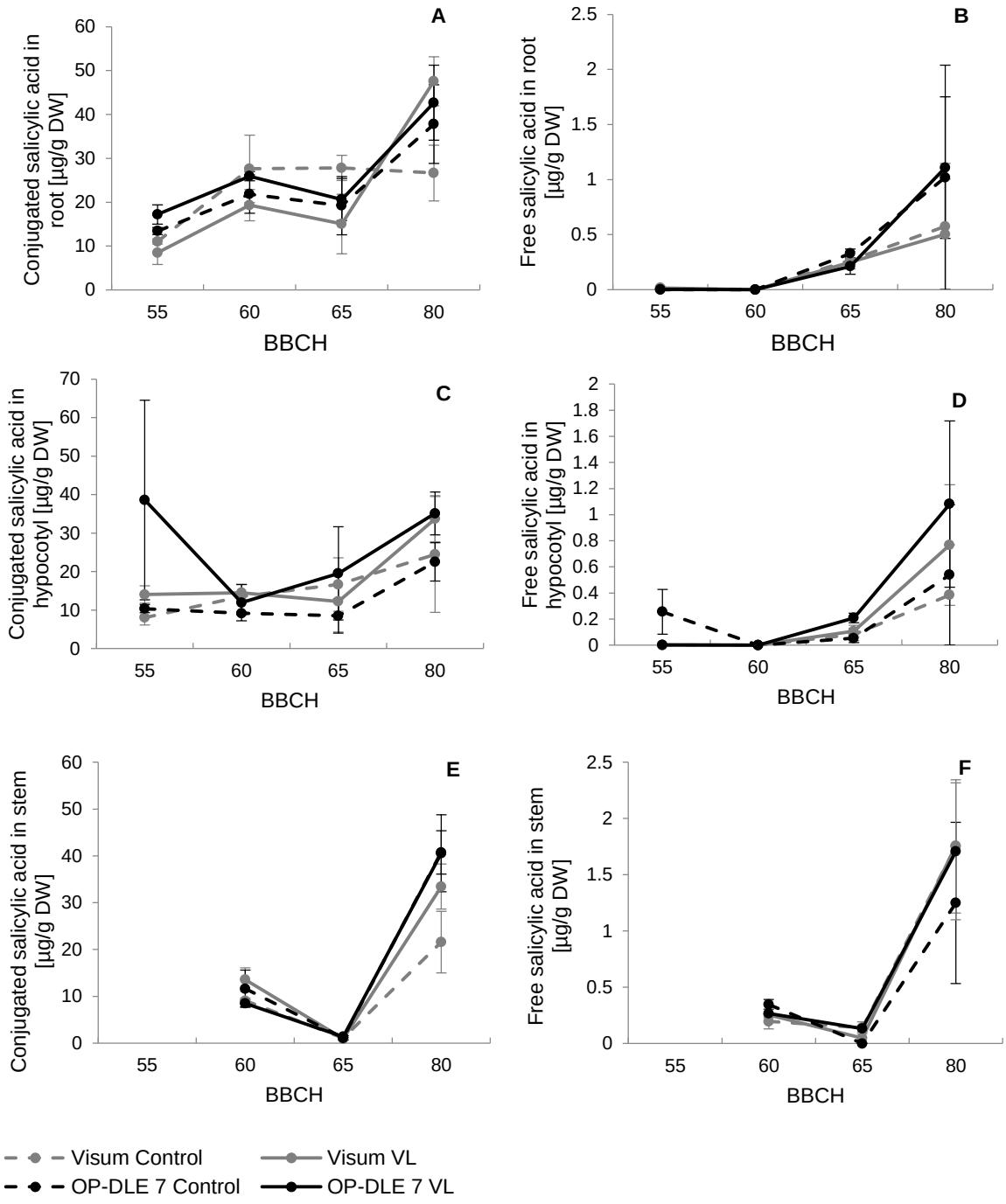

Figure 2.7 Conjugated (A, C, E) and free (B, D, F) salicylic acid content in root (A, B), hypocotyls (C, D) and stem (E, F) of spring oilseed rape (*Brassica napus*) at different growing stage. Bars indicate standard errors.

2.3.2 Disease assessment

2.3.2.1 Disease assessment on stubbles of winter oilseed rape after harvest

Significantly higher disease incidence and disease severity ($p<0.01$) were observed both in the roots and stems of the susceptible cultivar Falcon (86%, 2.78±0.07; 54%, 1.86±0.07) compared to the resistant cultivar SEM (59.5%, 1.98±0.07; 19%, 1.24±0.04). In addition, a higher proportion of root samples (~ 65%) from the susceptible cultivar

showed advanced development of microsclerotia under the epidermal and in pith tissues (Fig. 2.8). Among these samples, just half of them further contain high density of microsclerotia in the stems. In the resistant cultivar, although about 30% of the root samples were severely infected by *V. longisporum*, distribution of microsclerotia was largely restricted in the root tissues and only very scarcely (8%) reached the stems and formed high abundance of microsclerotia.

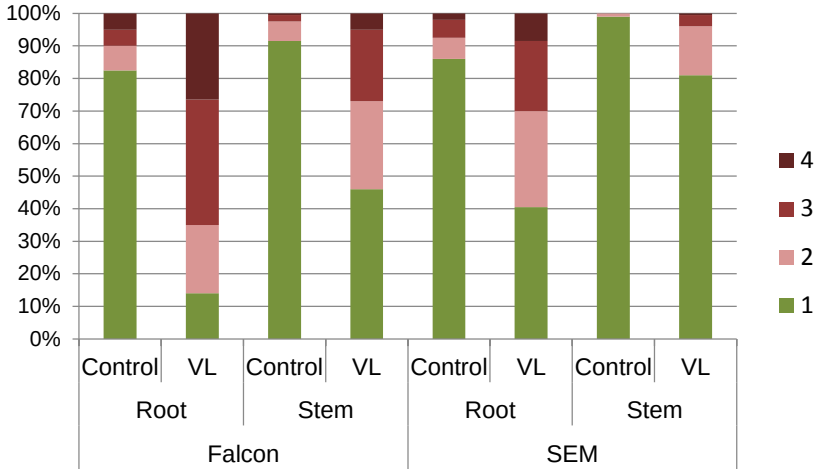

Figure 2.8 Disease severity (DS) on stubbles harvested from the field trial with winter oilseed rape in the season 2015/16. DS was assessed based on the amount of microsclerotia of *Verticillium longisporum* visible on the samples, in four classes: 1, healthy; 2, slight infection; 3, advance infection; 4, severe infection

2.3.2.2 Disease assessment on stubbles of spring oilseed rape after harvest

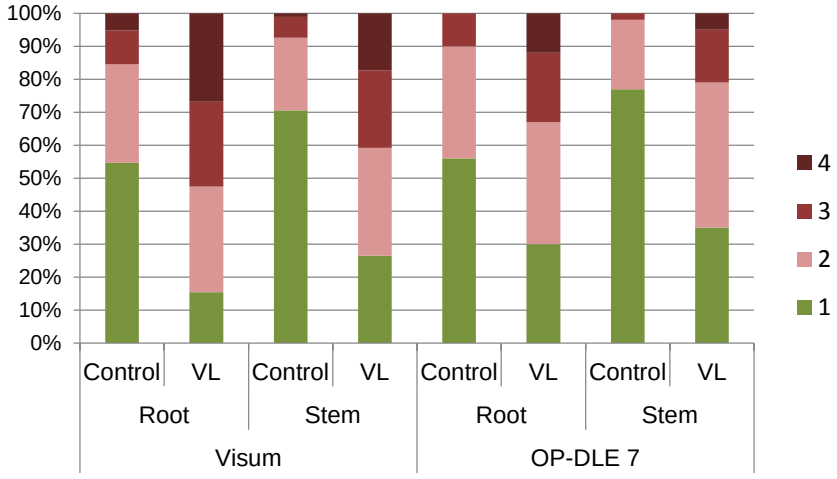

Figure 2.9 Disease severity (DS) on stubbles harvested from the field trial of spring oilseed rape in the season 2016. DS was assessed based on the amount of microsclerotia of *Verticillium longisporum* visible on the samples, in four classes: 1, healthy; 2, slight infection; 3, advance infection; 4, severe infection.

In spring oilseed rape, more samples from the susceptible cultivar Visum than the resistant cultivar OP-DLE 7 were infected by *V. longisporum* and formed microsclerotia in

the root (84% vs.70%) and stem (73% vs. 65%) tissues. Significantly higher disease severity ($p<0.01$) was observed in the roots of the susceptible cultivar (2.64±0.11) compared to the resistant cultivar (2.15±0.10). More than 50% of the collected samples from the susceptible cultivar showed a high density of microsclerotia on the subepidermal and in pith tissue of the roots. A slight reduction of disease severity on stems was found in the resistant cultivar (1.91±0.08), compared with the susceptible cultivar (2.32±0.11). However, compared with winter oilseed rape, more disease symptoms were found in stem tissues of spring oilseed rape. Therefore, the difference of disease severity on the stems and roots was not obvious (Fig. 2.9).

2.3.3 Yield and seed quality of winter oilseed rape

A 15% yield reduction was found in susceptible cultivar Falcon inoculated with *V. longisporum,* whereas yield in the resistant cultivar SEM was not affected (Fig. 2.10). However, *V. longisporum* infection had no effects on seed quality, i.e., content of protein, oil, fatty acid and glucosinolates, in both susceptible and resistant cultivars (Tab. 2.3).

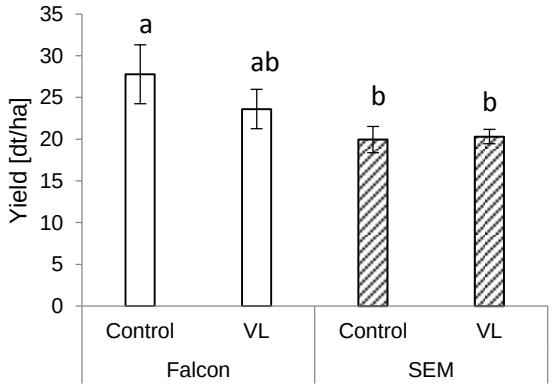

Figure 2.10 Yield of control and *Verticillium longisporum* inoculated winter type *Brassica napus* cv. Falcon (susceptible) and SEM (resistant) in the season 2015/16. Different letters indicate significant differences among the treatments (LSD test, $P<0.05$).

Table 2.3 Seed quality of control and *Verticillium longisporum* inoculated winter oilseed rape in the season 2015/16. Different letters indicate significant differences among each parameter (LSD test, $P<0.05$).

Treatment	Protein [%]	Oil [%]	Fatty acid			Glucosinolates [µmol/g DW]
			Oleic [% in oil]	Linolenic [% in oil]	Erucic acid [% in oil]	
Falcon						
Control	18.3±0.29 b	42.1±0.39 b	60.33±0.11 b	10.54±0.07 a	<2 a	10.82±0.34 a
VL	18.0±0.07 ab	42.4±0.15 b	60.00±0.11 b	10.60±0.05 a	<2 ab	10.95±0.29 a
SEM						
Control	18.6±0.03 a	43.2±0.12 a	63.02±0.24 a	10.32±0.03 b	<2 b	9.28±0.21 b
VL	18.7±0.04 a	43.3±0.09 a	63.16±0.09 a	10.35±0.03 b	<2 ab	9.19±0.27 b

2.4 Discussion

In the last decades, 'Verticillium stem striping' has become a severe threat to the main cropping regions of winter oilseed rape in Europe and as well of spring-sown Canola in Canada. However, a systematic monitoring of colonization and development of *V. longisporum* covering the whole growing stage is lacking. Different from artificial inoculation of *V. longisporum* under greenhouse conditions, which showed half leaf yellowing, vascular discoloration and stunting (Zeise and von Tiedemann 2002; Eynck et al. 2007; Floerl et al. 2008), up to BBCH 80, no such symptoms were observed in the field. Symptoms of 'Verticillium stem striping' in the field can be confused with natural senescence in the late developmental stages or discolorations of the epidermis induced by *Leptosphaeria maculans* (Phoma stem canker) unless massive microsclerotia are produced (Zhou et al. 2006). Therefore, qPCR is an essential tool for verification and quantification of *V. longisporum* in field-grown plants without visual symptoms (Knüfer et al. 2017). Similar to the results under greenhouse conditions described in previous studies (Zhou et al. 2006; Lopisso et al. 2017), *V. longisporum* was detected in the roots of field-grown winter oilseed rape before winter at BBCH 14. However, in the roots of field grown spring oilseed rape, *V. longisporum* was first detectable only at BBCH 60. From the aspect of time, two months were required for *V. longisporum* to get into the roots of spring oilseed rape, while only one month was needed to colonize the roots of winter oilseed rape. The stubbles used as inoculum were collected from the winter oilseed rape from last season in Fehmarn, Germany, which may lead to this different aggressiveness of *V. longisporum* toward winter and spring oilseed rape. However, once *V. longisporum* is established in the roots, less time was needed for the pathogen in spring oilseed rape to spread further into the hypocotyl and stem. The colonization of *V. longisporum* increased significantly in winter oilseed rape with an increased soil temperature of 1.6°C or 3.2°C (Siebold and von Tiedemann 2013). When the soil temperature was lower than 12°C, the growth of *V. longisporum* in both winter and spring oilseed rape was suppressed (unpublished). In the present study, the average soil temperatures were below 15°C during the growth stages of winter oilseed rape from BBCH 14 to BBCH 55. Therefore, the development of *V. longisporum* may have been delayed during this period. In contrast, soil temperature was consistently above 15°C enabling colonization of spring oilseed rape with *V. longisporum*. This indicates that soil temperature plays a crucial role in the spread of *V. longisporum* in plants. The flowering stage seems to be particularly important for *V. longisporum* to spread in both winter and spring oilseed rape plants, which was also found by Zhou et al. (2006) under controlled conditions in the greenhouse with root dipping inoculation of *V. longisporum*.

Clear differences were found in development of *V. longisporum* in the resistant and susceptible cultivars of winter oilseed rape. *Verticillium longisporum* was able to colonize the entire plant of both cultivars. However, the levels of *V. longisporum* DNA were significantly lower in the resistant cultivar. *Verticillium longisporum* was capable of spreading quicker in the susceptible winter oilseed rape from roots to stem, and could establish and develop well in the stem. In contrast, instead of stem, relative higher amounts of *V. longisporum* were found in the hypocotyl of the resistant cultivar, which indicates a restriction barrier existing in the hypocotyl against vascular spread of *V. longisporum* as described previously (Eynck et al. 2009). Symptom development in the greenhouse strongly correlates with fungal colonization of oilseed rape in the field as determined by qPCR at growth BBCH 80 (Knüfer et al. 2017). However, in contrary to winter oilseed rape, resistant and susceptible spring oilseed rape cultivars, which were selected from greenhouse screening for significant differences in susceptibility to *V. longisporum* (not shown), showed only slight differences in disease responses in the field trial. In both spring cultivars, *V. longisporum* colonized and accumulated quickly in the roots followed by the hypocotyl, but remained at low levels in the stem. After harvest, *V. longisporum* was able to form microsclerotia in the entire plant, with differences between susceptible and resistant cultivars being well detectable. However, in contrast to the qPCR data at BBCH 79-80, roots of susceptible winter oilseed rape at harvest were more severely infected by *V. longisporum* microsclerotia than stem. In spring oilseed rape, post-harvest formation of microsclerotia in the roots and stem did not differ as much as indicated by the qPCR data at BBCH 80. Taken together, these results suggest that the final formation of microsclerotia on the stubbles is not completely based on the colonization level of *V. longisporum* before ripening.

Infection with *V. longisporum* induced increased endogenous levels of free and conjugated SA under greenhouse conditions (Ratzinger et al. 2009; Kamble et al. 2013). In the present study, an increase of conjugated SA was observed in the early stage of *V. longisporum* infected resistant oilseed rape under field conditions. Conjugated SA was increased later in susceptible cultivar as well. Accordingly, an early response of SA may be necessary in the resistance of *B. napus* to *V. longisporum*. However, once the pathogen is established, the increase of SA was no more sufficient as a defense response. At BBCH 55, even a higher level of conjugated SA was recorded in the susceptible winter type cultivar, while free SA remained in a low level. In this stage, plants trend to convert free SA into conjugated form and the higher level of conjugated SA in susceptible cultivar seems to be an indicator for susceptibility. Furthermore, a strong reduction of SA content was observed during the flowering stage, which confirmed that SA

may play a role in flowering (Martínez et al. 2004). Higher accumulation of SA may happen in bud but not in other parts of the plants. Besides, lacking of SA may contribute to the spread of *V. longisporum* in this growing stage. In contrast to winter oilseed rape, instead of decreasing, levels of free SA were increased with the time. However, no clear difference was found between two genotypes of spring oilseed rape. Phloem is used for endogenous synthesis and allocation of SA, while xylem may contribute to long-distance transport of SA toward distal parts of the plants for response to disease (Rocher et al. 2006; Ratzinger et al. 2009). The highest level of SA was recorded in the *V. longisporum* infected hypocotyls of winter type oilseed rape.

Previous studies found yield reductions of single plant from 20% to 80% in greenhouse and assumed that a severe overall yield loss ranging from 10% to 50% could occur when 60% of plants have severe disease symptoms (Paul 2003; Dunker et al. 2008). In the present study, susceptible winter oilseed rape, 65% of which was affected by severe *V. longisporum* infection, had 15% total yield loss. However, the disease incidence of the resistant cultivar was less than 55%, of which single plant losses may be compensated by the whole plant stand avoiding an overall yield reduction that confirms previous studies on the compensation of damage to oilseed rape (Tatchell 1983; Dunker et al. 2008).

As described by Rygulla et al. (2007), numbers of zero erucic acid re-synthesized oilseed rape showed resistance to *V. longisporum* and were combined with variable seed glucosinolates content, among which lines with zero erucic acid and moderate seed glucosinolates content are ideal for quantitative resistance against *V. longisporum*. Although both of resistant cultivar breeding line SEM and susceptible cultivar Falcon produced seeds in ‘double low’ quality, seeds of the susceptible cultivar have higher glucosinolates content, which could be one of the reasons leading to its susceptibility. However, both erucic acid and glucosinolates content in seeds were not affected by the infection of *V. longisporum*. Furthermore, other seed quality indicators, such as oil content and its composition, were also not affected by *V. longisporum* infection, which confirms the previous study described by Dunker et al. (2008).

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Chapter 3: Seed transmission of *Verticillium longisporum* in oilseed rape (*Brassica napus* L.)

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Abstract: *Verticillium longisporum* is a soil-borne vascular pathogen, which has become an increasing threat to oilseed rape production in the last decades. Although this pathogen is presently wide spread in Europe and Canada, no clear evidence for its dissemination by seed transmission has been provided so far. To evaluate the potential of seed transmission of *V. longisporum*, resistant and susceptible cultivars of oilseed rape were artificially inoculated with *V. longisporum*. A DsRed labeled isolate of *V. longisporum* was used to verify the presence of seed transmission. Fungal spread in plants from greenhouse and field experiments was followed with qPCR. The incidence of *V. longisporum* in seeds *in vitro* grown on PDA or SNA medium ranged from 0.08% to 13.3%. Among three tested cultivars, the highest incidence of seed transmission was recorded in the susceptible cultivar Falcon. However, in the second generation from these seed lots, only about 1% of plants were colonized by microsclerotia on the stem. Besides, small amounts of *V. longisporum* DNA were also detectable in the seeds harvested from diseased field-grown plants by qPCR, although it was not successful to yield any *V. longisporum* isolates from these seeds in the *in vitro* test.

Keywords: winter oilseed rape, VL43, DsRed, *in vitro*, qPCR, field, greenhouse, second generation

3.1 Introduction

Verticillium species are soil-borne ascomycetes, most of which frequently cause Verticillium wilt disease performing wilting, stunting, chlorosis, vascular discoloration and premature senescence on more than 200 plant species. In contrast to *V. dahliae* which has a relatively wide host range, *V. longisporum, V. albo-atrum*, *V. alfalfa* and

Zheng X, Lopisso DT, Eseola AB, Koopmann B, von Tiedemann A (2019). Potential for seed transmission of *Verticillium longisporum* in oilseed rape (*Brassica napus* L.). Plant Disease 103 (8): 1843-1849. DOI: doi.org/10.1094/PDIS-11-18-2024-RE.

V. nonalfalfae have more restricted host ranges (Fradin and Thomma 2006; Johansson et al. 2006; Inderbitzin et al. 2011b; Depotter et al. 2016). The taxonomic history of *V. longisporum* is complicated. Due to the similarity in morphology, *V. longisporum* was first described as a variety of *V. dahliae* (Stark 1961). Thirty-seven years later, *V. longisporum* was first distinguished from *V. dahliae* and elevated to be a distinct species due to its elongate shape of microsclerotia, longer size of conidia, lacking activity of extracellular polyphenol oxidase and culture filtrate fluorescence, and its host specificity on Brassicaceae (Stark 1961; Karapapa et al. 1997; Bhat and Subbarao 1999; Zeise and von Tiedemann 2001, 2002; Steventon et al. 2002; Inderbitzin et al. 2011a). *Verticillium longisporum* is considered as an amphidiploid hybrid resulting from hybridizations at least including four different ancestors, the unknown species A1, D1 and *V. dahliae* D2, D3, forming three different hybrid lineages A1/D1, A1/D2 and A1/D3 (Inderbitzin et al. 2011b; Inderbitzin and Subbarao 2014). These lineages have different pathogenicity and virulence to various Brassicaceae. Maybe due to hybridization, *V. longisporum* and *V. dahliae* do not clearly differ in their host ranges. Both of them were reported to be equally virulent on non-brassicaceous plants, such as eggplant, horseradish, tomato and lettuce (Steventon et al. 2002; Babadoost et al. 2004; Novakazi et al. 2015). However, *V. longisporum* is more aggressive on brassicaceous hosts than *V. dahliae*. Lineage A1/D1 is the most pathogenic lineage on oilseed rape (Novakazi et al. 2015). The first report of oilseed rape as host of *V. longisporum* originates from West and South of Scania, southern Sweden in 1969 (Kroeker 1970).

Verticillium longisporum is one of the major pathogens of oilseed rape and well established in main oilseed rape producing regions, except Asia, where this pathogen has been only reported from Chinese cabbage (Karapapa et al. 1997; Gladders et al. 2011; Zeise and von Tiedemann 2002; Yu et al. 2015). The infection process of *V. longisporum* is highly similar to *V. dahliae*, both of which are monocyclic vascular diseases (Eynck et al. 2007; Klosterman et al. 2011). Under field conditions, *V. longisporum* germinates from microsclerotia and colonizes the roots of oilseed rape already before winter (unpublished data). 'Verticillium stem striping' occurs only in late growth stages of oilseed rape shortly before the onset of crop maturity (Heale and Karapapa 1999). Shortly before harvest, black pigmented microsclerotia are formed under the epidermis and in the stem pith (Eynck et al. 2009). Yield losses caused by *V. longisporum* infection are estimated to range between 10% and 50% with high disease incidence (Eastburn and Paul 2007; Dunker et al. 2008). However, no significant effect was observed so far on seed quality, such as seed weight, oil content and oil composition (Dunker et al. 2008).

While the modes of spreading of *V. longisporum* in oilseed rape are well described in general (Zeise and von Tiedemann 2001; Eynck et al. 2007), the specific aspects of pathogen transmission from on to the next season on field require closer investigation. As described by Zhou et al. (2006), no *V. longisporum* was isolated from seeds harvested from disease plants. Although no clear evidence for seed transmission of *V. longisporum* has been provided so far, seed transmission of *V. dahliae* in olive, cotton eggplant, tomato, spinach and lettuce was verified by molecular analysis or *in vitro* test, and has been reported to show a range from 16% to 66% infection incidence of seeds harvested from diseased plants (Kadow 1934; Snyder and Wilhelm 1962; Vallad et al. 2005; Karajeh 2006; Göre et al. 2011). Vascular fungi, such as *V. dahliae* and *Fusarium oxysporum*, cause systemic infection potentially reaching the seeds via the vascular system be dependent on genotype (Karajeh 2006). However, the precise location of *V. dahliae* in seeds, i.e. embryo, endosperm or cotyledons has not yet been explored.

In recent years, *V. longisporum* has more often been reported to appear in previously uninfected regions. Thus seed transmission could be a potential means for long distance spread of this pathogen. Understanding the seed transmission of *V. longisporum* in oilseed rape is essential for understanding the epidemiology of 'Verticillium stem striping'. The objectives of the present study were (1) to explore whether *V. longisporum* may be transmitted via seeds, (2) to assess the epidemic potential of *V. longisporum* infected seed under greenhouse and field conditions, and (3) to estimate the probability of disease transmission into the next generation.

3.2 Materials and methods

3.2.1 Plant material

Three winter oilseed rape cultivars, *B. napus* L. cv. Falcon, Visby (Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, NPZ, Hohenlieth, Germany) and Treffer (KWS Saat SE, Einbeck, Germany) were used. Cultivar Falcon is a German commercial variety, which is susceptible to *V. longisporum*. Seeds of the three cultivars were surface sterilized with 70% ethanol for 2 min and subsequently rinsed twice with sterilized ddH_2O. Seeds were then sown in multipot trays filled with quartz sand and grown in the greenhouse at a temperature of 22±2˚C for 14 days before vernalization for 10 weeks at 4°C. During vernalization, 3 g/l Hakaphos® blau was applied twice as fertilizer.

To evaluate seed transmission of *V. longisporum* under field conditions, eight plants of Aristoteles (Limagrain GmbH, Germany) with severe *V. longisporum* disease symptoms were collected from an experimental field in the northwestern part of Göttingen, Lower

Saxony, Germany (51°33'31.9'' N, 9°55'09.8'' E) in the season 2016/17. Seeds collected from individual plants were used for analysis.

3.2.2 Fungal preparation and inoculation

Verticillium longisporum isolate VL43 obtained from a diseased *B. napus* plant and a DsRed labeled VL43 isolate (Eynck, unpublished data) were used for the experiment under greenhouse conditions. Conidial suspension of VL43, which had been kept in 25% glycerol at -80˚C, was used to initiate fresh cultures. For preparation of the fungal culture, stock conidial suspension was plated on potato dextrose agar containing 250 ppm streptomycin, and incubated at 22˚C for 10 days. Conidia were harvested by flooding the plate with sterilized ddH_2O and sweeping the colony surface with a sterile loop. The resulting suspension was filtered through sterile gauze to remove mycelia.

Roots of vernalized seedlings were washed and cut with sterile scissor, and subsequently inoculated by dipping in 1 x 10^7 cfu/ml of conidia suspension or water for 30 min. The treated plants were replanted in plastic pots (1.5 l) with sterile soil-sand mixture (3:1) and kept in the greenhouse with a 16 h photoperiod and a temperature of 22±2˚C.

3.2.3 Assessment of disease development in the first and second generation

Disease severity (Tab. 3.1) and plant height were quantified in the greenhouse weekly from 14 to 49 dpi as described by Eynck et al. (2009).

Table 3.1 Assessment key for scoring foliar symptoms induced by *Verticillium longisporum* on *Brassica* species inoculated with root dip method (Eynck et al. 2009).

Score	Symptom development
1	No symptoms
2	Weak symptoms on the oldest leaf (yellowing, black veins)
3	Weak symptoms on the next younger leaves
4	About 50 % of the leaves have symptoms
5	More than 50 % of the leaves have symptoms
6	Up to 50 % of the leaves are dead
7	More than 50 % of the leaves are dead
8	Only apex is still alive
9	The plant is dead

The area under the disease progress curve (AUDPC) was conducted and calculated from the disease severity according to the formula followed:

$$\mathrm{AUDPC} = \sum_{i=1}^{n} \left(\frac{y_i + y_{i+1}}{2}\right) \times (t_{i+1} - t_i)$$

Where y_i is the disease severity score for observation number *i*, t_i is the corresponding number of days post inoculation of an observation, and *n* is the number of observations.

After harvest, visual and microscopic investigations for the presence and intensity of microsclerotia on subepidermal and in pith tissue of stems were conducted to determine disease severity on plants according to the following assessment key with four scales (Tab. 3.2).

Table 3.2 Assessment key for evaluation of *Verticillium longisporum* disease severity on stubble of *Brassica* spp. (Knüfer et al. 2017).

Score	Description
1	Healthy tissue: no microsclerotia visible in tissue
2	Slight infection: low abundance of microsclerotia
3	Advanced infection: high abundance of microsclerotia
4	Severe infection: tissue heavily infested with microsclerotia, epidermis is peeling off from the stubble

Based on these score and its number of plants, a disease index (DI) was calculated for each accession as follows:

$$\mathrm{DI} = \frac{(1 \times \mathrm{N1}) + (2 \times \mathrm{N2}) + (3 \times \mathrm{N3}) + (4 \times \mathrm{N4})}{(\mathrm{N1} + \mathrm{N2} + \mathrm{N3} + \mathrm{N4})}$$

where *Ni* is the number of plants in the respective score category.

3.2.4 Quantification of *V. longisporum* by qPCR

Hypocotyl, pod (BBCH 80) and seed (BBCH 99) samples were lyophilized or air-dried and ground to fine powder. Total DNA was extracted from about 50 mg or 100 mg ground sample using the cetyltrimethylammonium bromide (CTAB) method (Brandfass and Karlovsky 2008). The samples were homogenized in 1 ml CTAB with 2 µl *ß*-mercaptoethanol and 1 µl 1x proteinase K. The mixture was sonicated for 5 s and incubated for 10 min at 42°C, and subsequently for 10 min at 65°C. For degradation of RNA, 1.5 µl RNAse was added and incubated for 15 min at 37°C. For isolation of DNA from the mixture, 800 µl chloroform: isoamyl alchol (24:1) was added and incubated on ice for 10 min. After centrifugation at 12,000 rpm for 10 min, the supernatant was mixed in 200 µl 30% PEG and 100 µl 5 M NaCl for precipitation. The pellet after centrifugation for 5 min was washed with 70% ethanol. The washed pellet was dried in a centrifugal concentrator for 10 min at 30°C and dissolved over night in 100 µl TE buffer at 4°C.

The extracted total DNA stained with Midori Green was qualified with 1% agarose gel electrophoresis. A CFX384 real-time PCR detection system (Bio-Rad laboratories, Inc.) was used for the amplification and quantification of *V. longisporum* DNA using *ß*-tubulin primers forward (*5'-GCAAAACCCTACCGGGTTATG-3'*) and reverse (*5'-AGATATCCATCGGACTGTTCGTA-3'*) (Debode et al. 2011). The amplification mix

consisted of 5 µl premix (qPCRBIO SyGreen Mix Lo-Rox, Nippon Genetics Europe GmbH), 0.4 µM of each primer and 1 µl of template DNA and filled up to a total volume of 10 µl with ddH_2O. The PCR program considers a 3 min initial denaturation step, followed by 40 cycles with a denaturation step (5 s at 95°C), annealing (15 s at 68°C) and subsequent extension (15 s at 72°C). Fluorescence was detected after each elongation step and the program was completed with a final elongation step of 2 min at 72°C. Melting curve analysis was performed with 95°C for 10 s, followed by a 55°C step for 10 s and subsequent measurements within a range of 65°C to 95°C with every 5 s in 0.5°C temperature increments. A PCR for all treatment samples was performed with three biological and three technical replicates and data were analyzed using CFX Manager Software (Bio-Rad laboratories, Inc.).

3.2.5 *In vitro* test for seed transmission

For *in vitro* test of seed infection, 450 seeds per cultivar harvested from greenhouse grown plants and 280 seeds per plant collected from the experimental field were surface sterilized with 3% sodium hypochlorite for 2 min. Sterilized seeds were plated on synthetic nutrient-poor agar (SNA) and semi-*Verticillium*-selective medium, respectively, and incubated at 23°C in the dark for 28 days. Presence of *V. longisporum* was confirmed under the light microscope. To verify the observation, a Leica Leitz DMRB fluorescence microscope with a bandpass Cy3.5/DsRed filter system (excitation filter: 565 nm/30 nm, emission filter: 620 nm/60 nm, AHF Analysentechnik AG, Thübingen, Germany) was used for samples inoculated with the DsRed expressing strain.

3.2.6 Disease transfer to the second generation

About 360 seeds per cultivar consisted of three replicates were conducted for analysis of disease development in the second generation. Seeds harvested from inoculated plants were surface sterilized with 3% sodium hypochlorite for 2 min and sown in multipot trays filled with a sterile soil-sand mixture (3:1). Plants were vernalized as described above and transplanted into pots (11x11x12 cm) filled with sterile soil-sand mixture (3:1). According to the described assessment key (Tab. 3.2), the formation of microsclerotia on the stem after ripening was used to estimate the disease severity.

3.2.7 Statistical analysis

All data were analyzed using XLstat2017. Before testing for statistical significance, a probability plot with Shapiro-Wilk test was used to test the normality of the data. Suitable data were analyzed by factorial ANOVA, and Fisher LSD test was used for multiple comparisons. Whenever the data did not fit to normal distribution, Kruskal-Wallis test (two-tailed) and Conover-Iman procedure at P=0.05 were applied for multiple pairwise

comparisons among treatments. The experimental results were presented as means ± standard error at 5% significance level.

3.3 Results

3.3.1 Disease development in the first generation

Typical symptoms of *V. longisporum* infection in *B. napus* under greenhouse conditions, such as leaf yellowing, vein blackening, senescence of leaves, stunting and formation of microsclerotia were observed on plants inoculated with *V. longisporum*, whereas no such symptoms were present in mock-inoculated plants.

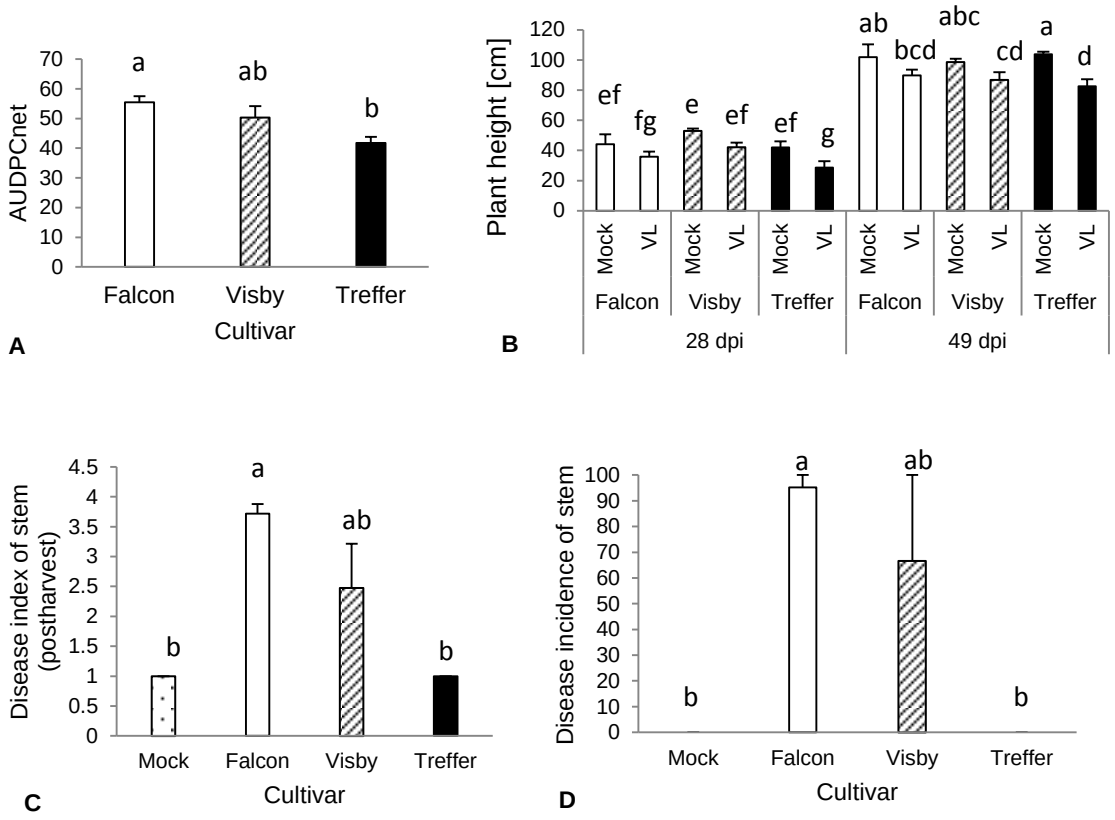

Figure 3.1 Response of three winter oilseed rape cultivars to *Verticillium longisporum* under greenhouse conditions determined by disease severity (A), plant height (B), disease index of stem postharvest (C) and disease incidence of stem (D). Mock, water control; VL, inoculated with *V. longisporum.* Bars indicate standard errors. Different letters indicate significant differences among the treatments (LSD test, $P<0.05$).

The first disease symptoms observed in the present study occurred at 14 dpi on the susceptible cultivars Falcon and Visby followed by Treffer at 21 dpi. By 28 dpi, more than 80% of inoculated plants had abnormal discoloration of leaves. Less net AUDPC calculated from 14 to 49 dpi was found in the resistant cultivar Treffer (Fig. 3.1A). However, in contrast to net AUDPC, a significant reduction of plant height was observed at 28 and 49 dpi (BBCH 80) on Treffer, when Falcon and Visby also revealed a slight stunting effect due to infection with *V. longisporum* (Fig. 3.1B). Microsclerotia formation

was recorded after harvest. None of the mock-inoculated plants and the Treffer plants showed any microsclerotia (Fig. 3.1C, D), while dense formation of microsclerotia was found under the epidermis and in the stem pith of the susceptible cultivars.

3.3.2 Quantification of *V. longisporum* in hypocotyls, pods and seeds of inoculated plants (first generation)

In hypocotyls and pods of plants grown in greenhouse conditions, *V. longisporum* was qualified and quantified by qPCR. In contrast to mock inoculation, *V. longisporum* was detectable in hypocotyls and pods of diseased plants (Fig. 3.2A, B). However, in seed samples, *V. longisporum* was found by qPCR only in the susceptible cultivars Falcon and Visby and not in the resistant cultivar Treffer (Fig. 3.2C).

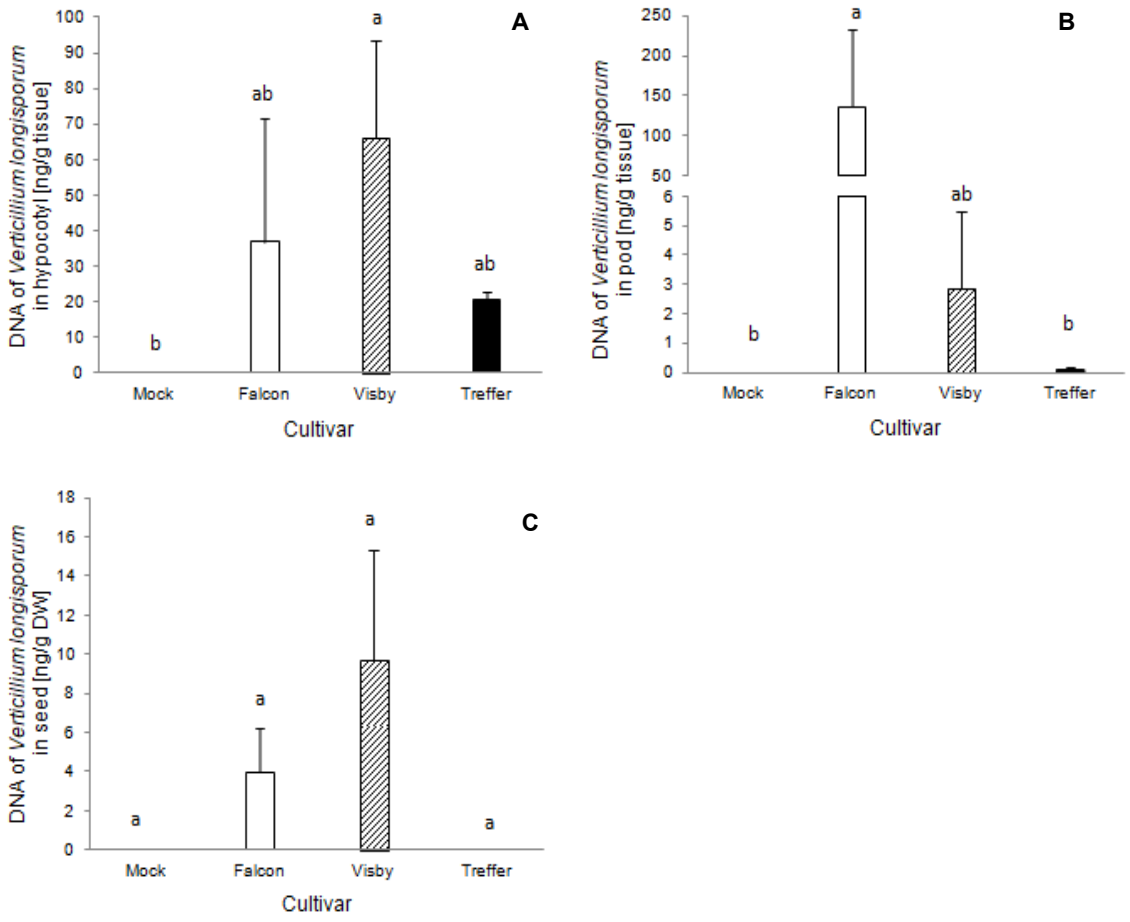

Figure 3.2 Quantification of *Verticillium longisporum* DNA in hypocotyls (A), pods (B) and seeds (C) of winter oilseed rape artificially inoculated with *V. longisporum* in the greenhouse. Bars indicate standard errors. Different letters indicate significant differences among the treatments (Kruskal-Wallis test, Conover-Iman procedure for multiple pairwise comparisons, $P<0.05$).

Seed samples that were harvested from diseased plants had a lower seed quality as seeds were smaller and slightly wrinkled. Besides, a reduction of thousand seed weight was found in the susceptible cultivar Falcon compared to mock-inoculated (not shown). In

the hypocotyl, a slight but insignificant increase in biomasses of *V. longisporum* was observed in the susceptible cultivars. Similar amounts of *V. longisporum* were found again in the seeds. However, the levels of *V. longisporum* DNA were about 10-fold lower. Except for Falcon, all inoculated plants had lower amounts of *V. longisporum* in the pods than in the hypocotyls. Furthermore, a significant difference of *V. longisporum* biomass between the susceptible and resistant cultivars was observed in the pods.

3.3.3 *In vitro* test for seed transmission

In order to determine incidence of seed transmission of *V. longisporum*, *in vitro* tests of surface sterilized seeds were conducted. No fungal growth was observed from seeds of mock-inoculated plants at 10 dpi, while white colonies were isolated from some seeds harvested from diseased plants. These colonies were further developed and formed dark pigmented microsclerotia (Fig. 3.3). Verticillate conidiophores and elongated conidia were observed under the light microscope.

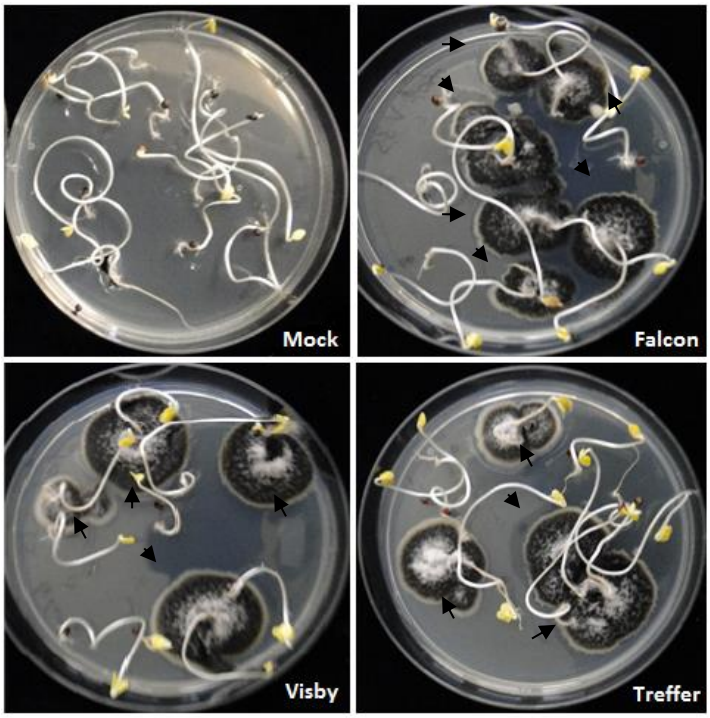

Figure 3.3 *In vitro* plate assay for determination of seed transmission of *Verticillium longisporum* in three winter oilseed rape cultivars infected with a DsRed-labeled strain of *V. longisporum* under greenhouse conditions. Surface sterilized seeds were incubated on SNA for 28 days at 23^{o}C in the dark. Typical *V. longisporum* mycelial colonies with dark microsclerotia are marked with arrows.

To further verify the identity of *V. longisporum*, seeds harvested from DsRed *V. longisporum* infected plants were tested *in vitro* on SNA medium as well. Colonies with similar character as described above were observed. Under fluorescence microscope with a Cy3.5/DsRed filter system, hyphae and conidia emitting red fluorescence were found.

Dark pigmented microsclerotia were also found among dense fluorescent hyphae (Fig. 3.4A, B). Thus, isolates from surface sterilized seeds were confirmed to be the identical *V. longisporum* strain, which had been inoculated to the plants.

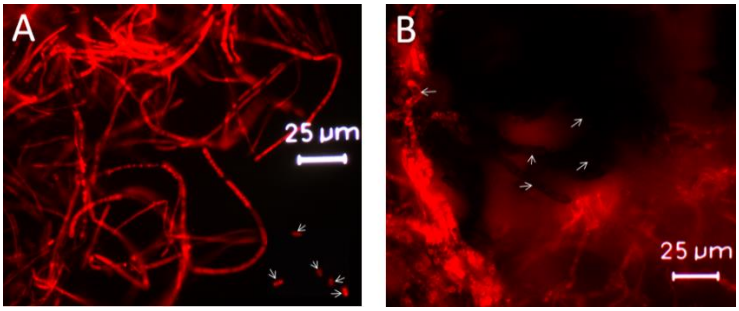

Figure 3.4 Microscopic image of DsRed labeled *Verticillium longisporum* isolated from seeds of diseased winter oilseed rape cultivars under greenhouse conditions. (A) Hyphae of *V. longisporum* with conidia (arrows). (B) Hyphae of *V. longisporum* with microsclerotia (arrows).

Around 13% and 12% of seeds harvested from infected susceptible cultivar Falcon showed positive presence of *V. longisporum* isolate VL43 and the DsRed labeled strain of VL43, respectively (Tab. 3.3) followed by Visby with lower frequency of infection. In contrast to the results of qPCR analysis, in the *in vitro* tests small scale of seeds obtained from the resistant cultivar Treffer were tested positive for the presence of *V. longisporum*.

Table 3.3 Frequency of *Verticillium longisporum* infection in seeds of winter oilseed rape cultivars inoculated with *V. longisporum* isolate VL43 and DsRed labeled VL43 under greenhouse conditions.

Cultivar	**Seed infection frequency [%]**		
	Mock	VL43	DsRed labeled VL43
Falcon	0	13.3	11.71
Visby	0	3.11	5.85
Treffer	0	0.08	3.17

3.3.4 Disease development in the second generation

A small percentage of plants grown up from seeds obtained from diseased plants inoculated in the greenhouse showed severe formation of microsclerotia in the roots and under epidermis and in the pith of stems among all tested cultivars (Fig. 3.5B). Although these plants showed high disease severity, the overall disease index was low (Fig. 3.5A), because it was compromised by the large number of non-infected healthy plants. Compared to the *in vitro* analysis, a lower number of seeds infected by *V. longisporum* further showed development of 'Verticillium stem striping' symptoms in mature plants under greenhouse conditions. Furthermore, unlike the *in vitro* test of seed transmission, no significant difference was found among three tested cultivars.

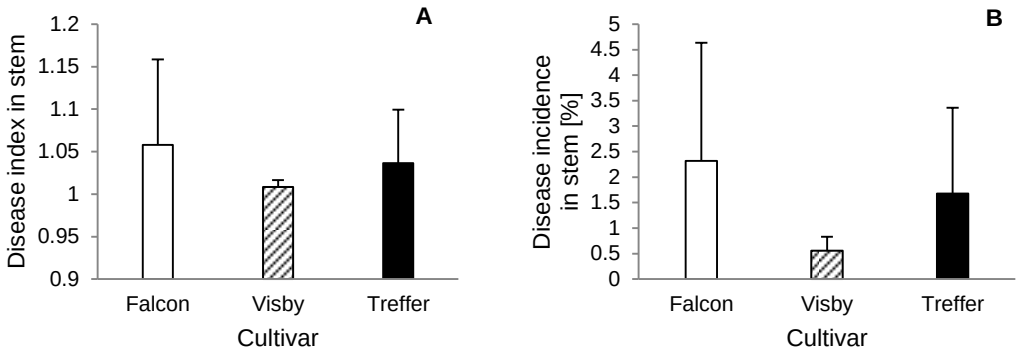

Figure 3.5 Disease development of *Verticillium longisporum* in the second generation of winter oilseed rape cultivars (first generation) that were inoculated with *V. longisporum* under greenhouse conditions. Disease index (A) and disease incidence (B) in stem are shown. Bars indicate standard errors. No significant difference was found among cultivars (Kruskal-Wallis test, Conover-Iman procedure for multiple pairwise comparisons, $P<0.05$).

3.3.5 Quantification of *V. longisporum* in seeds from field-grown *B.napus*

Seeds were harvested individually from eight field grown plants with severe disease symptoms. *Verticillium longisporum* was detected by qPCR on all of these seed samples (Fig. 3.6). However, the amounts of *V. longisporum* in seeds were variable and did not correlate with disease severity observed on stems. Interestingly, diseased plants collected from the field had similar levels of disease severity with regard to the formation of microsclerotia on stems as artificially inoculated greenhouse grown plants, whereas clearly less biomass of *V. longisporum* was detected in seeds collected from field grown plants.

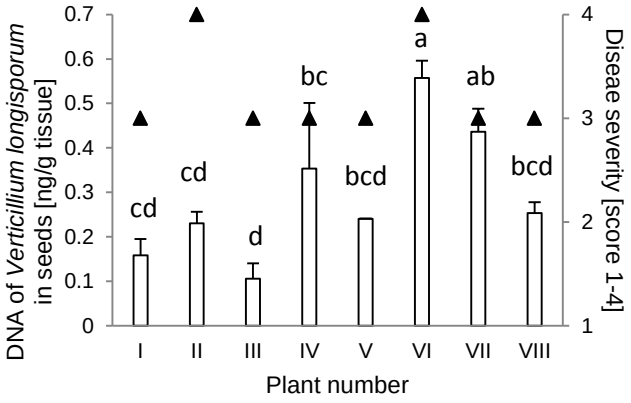

Figure 3.6 Quantification of *Verticillium longisporum* biomass in seeds of eight field grown diseased winter oilseed rape plants. The triangles present the disease severity of collected plants and columns present biomass of *V. longisporum*. Bars indicate standard errors. Different letters indicate significant differences among the treatments (LSD test, $P<0.05$).

Although quantification of *V. longisporum* in the seed samples from field showed positive results using qPCR, re-isolation of *V. longisporum* from these samples by *in vitro*

incubation failed due to the overgrowth of unwanted fungi, such as *Penicillium* spp., *Cladosporium* spp. and *Fusarium* spp. etc.

3.4 Discussion

Seeds are the basic units in plant production and may play a vital role in transmission of pathogens from one season to the next or from one location to another. Oilseed rape represents the second biggest production of world oilseed supply with frequent imports and exports among Europe, Asia and America on the world trading market (USDA 2017). Seed transmission of *V. longisporum* is a potential medium which allows for the local or long distance spread of 'Verticillium stem striping'. As *V. longisporum* grows systemically in the plant vasculature, it may also invade the seeds and cause internal seed-borne infection or a contamination around the seeds. Previously, one study has reported that no fungal contamination from *V. longisporum* was observed in seed coats or seedling (Zhou et al. 2006). In contrast, external seed contamination was confirmed by a more recent study (Gladders et al. 2013). In addition to this result and due to its similarities to *V. dahliae*, which has been confirmed to be partly seed-borne in several crops, transmission of *V. longisporum* via seeds seems to be a possible option.

In the present study, a seed test on *V. longisporum* and its DsRed labeled strain under sterile and controlled conditions confirmed that *V. longisporum* is able to establish in seeds as a result of systemic colonization of root-inoculated plants. The pathogen was also isolated from some seeds of the non-symptomatic resistant cultivar. The incidence of *V. longisporum* infection in the present study ranged from 0.08% to 13.3%, which was much lower than the incidence of seed transmission of *V. dahliae* in lettuce (Vallad et al. 2005). Nevertheless, even low proportions (~1%) of seeds harvested from diseased plants could be the source of 'Verticillium stem striping' disease in another field in the next season, where there has been no previous history of *V. longisporum*. Although plating is an easy-to-be-used method, a low detection power which can result in a high proportion of false negatives is the disadvantage of this commonly used method (Karajeh 2006). An optimized qPCR with specified primers is a more sensitive method to detect and quantify the very small amounts of fungus (Nazar et al. 1991; Knüfer et al. 2017). In contrast to the greenhouse experiment, no seed transmission could be observed from seeds obtained from plants infected under field conditions, which may be attributed to the variability in the environment (Sackston 2009). The slower growth of *V. longisporum* compared to other fungi represents a general difficulty to detect and isolate the pathogen from seeds harvested from field grown plants. However, qPCR analysis indicated the presence of *V. longisporum* in seeds from field-grown oilseed rape. Using sterilized seed samples obtained from diseased plants for qPCR detection may lead to a higher estimation of

V. longisporum amounts, because fungal biomass externally attached to the seed surface may produce qPCR signals without representing biologically active material.

This is the first report on the potential of seed transmission of *V. longisporum* in oilseed rape. As described in previous studies, *V. longisporum* is a vascular pathogen, which directly penetrates the root cortex and grows towards xylem vessels (Eynck et al. 2007; Depotter et al. 2016). Fungal hyphae occupy the xylem and spread upwards towards the top of the plants (Eynck et al. 2007; unpublished data). *Verticillium longisporum* may establish in pods of oilseed rape already before the onset of maturity. Similar to seed transmission of *V. dahliae* in spinach, seeds of oilseed rape may be infected systemically by *V. longisporum* via the vascular system (du Toit et al. 2005). However, further studies are required to support this assumption and to determine the exact location of *V. longisporum* in the seed.

Besides the local and long distance spread of 'Verticillium stem striping' in oilseed rape, seed-borne *V. longisporum* may have a direct economic impact on oilseed rape production by producing a high proportion of abnormally small seeds. One study from Dunker et al. (2008) showed that there is no effect of *V. longisporum* infection on the thousand seed weight under greenhouse conditions, but yield losses could be observed on individual plants. In contrast, 12% to 24% reduction of thousand seed weight was observed in several commercial fields, when more than 50% disease incidence occurred on stems (Gladders et al. 2013).

The lower disease severity and lower amounts of *V. longisporum* biomass in pods of the resistant cultivar indicate that breeding for resistant cultivars is also a good strategy to reduce the risk of seed transmission. This has been demonstrated for *V. dahliae* in cotton preciously (Göre et al. 2011). However, in our greenhouse trial, *V. longisporum* was able to colonize seeds of both the susceptible and the resistant cultivars, and to cause disease in the second generation without any differences made between cultivars.

Seed samples containing abnormally small seeds should be prevented from being sown in the field. During storage of harvested seeds or home-saved seeds, moderate temperature, which has no effect on the viability of seeds, applied for several weeks may reduce the incidence of *V. longisporum* infection in seeds (Vallad et al. 2005). The effectiveness of seed treatment with chemicals is not known yet. Since commercial oilseed rape fields are planted with seeds treated with fungicides, this appears to be a crucial question in the risk assessment of *V. longisporum* dissemination by seeds. The application of antagonistic bacteria or fungi may be another option to control seed-borne *V. longisporum* (Abuamsha et al. 2011). For plant quarantine regions where no *V. longisporum* has been recorded so

far, due to the low potential of seed transmission, further research needs to be considered to figure out a magnitude of infection level in order to reduce the risk of causing 'Verticillium stem striping' by transferring large amounts of *V. longisporum* transmitted seeds from diseased areas (Göre et al. 2011).

Reference

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Chapter 4: Effect of salicylic acid on *Verticillium longisporum* and *Brassica napus*

4.1 Introduction

Verticillium longisporum is an important soil-borne hemibiotrophic pathogen and host-specialized on Brassicaceae causing 'Verticillium stem striping' on *Brassica napus* under field conditions (Depotter et al. 2016). This disease is widely spread in Europe, North America and some regions in Asia (Gladders et al. 2011; Yu et al. 2015; CFIA 2017). A potential yield loss of 10-50% caused by early establishment of *V. longisporum* was estimated, however, it has not been confirmed or reported in large growing area yet (Dunker et al. 2008). Under controlled conditions and with artificial inoculation, infected *B. napus* plants show abnormal discoloration of leaves and stunting (Eynck et al. 2009; Lopisso et al. 2017). Because this pathogen grows in the vascular system of its host plants, it is not affected by chemical treatment. Hence, it is difficult to manage this disease. Therefore, breeding of resistant cultivars is the major strategy of control to avoid negative effects caused by *V. longisporum*.

Salicylic acid (SA) belongs to the group of plant phenolics, which displays an essential role in physiological and biochemical processes during plant development, such as negative regulator of seed germination (Rajjou et al. 2006; Xie et al. 2007), regulator of photosynthesis (Uzunova and Popova 2000), direct or indirect impact factor on nitrogen metabolism (Mabood and Smith 2007; Hayat et al. 2012), endogenous inducer of thermogenesis (Vlot et al. 2009), regulator of flowering (Khurana and Cleland 1992) and inducer of senescence (Morris et al. 2000). Besides, SA was reported to play a crucial role in defense of abiotic and biotic stresses in various plant species (Durner et al. 1997; Martínez et al. 2004; Anand et al. 2008; Chitra et al. 2008; Khan et al. 2015). As a mobile signal, SA is produced in roots and transported via the phloem to all plant parts to trigger local and systemic acquired resistance against viruses or biotrophic pathogens (Ben-Tal and Cleland 1982; Rocher et al. 2006; Rivas-San Vicente and Plasencia 2011). After infection of root or stem base vascular pathogens, such as *V. longisporum* and *Fusarium* spp., SA is accumulated in xylem vessels and transported with the transpiration stream from roots to shoots (Ratzinger et al. 2009).

Previous researches showed that SA was able to suppress the growth rate of *Phytophthora* spp., *Aspergillus* spp. and *Harpophora* spp*. in vitro* (Christie 2012; Degani et al. 2015; Panahirad et al. 2014). In order to avoid the negative effect of SA, *Ustilago maydis* targets the SA synthesis pathway in plants by secreting a chorismate mutase to channel chorismate into the phenylpropanoid pathway for avoidance of SA

accumulation during infection (Rabe et al. 2013) (Djamei et al. 2011; Chanclud and Morel 2016). In contrast, *Moniliophthora* produces SA, which not only has direct positive effects on fungal growth *in vitro*, but also contributes to manipulate the hormonal pathways involved in the host defense responses during fungal invasion (Chaves and Gianfagna 2006; Kilaru et al. 2007).

The level of SA was also elevated after infection of *B. napus* with *V. longisporum* (Kamble et al. 2013). Exogenous application of SA or its analogues may enhance the resistance of plants to pathogens (Achuo et al. 2004). After infection by pathogens, endogenous SA may increase to 10-100 µM locally (Strobel and Porter 2005). For the experimental enhancement of plant disease resistance, 100 µM-10 mM exogenous SA is usually applied to plants as a foliar spray, by direct root dipping or by soil drench (Esmailzadeh et al. 2008; Nazar et al. 2015; Kadotani et al. 2016).

The aim of this study is to reveal the direct effect of SA on the growth of *V. longisporum in vitro*. Furthermore, this study aims to find out a suitable dosage of exogenous application of SA for enhancement of resistance of *B. napus* against infection of *V. longisporum*.

4.2 Materials and methods

4.2.1 Fungal culture

Verticillium longisporum isolate VL43 obtained from a diseased *B. napus* plant (Zeise and von Tiedemann 2002) was used for inoculation. A conidial suspension, which had been kept in 25% glycerol at -80˚C, was used to initiate fresh culture. For preparation of the fungal culture, 400 µl of the stock conidial suspension were added into 250 ml autoclaved (121˚C, 20 min) potato dextrose broth (PDB), and then incubated on a rotary shaker with 80 rpm at 22˚C for 10 days. The resulting suspension was filtered through sterile gauze to remove mycelia. The conidial density was adjusted with sterile ddH_2O to 1 x 10^6 cfu/ml.

4.2.2 Determination of colony growth

After filtration through a polyethersulfone (PES) filter with 0.45 µm pore size (Minisart® High Flow Syringe Filter, Sartorius, Germany), the SA solution was mixed with autoclaved PDA medium (ca. 45°C) to make different concentrations (0, 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2 and 2.5 mM). The pH values of different concentrations of SA were measured. 2.5 µl of conidial suspension (1 x 10^6 cfu/ml) were placed at the center of the plate. Four replicates were conducted for each concentration, and the experiment has been done twice. The inoculated PDA media were incubated in dark at 20±2°C for 24 days. The diameter of each colony was measured every three or four days.

4.2.3 Plant material

Brassica napus L. cv. Drakkar was used in this study. Seeds were surface sterilized with 70% ethanol for 1 min under constant shaking, and subsequently rinsed twice with sterilized ddH_2O. Seeds were then sown in quartz sand and grown in a climate chamber with a 16 h photoperiod and a temperature of 22±2˚C for 12 days before treatments

4.2.4 Exogenous application of salicylic acid

Before inoculation of *V. longisporum* with the root-dip method, the roots of oilseed rape plants were directly dipped in 0, 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5 or 5 mM of SA for 24 h. After treatments, plants were rinsed with sterile tap water several times and dried on clean filter papers.

4.2.5 Inoculation of *V. longisporum* on *B. napus*

Twelve-day-old seedlings, of which cotyledons were completely unfolded, were inoculated or mock-inoculated with the root-dip method. The roots of the plants were dipped in a conidial suspension (1 x 10^6 cfu/ml) or water for 50 min and replanted in pots (7 x 7 x 8 cm) with a fresh soil-sand mixture (3:1).

4.2.6 Disease assessment

The plant height was measured at 14, 21, 26, 35 and 56 dpi. According to the discoloration of hypocotyl caused by *V. longisporum* (Fig. 4.1), the following formula was used to calculate the relative area of discoloration in hypocotyl of experimental plants:

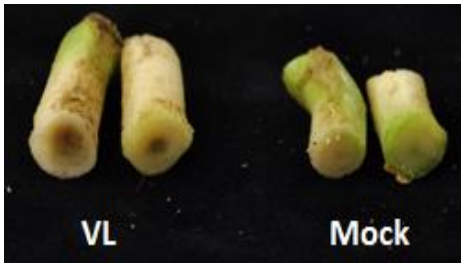

Figure 4.1 Symptoms on hypocotyls of *Brassica napus* caused by infection of *Verticillium longisporum*.

$$\text{Relative area of discoloration [\%]} = \left(\frac{\text{Mean diameter of discoloration ^2}}{\text{Mean diameter of cross section of hypocotyl ^2}}\right)\text{x}100\%$$

$$\text{AUDPC} = \sum_{i=1}^{n}\left(\frac{y_i + y_{i+1}}{2}\right) \times (t_{i+1} - t_i)$$

Where AUDPC is area under the disease progress curve, *yi* is the disease severity score for observation number *i*, *ti* is the corresponding number of days post inoculation of an observation, and *n* is the number of observations.

4.2.7 Extraction and quantification of DNA of *V. longisporum*

Total DNA from hypocotyl samples was extracted using a cetyltrimethylammonium bromide (CTAB) method (Brandfass and Karlovsky 2008). Ten hypocotyls samples were collected from each treatment. Three replicates were conducted. After lyophilization, 50 mg fine grounded hypocotyl samples were used for DNA extraction.

The extracted total DNA stained with ethidium bromide (EB) was qualified and quantified with 1% agarose gel electrophoresis. A CFX384 real-time PCR detection system (Bio-Rad laboratories, Inc.) was used for the amplification and quantification of *V. longisporum* DNA using primers OLG70 (*5'-CAGCGAAACGCGATATGTAG-3'*) and OLG71 (*5'-GGCTTGTAGGGGGTTTAGA-3'*) (Lopisso et al. 2017). The amplification mix was set up according to the manufacturer's protocol (Bioline, UK). The PCR-program considers a 4 min initial denaturation step, followed by 40 cycles with a denaturation step (10 s at 95°C), annealing (15 s at 60°C) and subsequent extension (15 s at 72°C). Fluorescence was detected after each elongation step and the program was completed with a final elongation step of 2 min at 72°C. Melting curve analysis was performed with 95°C for 10 s, followed by a 55°C step for 10 s and subsequent measurements within a range of 55°C to 95°C with every 5 s in 0.5°C temperature increments. PCR for all treatment samples were performed with three biological and three technical replicates and data were analyzed using CFX Manager Software (Bio-Rad laboratories, Inc.).

4.2.8 Determination of endogenous salicylic acid

Salicylic acid was extracted from hypocotyl samples according to a modified method described by Enyedi et al. (1992). The hypocotyl samples used for SA analysis were the same as used for DNA extraction. About 50 mg ground samples were homogenized and hydrolyzed with 0.8 ml of 2 M NaOH. The mixture was heated in a water bath at 80°C for 2.5 h. For acidification of the mixture, 0.64 ml concentrated HCl (37%) was added and the mixture was incubated at 80°C in a water bath for 60 min. To extract SA, 1.6 ml ethyl acetate was added and the mixture was transferred to a fresh tube with subsequent evaporation to dryness using speed vacuum centrifugation at 35°C. The residue was dissolved in 200 µl of HPLC grade methanol. The final solution were stored at -20°C till measurement. A dilution series with 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5, 7.5, 10 and 20 µM of SA was used as internal standard. Peak of SA was identified by comparing retention times of samples and standards and confirmed by addition of standard SA to the samples. Before loading into a HPLC vial, all samples or standards were centrifuged at 500 rpm for 5 min to precipitate undissolved particles to prevent injection problems.

The HPLC-fluorescence system consisted of a Varian 410 automatic injector, two Varian 210 pumps with 10 W SS head, a LiChrospher RP-18 column (250 x 4 mm, 5 µm) protect by a Security Guard™ Carbo-H precolumn (4 x 3 mm, 5 µm) keeping in column oven at 30°C and a Varian 363 fluorescence detector with excitation wavelength at 315 nm and emission wavelength at 405 nm. Each sample was analyzed for 33 min under a bi-mobile phase (A) 20 mM sodium acetate, pH 5.0 and (B) methanol with a flow rate of 1 ml/min with following process: initial 10% B for 2 min, linear gradient to 38% B in 13 min, increased to 98% B in 30 s and held for 9 min, equilibrated to initial condition in 30 s and hold for 8 min. The injection volume was 10 µl.

4.2.9 Statistical analysis

The experimental data were analyzed as completely randomized designs with four replications using STATISTICA 13.2. Before testing for statistical significance, a probability plot with Shapiro-Wilk test was used to test for normality of the data. Suitable data were analyzed using one-way ANOVA. A multiple comparison was analyzed by Fisher LSD test. Whenever data did not fit to normal distribution, nonparametric test Kruskal-Wallis ANOVA and multiple comparison of mean was applied. The experimental results are presented as means ± standard error at 5% significance level.

4.3 Results

4.3.1 Effect of salicylic acid on growth of *V. longisporum in vitro*

The application of different concentrations of SA, except for 5 mM, did not significantly affect the pH value of the medium (Tab. 4.1).

Table 4.1 pH value of medium containing different concentrations of salicylic acid. Different letters indicate significant differences among the treatments (Kruskal-Wallis test, $P<0.05$).

Concentration of SA	pH	Concentration of SA	pH
0	5.55±0.01 a	1 mM	5.28±0.02 ab
0.1 mM	5.54±0.01 a	1.5 mM	5.06±0.01 ab
0.25 mM	5.48±0.02 ab	2 mM	4.95±0.01 ab
0.5 mM	5.46±0.01 ab	2.5 mM	4.84±0.01 ab
0.75 mM	5.40±0.01 ab	5 mM	4.18±0.01 b

No growth of *V. longisporum* occurred on PDA medium amended with 2.5 and 5 mM SA throughout the experiment. In contrast, 0.1, 0.25, 0.5, 0.75 and 1 mM SA did not show any effect on the growth rate of *V. longisporum* (Fig. 4.2). A suppressive effect on fungal growth rate caused by 1.5 mM SA was found at 3 dpi.

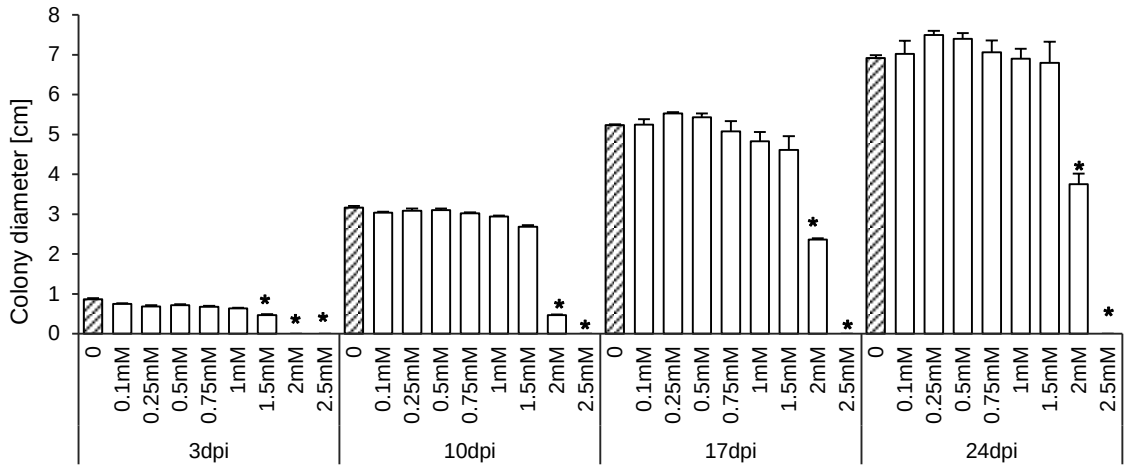

Figure 4.2 Growth of *Verticillium longisporum* on PDA medium containing different concentrations of salicylic acid at 3 to 24 dpi. Bars indicate standard errors. Asterisks on the bars indicate significant differences between two genotypes at the same time point (Kruskal-Wallis test within each time point without 2.5 mM, $P<0.05$).

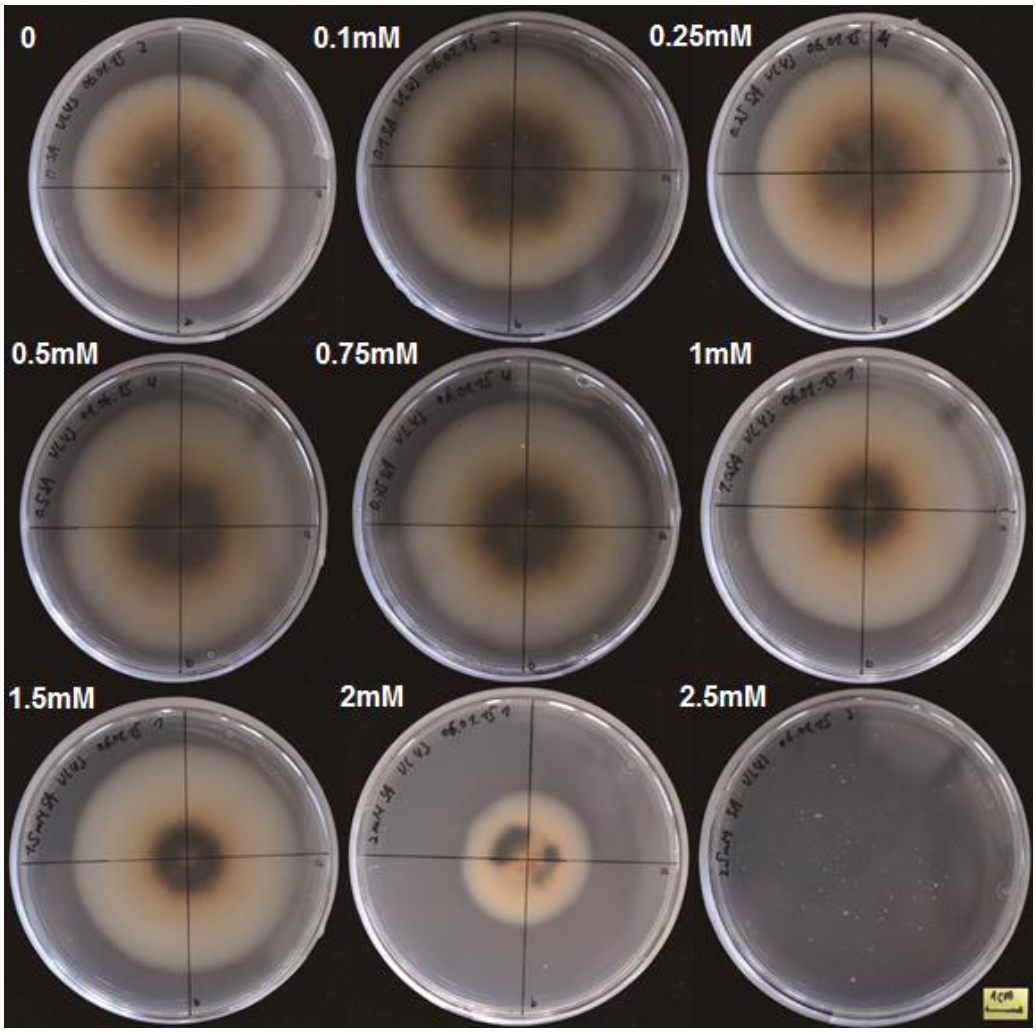

Figure 4.3 Cultural characteristics of *Verticillium longisporum* grown on PDA containing different concentrations of salicylic acid at 24 dpi. The comparing scale present is one centimeter.

However, it quickly recovered after 7 dpi. On medium with 2 mM SA, the growth of fungal mycelium partially recovered by 10 dpi, although it was still significantly slower than the water control. Nevertheless, compared to the control, *V. longisporum* grew on PDA with 1 and 1.5 mM SA formed less pigmented microsclerotia (Fig. 4.3). In contrast, low concentrations of SA (0.25 and 0.5 mM) had a slight growth promoting effect on *V. longisporum.*

4.3.2 Effect of salicylic acid on *B. napus* and growth of *V. longisporum in planta*

4.3.2.1 Phytotoxic effect of salicylic acid on *B. napus*

After dipping the roots of *B. napus* in SA solution for 24 h, a phytotoxic effect of SA was found in 1.5, 2, 2.5 and 5 mM SA. All plants from these treatments showed dried-up parts in the leaf margin, especially for the youngest leaf (Fig. 4.4A). Besides, the hypocotyls dipped in 2.5 and 5 mM SA solutions were lacking of pigment and constrictive (Fig. 4.4B). After transplanting the treated plants into soil, 100% of plants treated with 5 mM SA and more than 50% of plants treated with 2.5 mM SA quickly died after 3 days. Plants treated with 1, 1.5 and 2 mM SA were relatively smaller compared with control plants treated with water (Fig. 4.4C).

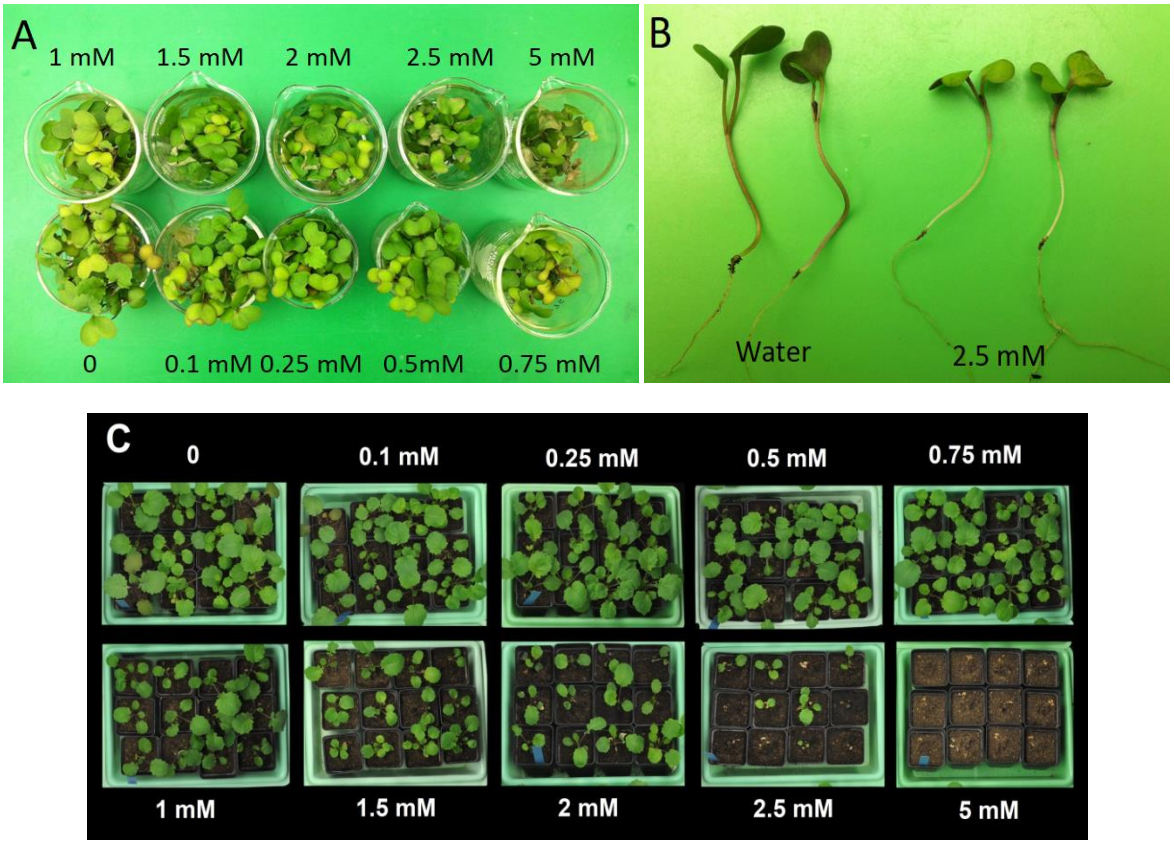

Figure 4.4 Phytotoxic effects of salicylic acid on *Brassica napus*. A, toxic symptoms on leaves; B, toxic symptoms on hypocotyl; C, growth of plants with salicylic acid treatment without inoculation of *Verticillium longisporum* at 21 days post treatment.

4.3.2.2 Effect of salicylic acid on *B. napus* and growth of *V. longisporum*

Infection of *V. longisporum* still caused plant height reduction in plants, which were pretreated with 0.1, 0.25 and 0.75 mM SA before inoculation with *V. longisporum*. However, the stunting effect was not found in plants that were pretreated with 0.5, 1, 1.5, 2 and 2.5 mM SA (Fig. 4.5).

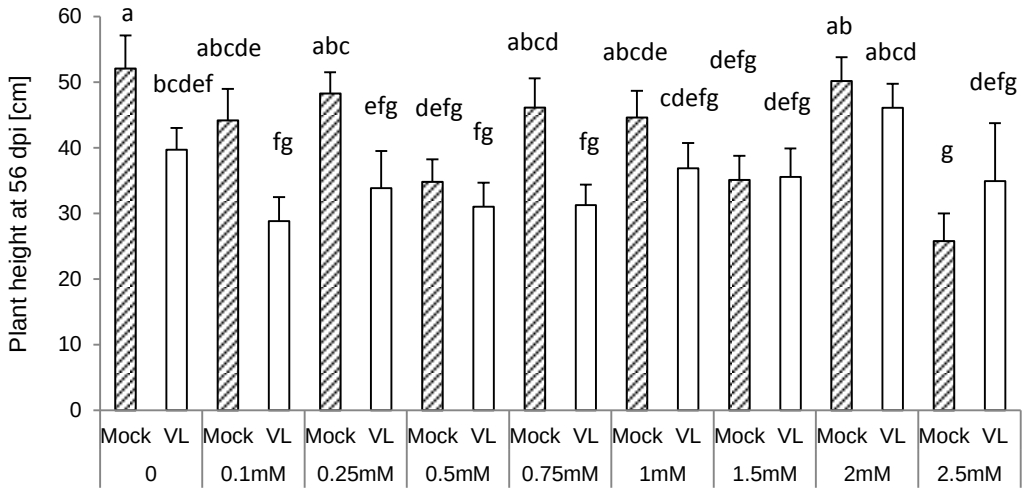

Figure 4.5 Plant height of *Brassica napus* plants pretreated with salicylic acid an inoculated with *Verticillium longisporum*. Bars indicate standard errors. Different letters indicate significant differences among the treatments (LSD test, *P*<0.05).

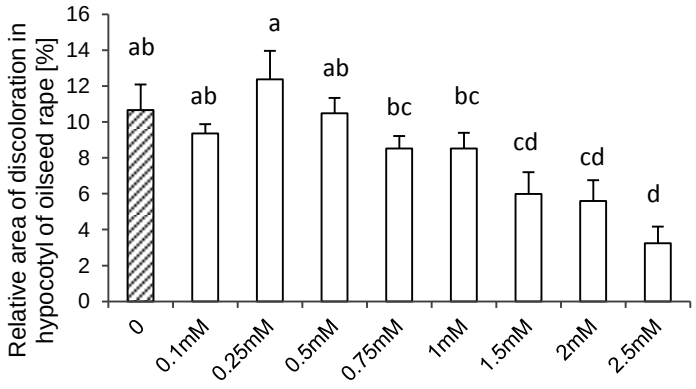

Figure 4.6 Effect of salicylic acid treatment on lesion severity in the hypocotyl after infection with *Verticillium longisporum* at 56 dpi. Different letters indicate significant differences among the treatments (LSD test, *P*<0.05).

Plants amended with more than 1.5 mM SA had significantly smaller lesions in the hypocotyl compared to the water control, while plants treated with lower concentrations of SA did not show such a clear effect (Fig. 4.6). Generally, *B. napus* cv. Drakkar is not susceptible to *V. longisporum*. The biomass of *V. longisporum* in the hypocotyls was

relatively low, only around 0.01 pg/ng total DNA were found. No significant reduction of *V. longisporum* DNA was found among SA treatments, except for the plants that pretreated with 1.5, 2 and 2.5 mM SA (Figure 4.7).

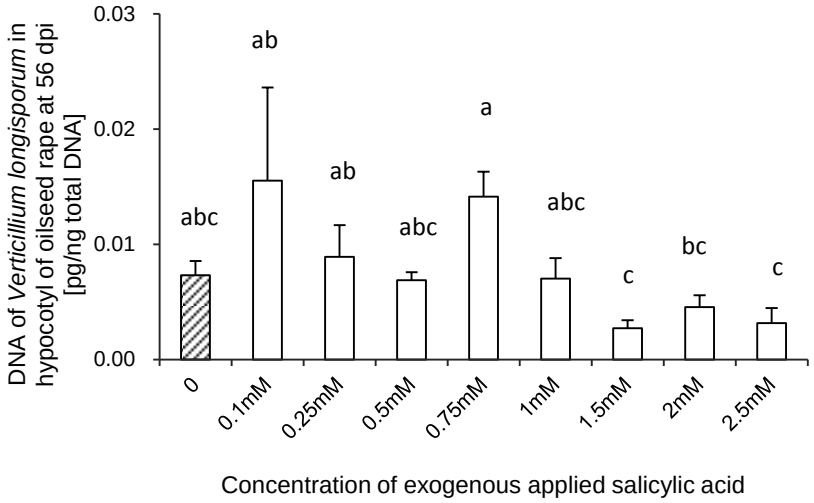

Figure 4.7 Development of *Verticillium longisporum* in *Brassica napus* after exogenous application of salicylic acid. Bars indicate standard errors. Mean data obtained from three biological replicates. Different letters indicate significant differences among the treatments (LSD test, $P<0.05$).

Total salicylic acid levels significantly increased after inoculation with *V. longisporum*, while exogenous root application of SA did not have such an effect on endogenous SA levels at 56 dpi (Fig. 4.8). The plants, which were treated with 1.5, 2 and 2.5 mM, showed less or even not induced SA level by *V. longisporum* inoculation.

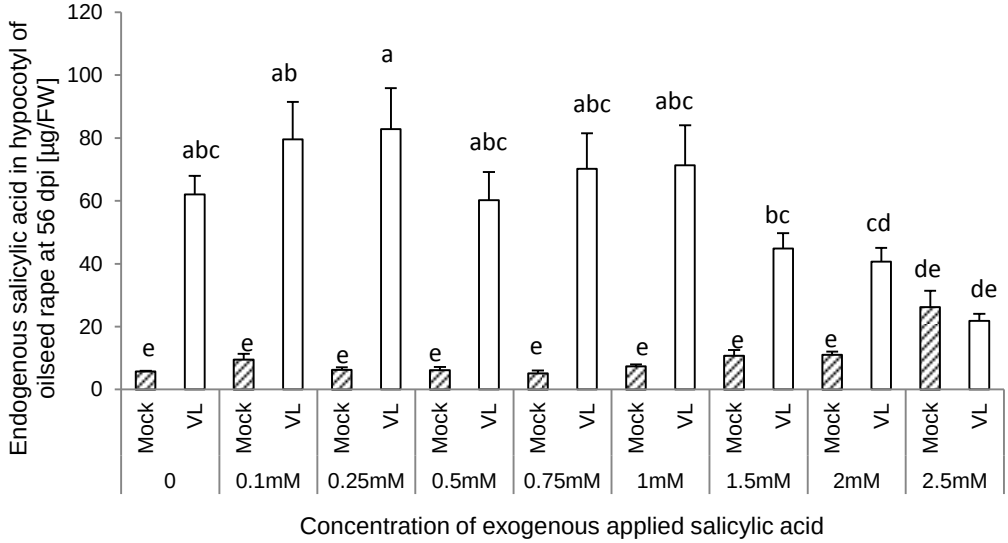

Figure 4.8 Endogenous total SA in hypocotyls of *Brassica napus* at 56 dpi. Different letters indicate significant differences among the treatments (LSD test, $P<0.05$).

4.4 Discussion

Numerous previous studies have investigated the role of salicylic acid in defense of plants against pathogens. There are also some studies about the direct effect of SA on pathogens under *in vitro* conditions. However, this study is the first to fill the gap of studies on direct effects of SA on *V. longisporum*. Excluding the potential effect of pH shift, SA had a direct antifungal effect on *V. longisporum* at concentrations of 1.5, 2, 2.5 and 5 mM, which is equivalent to the commonly used concentrations for the experimental induction of disease resistance in plants (Strobel and Porter 2005). However, 1.5 mM SA only partially suppressed or poisoned the conidia of *V. longisporum*, therefore, the fungus may grow further and overcome the inhibiting effect. The extremely high concentration of SA entirely inhibited *V. longisporum* grown *in vitro* leading to unrecoverable damage in the growth of the colony. As described by Ratzinger et al. (2009), the concentrations of endogenous SA found in xylem sap of healthy and *V. longisporum* infected plants of *B. napus* were 0.06-0.11 µM and 0.2-1.5 µM, respectively. In the plant tissue of *B. napus*, SA can be induced by infection of *V. longisporum* reaching 4 µg/g fresh weight in the hypocotyl and 7 µg/g fresh weight in leaves (Kamble et al. 2013). The concentration of SA equivalent to the one reported in *V. longisporum* infected plant tissues did not inhibit the growth of *V. longisporum in vitro*, in contrast, it induced a weak to moderate promoting effect on fungal growth. Thus, it seems unlikely that *V. longisporum* induced endogenous levels of SA are capable to suppress the growth of this pathogens and act as a direct antifungal compound in plant defense (Rüffer et al. 1995). This implies that SA has a role in disease resistance based on its signaling function as trigger or modulator of defense pathways.

High concentrations of exogenously applied SA not only had an antifungal effect on *V. longisporum* but were also phytotoxic to *B. napus*. As the effects on the fungus, phytotoxic effects on *B. napus* caused by extremely high concentrations of SA was irreversible. Plants that survived from such high concentrations of SA were stunted and thickening their hypocotyls and stems. In *B. juncea*, concentrations of 0.5 and 1 mM of SA applied on leaves significantly reduced the infection by *Alternaria brassicae* (Kamble and Bhargava 2007). *ß*-aminobutyric acid pretreatment on *B. napus*, which has been reported to induce resistance via the SA signaling pathway (Zimmerli et al. 2001), was able to reduce the establishment of *V. longisporum* in *B. napus* plants, while increase of endogenous SA was weakened by this pretreatment (Kamble et al. 2013). The growth stage used in the present study for detecting the effect of SA on *V. longisporum* is the flowering period, which was reported to be essential for accumulation of *V. longisporum* on *B. napus* (Zhou et al. 2006). However, the exogenous pretreatments of SA did not have an influence on the development of *V. longisporum in planta* during the flowering

period. As indicated in previous reports, the present study showed that the endogenous SA levels were induced by the infection of *V. longisporum*, except for the pretreatment with 2.5 mM SA. Plants which suffered from phytotoxic effects may already have high concentrations of SA in roots before *V. longisporum* infection. Therefore, such high concentration of SA may have direct antifungal effects on the growth of *V. longisporum* during adhesion on and penetration of roots. Similar consequences occurred in plants treated with 1.5 and 2 mM SA. Since these concentrations confirmed to also suppress the growth of *V. longisporum in vitro* directly, a lower increase of endogenous SA due to the reduction of fungal growth founded in the hypocotyls. Thus, it seems that endogenous SA or pretreatment of SA may have an effect on the growth of the fungus at early time points of infection, however, the exact role of salicylic acid in the early phase of *V. longisporum*-*B. napus* interaction for defense needs to be further investigated.

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Role of Salicylic Acid and Components of the Phenylpropanoid Pathway in Basal and Cultivar-Related Resistance of Oilseed Rape (*Brassica napus*) to *Verticillium longisporum*

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Abstract: Enhanced resistance is a key strategy of controlling 'Verticillium stem striping' in *Brassica napus* caused by the soil-borne vascular pathogen *Verticillium longisporum*. The present study analyses the role of a broad range of components in the phenylpropanoid and salicylic acid (SA) pathways in basal and cultivar-related resistance of *B. napus* towards *V. longisporum*. A remarkable increase of susceptibility to *V. longisporum* in SA-deficient transgenic *NahG* plants indicated an essential role of SA in basal resistance of *B. napus* to *V. longisporum*. Accordingly, elevated SA levels were also found in a resistant and not in a susceptible cultivar during early asymptomatic stages of infection (7 dpi), which was associated with increased expression of *PR1* and *PR2*. In later symptomatic stages (14 or 21 dpi), SA responses did not differ anymore between cultivars varying in resistance. In parallel, starting at 7 dpi, an overall increase in phenylpropanoid syntheses developed in the resistant cultivar, including the activity of some key enzymes, phenylalanine ammonium lyase (PAL), cinnamyl alcohol dehydrogenase (CAD) and peroxidase (POX) and the expression of key genes, *PAL4*, *CCoAMT*, *CCR*, *POX*. As a consequence, a remarkable increase in the levels of phenolic acids (*t*-cinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid) occurred associated with cultivar resistance. A principal component analysis including all 27 traits studied indicated that component 1 related to SA synthesis (*PR1*, *PR2*, *POX*, level of free SA) and component 2 related to lignin synthesis (level of free ferulic acid, free *p*-coumaric acid, conjugated *t*-cinnamic acid) were the strongest factors to determine cultivar-related resistance. This study provides evidence that both SA and phenolic acid synthesis are important in cultivar-related resistance, however, with differential roles during asymptomatic and symptomatic stages of infection.

Keywords: salicylic acid; phenolic acids; *NahG*; gene expression; enzymatic analysis

1. Introduction

Verticillium longisporum (VL) is a soil-borne vascular fungal pathogen with host specificity to Brassicaceae [1]. The pathogen widely occurs in oilseed rape production regions in Europe and North America [2–5]. Oilseed rape (*Brassica napus*) is the most important crop for oil production in Europe and Canada and the prevalent host of *V. longisporum* [6]. Due to the relatively short crop rotation and increased area of oilseed rape cultivation, incidence of 'Verticillium stem striping' is on the rise and threatens oilseed rape production. In the absence of their host, the melanized microsclerotia of *V. longisporum* remain dormant and viable in soil for several years [6–8]. As control of *V. longisporum* with

Zheng X, Koopmann B, von Tiedemann A (2019). Role of salicylic acid and components of the phenylpropanoid pathway in basal and cultivar-related resistance of oilseed rape (*Brassica napus*) to *Verticillium longisporum*. Plants 2019, 8, 491. DOI: doi.org/ 10.3390/plants8110491.

fungicides has not been successful [9], the most promising measure against this disease is breeding for effective resistance.

Salicylic acid (SA) is an important phytohormone which has been shown to play a role not only in local resistance but also in the activation of systemic acquired resistance (SAR) against pathogens [10]. SA can be synthesized either via the phenylalanine or isochorismate pathways in *Arabidopsis* [11,12]. Application of SA or its analogues (benzo (1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester, BTH) enhances the resistance of plants against pathogens, while in SA-deficient *NahG* transformed plants the susceptibility to pathogens was increased [13,14]. However, surprisingly, in *B. napus*, a positive correlation between the concentration of SA in shoot extracts and the biomass of *V. longisporum* colonizing the plant was reported [15].

In *Arabidopsis*, soluble phenylpropanoids are involved in defense responses to *V. longisporum* [16]. In oilseed rape, genomic loci (QTL) for a number of phenylpropanoids were found to co-localize with the QTL for *V. longisporum* resistance [17]. Lignin, an important compound among phenylpropanoids, is mainly deposited in secondary cell walls, which may provide strength for preventing direct penetration by the fungus. Besides, several phenolic acids, intermediates in the lignin biosynthetic pathway, were accumulated in vascular tissues after infection of oilseed rape with *V. longisporum [18]*.

Although previous studies have indicated a role of SA and lignin biosynthesis in resistance responses of oilseed rape to *V. longisporum*, the role of specific components and the interplay between these pathways during infection is not yet understood. Therefore, the aim of this study was to explore the role in resistance of a larger set of metabolites, enzymes and genes involved in the biosynthesis of SA and lignin during infection of *B. napus* with *V. longisporum*. More specifically, we followed three hypotheses, (1) that SA is required in basal resistance of *B. napus* to *V. longisporum*, (2) that the phenylpropanoid pathways related to lignin synthesis determine cultivar-related resistance to *V. longisporum* and (3) that biosynthesis of SA and phenolic acids are in a competitive relationship.

2. Results

2.1. V. longisporum Disease Development and Plant Colonization in NahG Transformed Oilseed Rape

Typical symptoms of *V. longisporum* infection on *B. napus* under greenhouse or climate chamber conditions were leaf yellowing, vein blacking, premature senescence of leaves and stunting. In the *NahG* transformants, severe symptoms were observed already two weeks after inoculation, while in the wild type plants, only relative mild symptoms occurred at 21 dpi. In addition, diseased transformant plants showed a severely crippled, deformed shoot growth at 21 dpi. Except for the first week after inoculation, significantly higher levels of disease severity were recorded in *V. longisporum* infected *NahG* transformants than in the wild type plants. However, exogenously supplied SA by root dipping prior to inoculation did not effectively reduce disease severity (Table 1). The stunting effect of *V. longisporum* was significantly stronger on *NahG* transformants than on wild type plants. No significant effects by *V. longisporum* on the dry weight of roots and shoots was observed at early time points (7, 14 dpi). However, roots of *NahG* transformants were comparatively more sensitive to the root application of SA, which induced a reduction of biomass in mock-inoculated plants at 7 dpi. At 21 dpi, a significant reduction of root and shoot biomass was observed in all *V. longisporum*-inoculated *NahG* transformants. Except for root biomass at 21 dpi without exogenous application of SA, biomass of wild type plants was not significantly reduced by *V. longisporum* inoculation. Correspondingly, significantly higher amounts of fungal DNA were found in the hypocotyls of *NahG* transformants compared to wild type plants (Figure 1A). Exogenous SA treatment did not significantly reduce the amount of fungal DNA (Figure 1B).

Table 1. Disease severity, plant height and dry weight of mock- and *V. longisporum*-inoculated oilseed rape. Different letters indicate significant differences within rows for each parameter (LSD test, $p < 0.05$). SA, salicylic acid; VL, *V. longisporum* inoculated.

			Water Treatment				0.5 mM SA Root Treatment			
			NahG		Wild Type		*NahG*		Wild Type	
			Mock	VL	Mock	VL	Mock	VL	Mock	VL
Disease severity [1–9]		7 dpi	1.1 ± 0.07 a	1.3 ± 0.12 a	1.0 ± 0.00 a	1.1 ± 0.05 a	1.2 ± 0.09 a	1.3 ± 0.10 a	1.0 ± 0.00 a	1.0 ± 0.00 a
		14 dpi	1.2 ± 0.09 b	4.3 ± 0.20 a	1.1 ± 0.05 b	1.2 ± 0.08 b	1.2 ± 0.09 b	3.2 ± 0.34 a	1.0 ± 0.00 b	1.3 ± 0.00 b
		21 dpi	1.4 ± 0.15 b	5.5 ± 0.15 a	1.2 ± 0.08 b	1.6 ± 0.18 b	1.4 ± 0.15 b	4.9 ± 0.18 a	1.2 ± 0.09 b	1.6 ± 0.20 b
Plant height [cm]			22.5 ± 1.3 c	6.7 ± 0.4 d	34.6 ± 2.8 a	23.2 ± 2.0 c	24.5 ± 1.5 bc	7.4 ± 0.4 d	31.5 ± 2.7 ab	21.0 ± 1.4 c
Dry weight [mg]	7 dpi	root	28 ± 1.1 a	30 ± 4.7 a	24 ± 3.2 ab	31 ± 1.6 a	23 ± 1.9 ab	17 ± 0.9 b	28 ± 1.5 a	26 ± 3.8 ab
		shoot	134 ± 5.1 ab	136 ± 18.7 ab	136 ± 9.3 ab	153 ± 6.9 a	115 ± 7.7 b	122 ± 15.9 ab	134 ± 6.8 ab	150 ± 6.3 ab
	14 dpi	root	74 ± 9.5 a	64 ± 9.8 a	70 ± 16.5 a	63 ± 9.4 a	61 ± 6.6 a	57 ± 7.0 a	87 ± 18.6 a	63 ± 4.3 a
		shoot	375 ± 64.2 a	309 ± 29.0 a	400 ± 51.8 a	342 ± 9.4 a	296±23.7 a	277 ± 25.6 a	409 ± 18.6 a	339 ± 4.3 a
	21 dpi	root	132 ± 11.1 b	70 ± 4.8 c	170 ± 8.3 a	128 ± 10.9 b	116±10.4 b	55 ± 5.2 c	140 ± 14.9 ab	131 ± 10.3 b
		shoot	662 ± 89.7 a	383 ± 80.6 b	1144 ± 192.2 a	847 ± 192.2 a	743±142.4 a	380 ± 88.1 b	1000 ± 156.4 a	684 ± 60.8 a

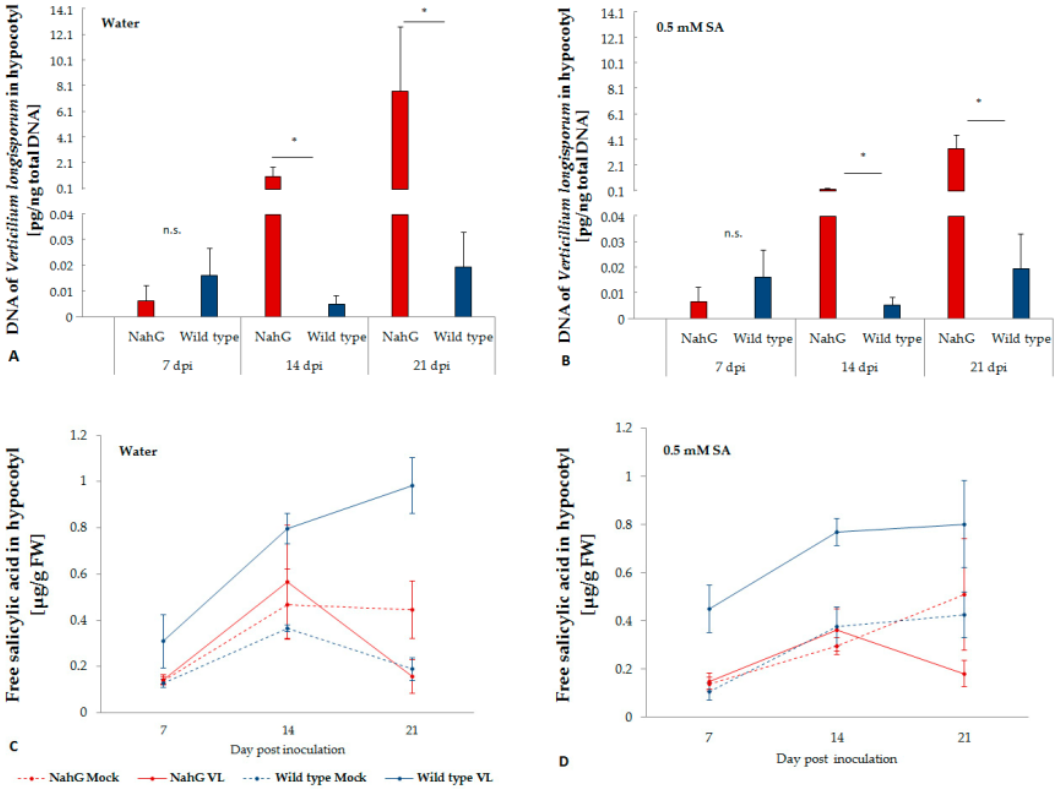

Figure 1. Colonization of in *B. napus* cv. Drakkar and its *NahG* transformant with *V. longisporum* with (**A**) or without (**B**) exogenous application of salicylic acid and the endogenous free salicylic acid content in the hypocotyl after infection (**C**,**D**). Bars indicate standard errors. Mean data were obtained from four biological replications. Asterisks on the bars indicate significant differences between two genotypes at the same time point (LSD, $p < 0.05$).

2.2. Endogenous SA in the Hypocotyl of Wild Type and NahG Transformant Plants

NahG transformants were not able to accumulate free SA upon infection with *V. longisporum*, while in wild type plants a significant increase of free SA was observed in *V. longisporum* infected plants, especially two weeks after inoculation (Figure 1C), when *V. longisporum* biomass in hypocotyls significantly increased in *NahG* transformants but not in wild type plants. Exogenous application of SA did not affect the level of free SA in plant tissue (Figure 1D). Free SA showed a slight negative correlation with logarithm of *V. longisporum* DNA ($r = -0.24$, $p = 0.09$).

2.3. V. longisporum Disease Development in Resistant and Susceptible Cultivars

No visual symptoms were observed during the first week after inoculation in either cultivar. However, two weeks after inoculation with *V. longisporum*, the susceptible cultivar Falcon began to display significantly more severe disease symptoms on leaves and a stronger stunting, while the resistant cultivar only exhibited a slight reduction of plant height (Figure 2A,C). A significant accumulation of *V. longisporum* DNA in the hypocotyl of the susceptible cultivar was detected which was lacking in the resistant cultivar SEM confirming the disease phenotyping data (Figure 2B).

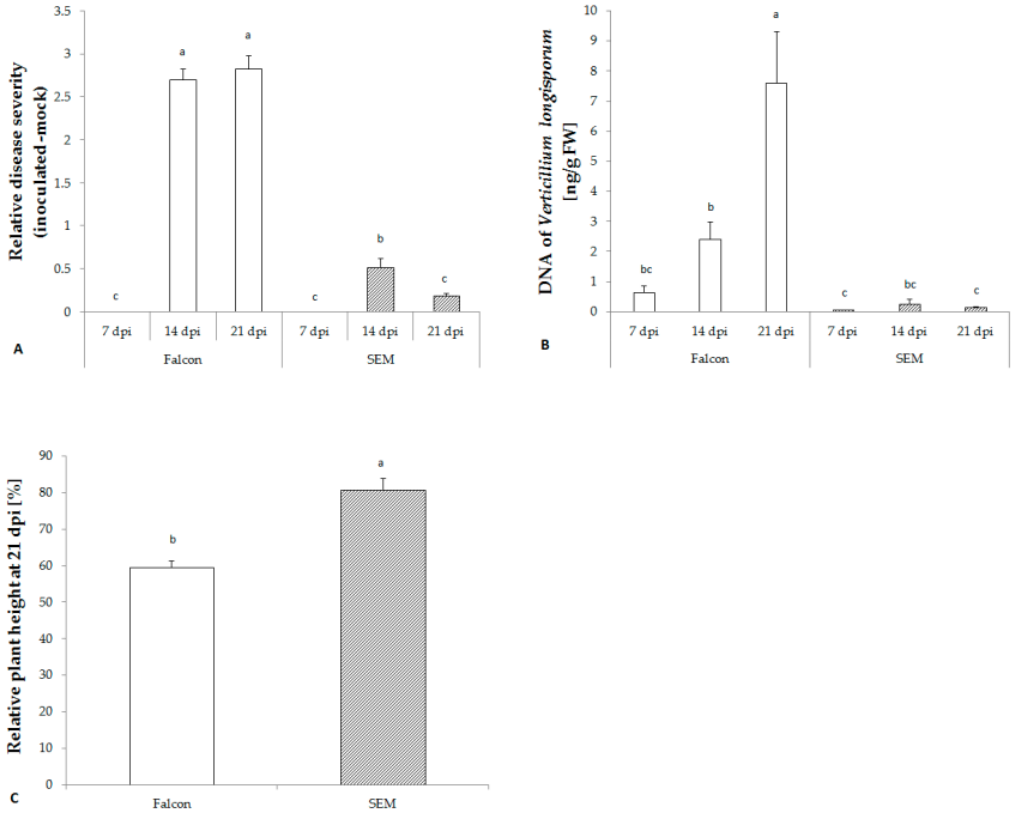

Figure 2. Development of *V. longisporum* in *B. napus* cv. Falcon and SEM determined by disease severity (**A**), fungal biomass in hypocotyls (**B**), and plant height at 21 dpi (**C**). Bars indicate standard errors. Different letters indicate significant differences among the treatments (LSD test, $p < 0.05$). FW, fresh weight.

2.4. Changes in the SA Biosynthetic Pathway of B. napus Infected with V. longisporum

In the absence of disease symptoms of *V. longisporum* infection, free benzoic acid (BA) in the resistant cultivar SEM was strongly used for the production of free SA which was evident by fast increase of free SA at the same time (Figure 3A,B). Similar to free SA, the activity of benzoic acid

2-hydroxylase (BA2H) was transiently enhanced at 7 dpi by infection. In general, responses in both metabolite levels and enzyme activities relative to fungal biomass in the susceptible cultivar Falcon were low (Figure 4B). Besides, a stronger increase in expression of SA mediated marker genes *PR1* and *PR2* relative to the fungal biomass was found in the resistant cultivar (Figure 5A,B), while the jasmonic acid mediated marker gene *PDF1.2* was down-regulated (Figure 5C).

2.5. Changes in Phenolic Acid Levels related to Lignin Biosynthesis in Response to V. longisporum Infection

Phenolic acids are formed in the lignin biosynthetic pathway in the following order: *trans*-cinnamic acid (*t*CA), *p*-coumaric acid (*p*CA), caffeic acid (CA), ferulic acid (FA), sinapic acid (SiA). In healthy *B. napus* plants, the contents of all conjugated phenolic acids increased with growth, whereas free phenolic acids were relatively stable in content all over the time of sampling (*data not shown*). In contrast to SA, no significant regulation of phenolic acids was found in either cultivar one week after inoculation with *V. longisporum*. Except CA and free SiA, significant increases of *t*CA, *p*CA, FA and conjugated SiA toward infection of *V. longisporum* were detected in the resistant cultivar at 14 dpi (Figure 3). The amount of CA was under detection limit until 14 dpi, but a clear increase was found in the resistant cultivar at 21 dpi (Figure 3G,H).

2.6. Activity of Key Enzymes in Lignin Biosynthetic Pathway

The activity of phenylalanine ammonia lyase (PAL) towards infection of *V. longisporum* was remarkably increased in the resistant cultivar at 7 dpi, while in the susceptible cultivar less activity was found (Figure 4A). Prior to production of phenolic acids, the activity of cinnamyl alcohol dehydrogenase (CAD) and peroxidase (POX) increased in the resistant cultivar at 7 dpi (Figure 4D,E). However, cinnamate 4-hydroxylase (C4H), which catalyzes conversion of *t*CA to *p*CA, did not differ between resistant and susceptible cultivars (Figure 4C).

2.7. Regulation of Genes of Key Enzymes involved in Lignin Synthesis

Similar to enzyme activity, expression of almost all genes involved in lignin synthesis (*PAL4*, *C4H*, *C3H*, *CCoAMT*, *CCR*, *CAD* and *POX*) was up-regulated relative to fungal biomass in the resistant cultivar already at 7 dpi. In contrast, *COMT* was strongly down - regulated in the resistant cultivar (Figure 5). No significant responses were found in the expression of *4CL* (Figure 5G).

2.8. Principal Component Analysis

Principal component analysis is a powerful statistical tool for multivariate experiments. Using principal components 1 and 2 based on 27 traits, a clear separation of resistant and susceptible cultivars is possible, with the resistant cultivar at 7 dpi being most distinct cluster (Figure 6A). The distributions of $\cos^2$ values among principal components indicate that gene expression of *PR1*, *PR2*, *POX* and level of free SA were the strongest contributors to the component 1, while component 2 was mainly determined by the level of free FA, free *p*CA, conjugated *t*CA (Figure 6B). These parameters seem to be the best variables to explain the variation in response of both genotypes to fungal infection.

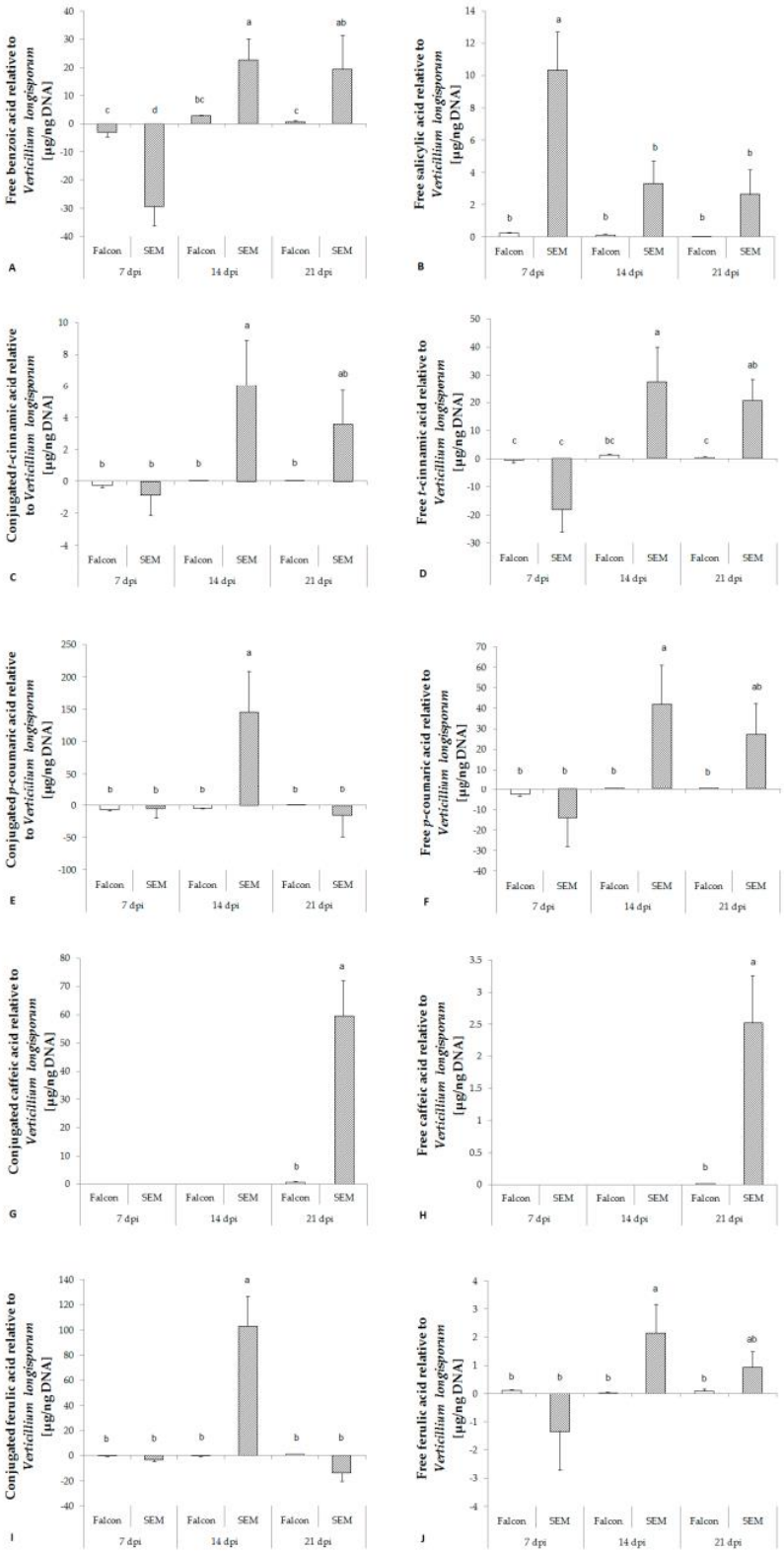

Figure 3. Response of free benzoic acid (**A**), salicylic acid (**B**) and different phenolic acids (**C–L**) in the hypocotyl of *B. napus* cv. Falcon and SEM upon infection of *V. longisporum*. Bars indicate standard errors. Different letters indicate significant differences among the treatments (LSD test, $p < 0.05$). FW, fresh weight; n.s., no significant difference.

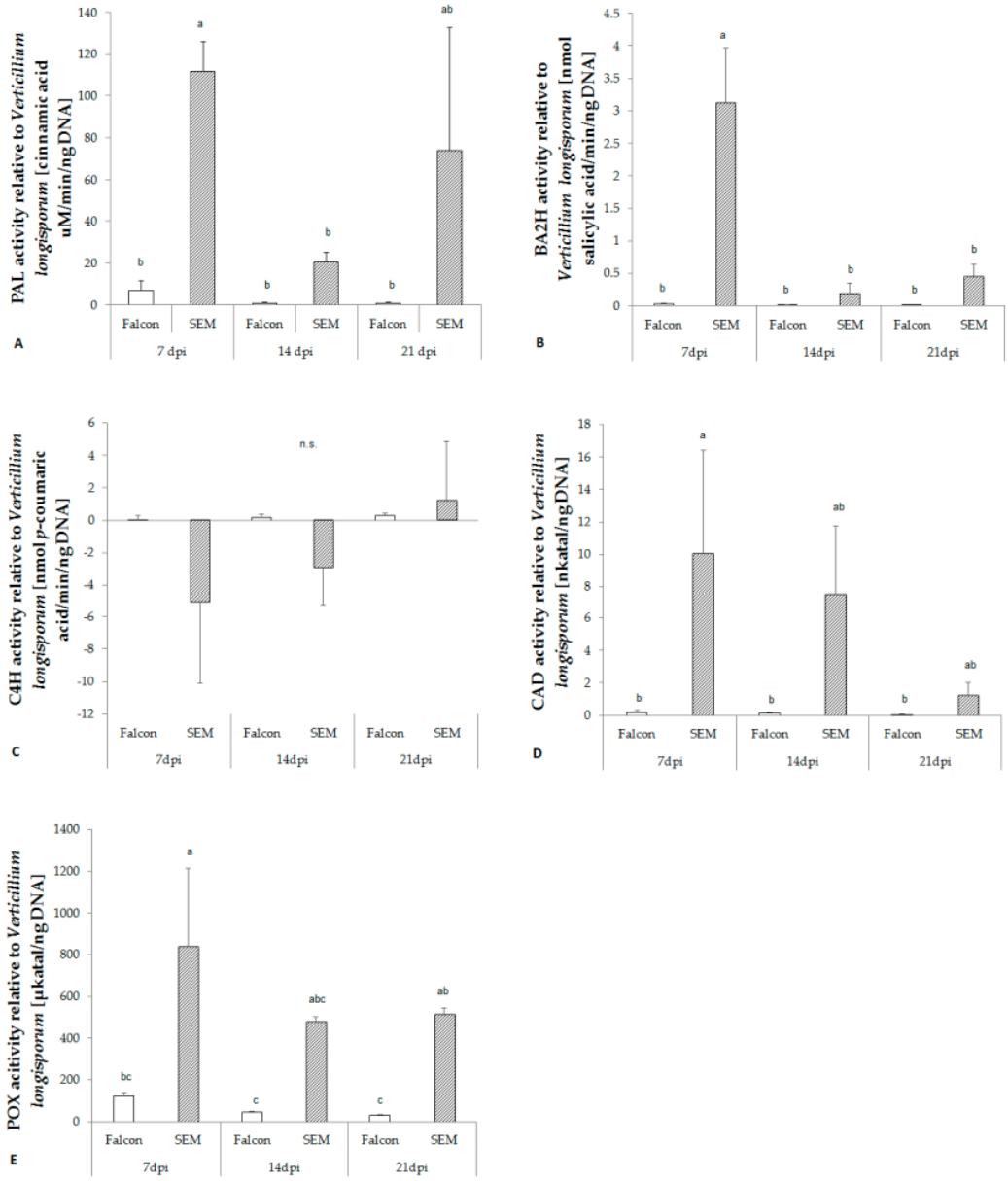

Figure 4. Enzyme activity in the hypocotyl of *B. napus* cv. Falcon and SEM inoculated with *V. longisporum*. (**A**) PAL, phenylalanine ammonia lyase; (**B**) BA2H, benzoic acid 2-hydroxylase; (**C**) C4H, cinnamate 4-hydroxylase; (**D**) CAD, cinnamyl alcohol dehydrogenase; (**E**) POX, peroxidase. Bars indicate standard errors. Different letters indicate significant differences among the treatments (LSD test, $p < 0.05$). n.s. means no significant difference.

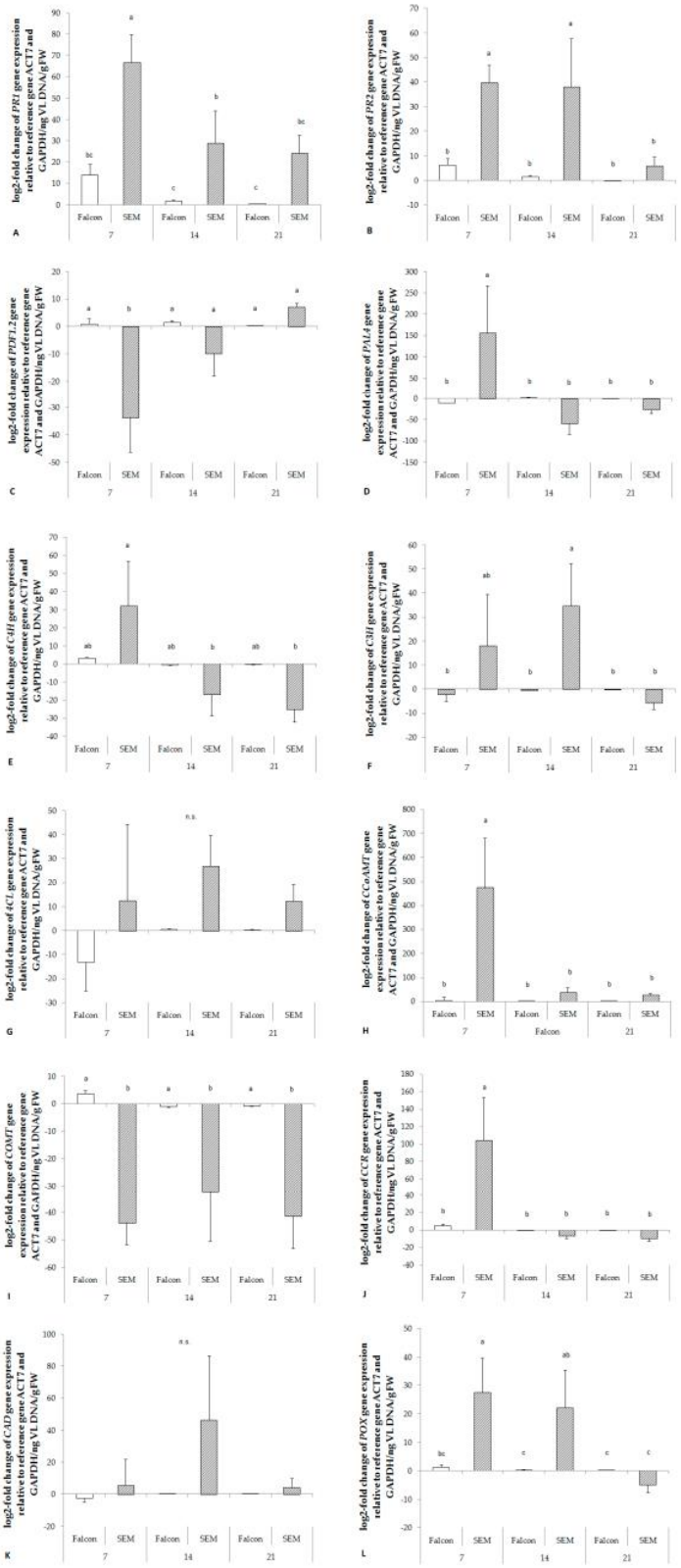

Figure 5. Fold change of salicylic acid mediated resistance marker genes (**A, B**), jasmonic acid mediated resistance marker gene (**C**), and genes of key enzymes involved in lignin synthesis (**D–L**) in the hypocotyl of *B. napus* after infection by *V. longisporum*. The change rates were derived from a comparison of mock-inoculated plants. Target gene expression was normalized to the expression of *ACT7* and *GAPDH*.

The log2-fold change was related to the fungal biomass. *PR1*, pathogenesis-related protein 1; *PR2*, pathogenesis-related protein 2; *PDF1.2*, plant defensin 1.2; *PAL4*, phenylalanine ammonia lyase; *C4H*, cinnamate 4-hydroxylase; *C3H*, *p*-coumarate 3-hydroxylase; *4CL*, 4-coumarate:CoA ligase; *CCoAMT*, caffeoyl-CoA 3-O-methyltransferase; *COMT*, catechol-O-methyl transferase; *CCR*, cinnamoyl-CoA reductase; *CAD*, cinnamyl alcohol dehydrogenase; *POX*, peroxidase; *ACT7*, actin 7; *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase. Bars indicate standard errors. Different letters indicate significant differences among the treatments (LSD test, $p < 0.05$). FW, fresh weight; VL, *V. longisporum*; n.s., no significant difference.

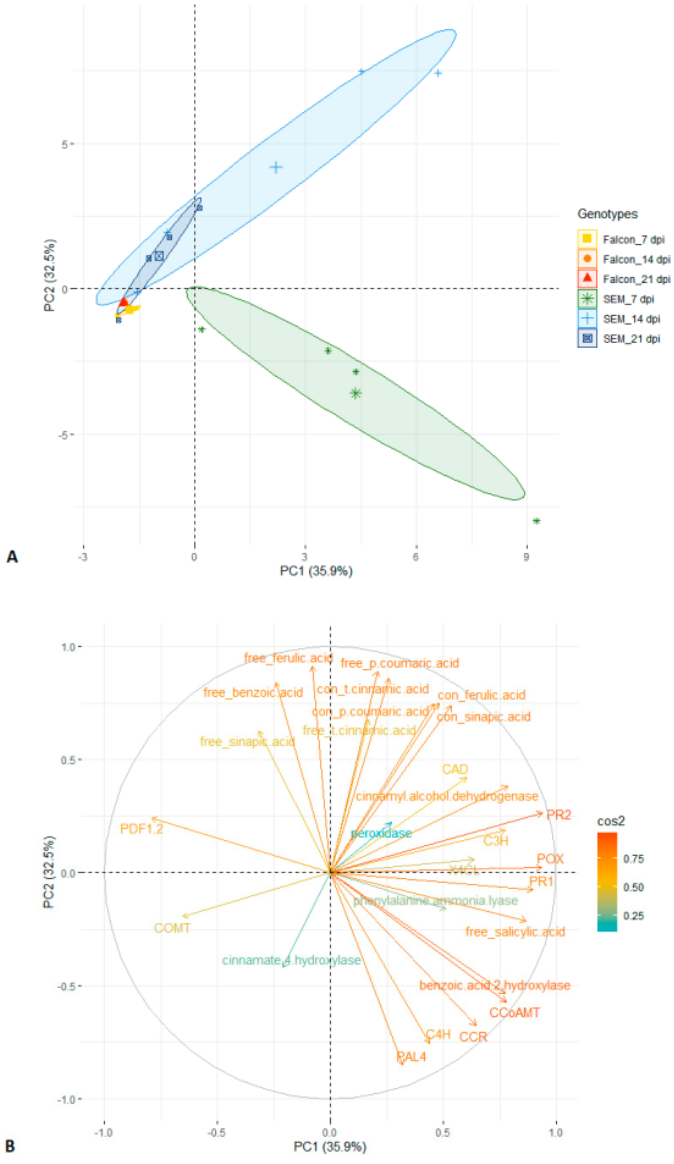

Figure 6. Principal component analysis of twenty-seven traits (metabolites, enzyme activities and gene expression) recorded in two *B. napus* genotypes with *V. longisporum* infection (**A**) and circles of cos^2 value of the variables (**B**). Labels of traits in capital letters are names of genes. Numbers of clusters were determined by elbow method. PC, principal component; con, conjugated form.

3. Discussion

3.1. SA Plays a Role in Basal Resistance of B. napus to V. longisporum

SA, an important phytohormone involved in disease defense, has been shown to play a role not only in local resistance but also in the activation of systemic acquired resistance (SAR) [10]. Most of the SA synthesized in plants is modified by glucosylation and methylation, which are induced upon pathogen infection [14]. Free SA can be released from this inactive storage form when necessary and maintain SAR over extended periods of time [19]. Methyl SA, a volatile ester, as well as free and conjugated SA in tobacco were induced after infection with avirulent strains of *Pseudomonas syringae* [20]. However, overexpression of glucosyl and methyl transferases in *A. thaliana* suppressed the accumulation of SA and SA-glucoside, and the *AtSGT1* and *OsBSMT1* mutants became more susceptible to disease [21,22]. Like SA and SA-glucoside concentrations in the xylem sap of *B. napus* [15], the concentrations of free SA in wild type plants measured in the present study were strongly modulated by infection of *V. longisporum*. *V. longisporum* penetrates the roots within 60 hpi and starts to colonize the xylem of the shoot three weeks after inoculation [23]. Accordingly, an increase of free SA in the hypocotyl was observed in wild type plants one week after inoculation in the present study. Previous studies showed that the SA-dependent defense pathway was not effective to increase resistance to all pathogens studied [13,14]. SA has been reported to be involved in basal defense and to induce resistance to *Oidium neolycopersici* in tobacco and to *Botrytis cinerea* in tomato; however, SA-deficient *NahG* transformed tobacco and tomato did not enhance the susceptibility to *B. cinerea* and powdery mildew, respectively [13,14]. As described by Johansson et al. [24], no enhanced susceptibility to *V. longisporum* was found in a *Nah*G mutant of *Arabidopsis*. The *Nah*G transformant in the present study showed a remarkable increase in susceptibility to *V. longisporum*, highlighting that SA probably plays an important role in basal defense of *B. napus* against *V. longisporum*. Although no linear correlation exists between SA levels and fungal growth, a threshold level of SA is required for resistance of *B. napus* to *V. longisporum*.

However, endogenous free SA was not accumulated by exogenous application of 0.5 mM of SA to the roots, which neither had direct negative effects on *V. longisporum* nor was it phytoxic to *B. napus* [25]. In *Arabidopsis*, SA pretreatment did not show any significant alterations in fresh weight loss or symptoms caused by *V. longisporum* [24]. Similarly, in our experiment, no clear reduction in *V. longisporum* biomass and in dry weight losses of roots and stems was observed in SA pretreated *Nah*G transformants and wild type plants.

3.2. Role of SA and Phenolic Acids in Cultivar Resistance of B. napus to V. longisporum

Cultivar-related resistance was associated with a significantly higher increase of SA relative to fungal biomass in the early asymptomatic interaction stage (7 dpi). The resistant cultivar reacted much stronger by shifting more BA to SA. SA can also directly bind to NPR1, the core compound of the SA signaling network, forming a copper-binding transcription-regulator to activate the expression of *PR1* [26]. In contrast to *A. thaliana* [17], *B. napus* showed a strong enhancement of expression of SA-mediated marker genes *PR1* and *PR2* upon *V. longisporum* infection, while JA-dependent *PDF1.2* was relatively stable or even down-regulated.

Lignin is an aromatic polymer that is deposited during secondary cell wall thickening providing a physical barrier against initial pathogen penetration and colonization. The biosynthesis of SA from phenylalanine and lignin synthesis both depend on PAL activity, which may lead to a competitive relationship between these two synthesis pathways [27]. The phenylalanine route branches into lignin synthesis from *t*CA catalyzed by the enzyme C4H, and the synthesized lignin monomers are transported to the cell wall and polymerized by POX [28]. In *Arabidopsis*, soluble phenylpropanoids were involved in the defense response against *V. longisporum* infection. Such accumulated soluble phenolic compounds may be toxic to pathogens [16]. Maury et al. [29] demonstrated that tobacco compromised in O-methyltransferase activity produced lower amounts of phenolic bacterial virulence

gene inducers and thus had smaller tumors caused by *Agrobacterium tumefaciens*. Previous studies showed that both conjugated and free phenolic acids were induced by *V. longisporum* infection, and higher levels were found in hypocotyls of resistant cultivars of *B. napus*, thus indicating a role in cultivar resistance [18].

In the present study, production of SA declined in the resistant cultivar starting from 14 dpi, which coincided with a strong increase in levels of phenolic acids. However, enzymes involved in lignin synthesis were already activated at 7 dpi, which occurred alongside with an enhanced expression of the related genes. Our study showed consistently higher activity of POX in the resistant cultivar from 7 to 21 dpi and a strong up-regulation of *POX* until 14 dpi indicating that the resistant cultivar may have accumulated sufficient amounts of lignin by 21 dpi after infection, which is in agreement with previously shown histochemical analyses [18].

V. longisporum is considered a hemibiotroph, which has a biotrophic life phase in the roots and the xylem and a late necrotrophic phase in the stem parenchyma [30]. As described in previous studies, some pathogen effectors, such as VdIsc1 secreted by *V. dahliae*, can target SA signaling in plants to enhance virulence by preventing SA accumulation [31]. Necrotrophic pathogens, such as *B. cinerea* and *Alternaria solani*, have been reported to enhance the SA signaling pathway in order to antagonize jasmonic acid and to promote disease development in tomato [32]. Accordingly, in the early biotrophic stage of infection, *V. longisporum* may be able to secrete an effector targeting SA synthesis and thus reducing SA levels in susceptible cultivars and allowing higher infection. Since SA is important for basal resistance in oilseed rape, and SA may be induced by infection with biotrophic or hemibiotrophic pathogens [31], avirulent strains of viruses or biotrophic fungi may be efficient biocontrol agents to prevent infection with *V. longisporum* by inducing enhanced levels of SA.

Until present, responses of *B. napus* to *V. longisporum* infection on the metabolomic and transcriptomic level were assessed in relation to plant but not to fungal biomass. This resulted in poor contrasts between responses in resistant and susceptible tissues as their expression was similar related to the same sample volume although responses relative to fungal biomass strongly differed. We believe that the latter is more relevant to accurately describe plant responses to a certain unit of pathogen biomass.

4. Conclusions

In summary, a remarkable increase of susceptibility to *V. longisporum* was observed on SA-deficient transgenic *Nah*G oilseed rape indicating that SA plays a role in basal resistance of *B. napus* to *V. longisporum*. In cultivar-related resistance, a stronger increase of SA relative to fungal growth was observed in the resistant cultivar, indicating an important role of elevated SA in defense during early stages of infection, while at later stages, when SA responses do not differ anymore between cultivars, higher levels of phenolic acids are associated with cultivar resistance.

5. Materials and Methods

5.1. Plant Material and Cultivation

Four cultivars and genotypes, *B. napus* cv. Drakkar, *NahG* transformed Drakkar, Falcon (Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, NPZ, Hohenlieth, Germany), SEM 05-500256 (SEM, Syngenta, Germany) were used in the study. Cultivar Falcon is a susceptible German commercial winter oilseed rape cultivar, and SEM is a winter type breeding line resistant to *V. longisporum* [18]. Seeds of these cultivars were surface sterilized with 70% ethanol for 1 min under constant shaking and subsequently rinsed with sterilized ddH_2O and pre-germinated in quartz sand. Plants were kept in a climate chamber with a 16 h photoperiod and a temperature of 22 ± 2 °C for 12 days before root inoculation with *V. longisporum*.

5.2. Treatments and Experimental Design

To study the basic function of SA and its role in cultivar-related resistance, pot experiments were conducted in a completely randomized block design under climate chamber conditions and repeated twice. The study on the basic function of SA consisted of a combination of three experimental factors, which were genotype (a spring oilseed rape genotype Drakkar and its *NahG* transformant), disease (mock-inoculated and *V. longisporum*-inoculated) and root-dip treatment with SA or water. Treatments were arranged with four biological replicates each composed of 10 plants grown independently in separate pots. The study of cultivar-related resistance consisted of a combination of two experimental factors, which were genotype (two winter oilseed rape genotypes Falcon and SEM) and disease (mock-inoculated and *V. longisporum*-inoculated). Treatments were arranged in a randomized pattern with four biological replicates each composed of 20 plants grown independently in separate pots.

5.3. Production of Transgenic B. napus Expressing the NahG Gene

Seeds of transgenic OSR carrying the *NahG* gene were kindly provided by Christian Möllers (Division of Plant Breeding, University of Göttingen). The plasmid used for transformation had been constructed by Corinna Thurow and Christiane Gatz (Institute of Plant Biochemistry, University of Göttingen). Briefly, hypocotyl segments of *B. napus* cv. Drakkar (spring type) were transformed by *Agrobacterium* mediated transformation [33,34]. A binary plasmid, pCAMBIA2300 (Figure 7) containing the *Nah*G gene of *Pseudomonas putida* ND6, which encodes salicylate hydroxylase [35] for degradation of SA to catechol, was used for construction of *Agrobacterium* strain AGLO. A northern blot analysis was performed for expression analysis of transformed plants. Endogenous SA levels in hypocotyl and shoot tissues of transformed plants were measured. One transformant, which had the strongest expression of the *Nah*G gene and low levels of SA, was used for the study.

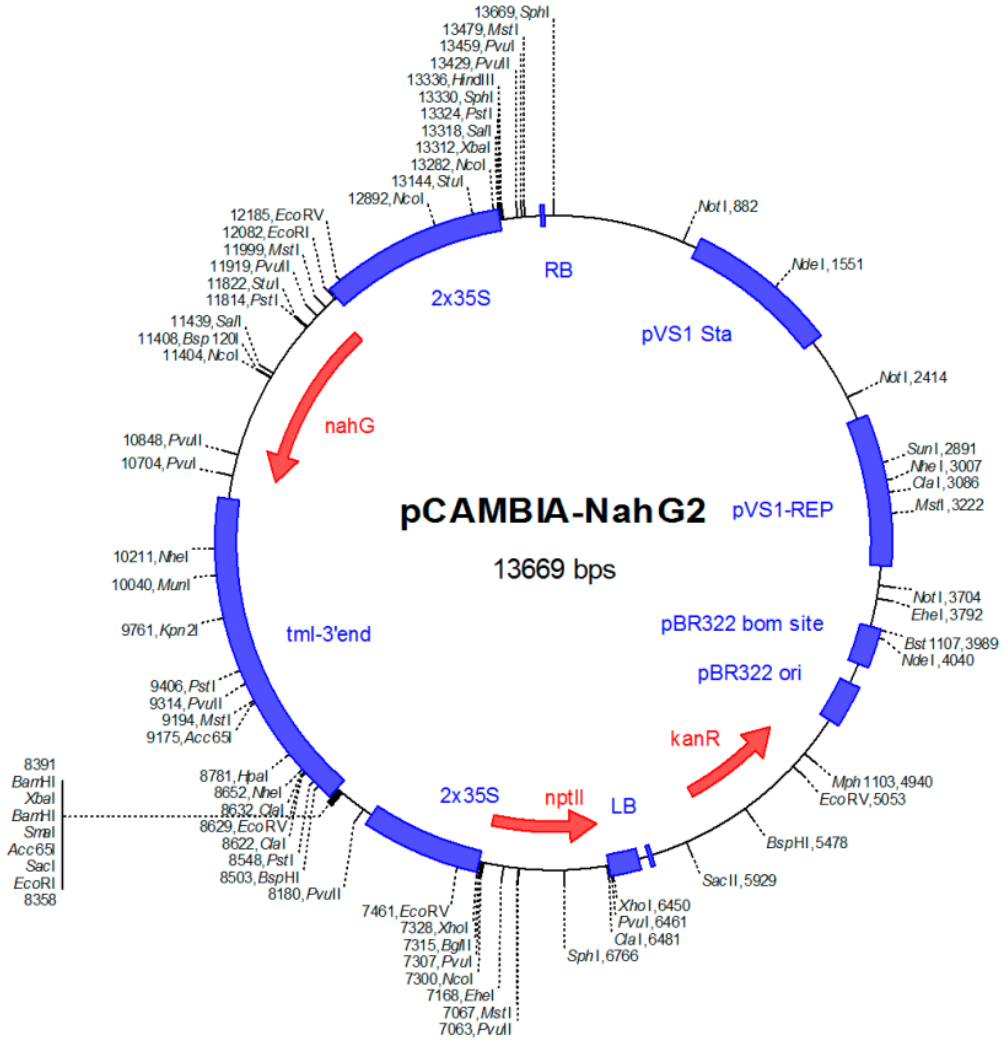

Figure 7. Structure of binary plasmid pCAMBIA2300 containing the *NahG* gene. A fragment of double 35S-*NahG* gene-tml-3´end from pCIB200-*NahG* was inserted into pCAMBIA2300 by using *Xba* I restriction enzyme recognition site. Kanamycin-resistance (*kan*R) marker was the selectable marker used for transformed plant cells. Neomycin phototransferase II (*npt* II) was the selectable marker for plant transformation.

5.4. Exogenous Application of SA

Prior to inoculation with *V. longisporum* by the root-dip method, roots of Drakkar and its *Nah*G transformant were dipped in 0.5 mM of SA or sterile tap water for 24 h. After treatment, plants were rinsed with sterile tap water several times and dried on clean filter papers.

5.5. Fungal Culture and Inoculation

V. longisporum isolate VL43 obtained from a diseased *B. napus* plant [4,36] was used for inoculations. Conidial suspension kept in 25% glycerol at −80 °C was used to initiate fresh cultures. Fungal cultures were prepared from 400 µl stock conidial suspension added to 250 ml of autoclaved (121 °C, 20 min) potato dextrose broth and then incubated on a rotary shaker at 80 rpm at 22 °C for 10 days. The resulting suspension was filtered through sterile gauze to remove mycelia.

Twelve-day-old seedlings with cotyledons completely unfolded were inoculated or mock-inoculated using a root-dip method [18]. Plant roots were rinsed with tap water and dipped in a

conidial suspension (1×10^6 cfu/ml) or water for 50 min and replanted in pots ($7 \times 7 \times 8$ cm) containing a sterile soil-sand mixture (3:1).

5.6. Disease Assessment

According to the assessment key (Table 2) described by Eynck et al. [37], disease severity (DS) was quantified at 7, 14 and 21 dpi. Plant height was measured at 21 dpi. Dry weight of roots and shoots of Drakkar and *NahG* transformants were determined at 7, 14 and 21 dpi.

Table 2. Assessment key for scoring foliar symptoms induced by *V. longisporum* on *Brassica* species inoculated with the root dip method.

Score	Symptom Development
1	No symptoms
2	Weak symptoms on the oldest leaf (yellowing, black veins)
3	Weak symptoms on the next younger leaves
4	About 50% of the leaves have symptoms
5	More than 50% of the leaves have symptoms
6	Up to 50% of the leaves are dead
7	More than 50% of the leaves are dead
8	Only apex is still alive
9	The plant is dead

5.7. Extraction and Quantification of Fungal DNA

Total DNA from hypocotyl samples was extracted using a cetyltrimethylammonium bromide (CTAB) method [38]. About 100 mg of ground fresh plant tissue was suspended in 1 ml CTAB with 2 µl *ß*-mercaptoethanol and 1 µl 1x proteinase K. The extracted DNA was dissolved in 200 µl TE buffer.

A CFX384 real-time PCR detection system (Bio-Rad Laboratories Inc., Kabelsketal, Germany) was used for the amplification and quantification of *V. longisporum* DNA using primers OLG70 (*5′-CAGCGAAACGCGATATGTAG-3′*) and OLG71 (*5′-GGCTTGTAGGGGGTTTAGA-3′*) [39]. The amplification mix consisted of 1× $(NH_4)_2SO_4$ buffer, 2.5 mM of $MgCl_2$, 100 µM of dNTPs, 0.02 U/µl of BioTaq DNA polymerase (Bioline, Luckenwalde, Germany), 0.1x SYBR Green I solution (Invitrogen, Karlsruhe, Germany), 0.3 µM each of primers OLG70 and OLG71 and 1 µl of template DNA and filled up to a total volume of 10 µl with ddH_2O. PCR conditions were as described in Table 3. PCR for all treatment samples were performed with four biological and three technical replicates and data were analyzed using CFX Manager Software.

Table 3. PCR program for quantification of DNA of *V. longisporum* and gene expression assay.

Step	qPCR for Quantification of *V. longisporum*	RT-qPCR for Gene Expression
Initial Denaturation	95 °C, 4 min	95 °C, 4 min
Denaturation	95 °C, 10 s	95 °C, 10 s
Annealing	60 °C, 15 s	64.8 °C, 15 s
Extension	72 °C, 15 s	72 °C, 15 s
Repeat Times	40 cycles	40 cycles
Melting Curve Analysis	55 to 95 °C	60 to 95 °C

5.8. Quantification of Endogenous SA

Free SA was extracted from hypocotyl tissue according to a method modified from Kamble et al. [40]. About 200 mg of liquid nitrogen ground fresh hypocotyl samples was suspended in 1.5 ml of acetone, shaked vigorously and centrifuged at 5500 rpm at 4 °C for 45 min. The supernatant was transferred 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 added subsequently. The upper phase from the

mixture was transferred and evaporated to dryness at 35 °C. The residue was dissolved again in 200 µl of HPLC grade methanol.

A dilution series of 100 nM to 20 µM of SA dissolved in HPLC grade methanol was used as internal standard. The peak of SA was identified by comparing retention times of samples and standards and confirmed by addition of standard SA to the samples. Before loading into a HPLC vial, all samples or standards were centrifuged at 5000 rpm for 5 min to precipitate unsolvable particles to prevent injection problems.

A HPLC-fluorescence system consisting of a Varian 410 automatic injector, two Varian 210 pumps with 10 W SS head, a Lichrospher RP-18 column (250 × 4 mm, 5 µm) protected by a Security Guard™ Carbo-H precolumn (4 × 3 mm, 5 µm) kept at 30 °C and a Varian 363 fluorescence detector with excitation wavelength at 315 nm and emission wavelength at 405 nm. Each sample was analyzed for 33 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: initial 10% B for 2 min, linear gradient to 38% B in 13 min, increased to 98% B in 30 s and held for 9 min, equilibrated to initial condition in 30 s and hold for 8 min. The injection volume was 10 µl.

5.9. Quantification of Phenolic Acids in Hypocotyls

Phenolic acids were extracted from hypocotyl following published protocols [18,41,42]. About 200 mg of hypocotyl samples ground in liquid nitrogen were suspended in 2 ml of 80% methanol with 0.2 mg/ml of 2,6-di-tert-butyl-4-methylphenol. The mixture was well mixed and sonicated for 10 s. After incubation for 30 min at room temperature, the mixture was centrifuged at 1000 rpm for 10 min. The procedure was repeated twice.

Free phenolic acids. The supernatant was transferred and evaporated in a speed vacuum centrifuge at 30 °C. The residue was dissolved in 1 ml demineralized water, and 0.5 µl of 37% HCl was added to adjust to pH 2–3. Ethyl acetate (1 ml) was used twice for extraction of free phenolic acids from the crude extract. After thoroughly mixing, the upper phase was transferred and evaporated to dryness at 35 °C. The residue was dissolved again in 200 µl of 80% HPLC grade methanol.

Methanol-insoluble ester bound phenolic acids. The pellet after methanol extraction was hydrolyzed with 1 ml of 2 M NaOH at 95 °C for 1 h and mixed four times in between. For acidification of the mixture, 218 µl of 37% HCl was added. Ester bound phenolic acids were extracted twice with 1 ml ethyl acetate. The supernatant was transferred and evaporated to dryness using speed vacuum centrifugation at 35 °C. The residue was dissolved in 200 µl of 80% HPLC grade methanol.

A standard series mix of CA, *p*CA, FA, SiA, *t*CA, BA and SA dissolved in 80% HPLC grade methanol, from 50 ppb to 10 ppm for each compound, was used as internal standards. Peak assignment of each component was made by comparing retention times of samples and standards as well as by comparing the UV absorption spectra (200~500 nm) of analytes to purchased standards.

The HPLC-fluorescence/DAD system consisted of a JASCO AS-2051 Plus intelligent sampler (4 °C), a DG-2080-54 4-line degasser, an LG-2080-04S quaternary gradient unit, a Kinetex EVO C18 column (250 × 4 mm, 5 µm) protected by a Gemini NX C18 guard column (4 × 3.0 mm, 5 µm) kept in a column oven at 40 °C (CO-2060 Plus Intelligent Column Thermostat), an FP-2020 Plus intelligent fluorescence detector with excitation wavelength at 315 nm and emission wavelength at 405 nm and a MD-2015 Plus multi-wavelength detector measuring over the range of 200~500 nm. Each sample was analyzed for 65 min in a bi-mobile phase run with (A) 0.1% phosphoric acid in water and (B) 0.1% phosphoric acid in acetonitrile with a flow rate of 1 ml/min following this protocol: initial 9% B for 5 min, linear gradient to 32% B in 39 min, increased to 98% B in 30 s and hold for 10 min, equilibrated to initial condition in 30 s and hold for 10 min. The sample injection volume was 10 µl.

All analytical data from metabolite assays were recorded as µg per g plant FW and related to fungal biomass measured in the plant tissue as ng fungal DNA per g plant FW.

5.10. Enzyme Assays

The hypocotyls were harvested from experimental plants (Falcon and SEM) at 7, 14, 21 dpi. Eight to twelve fresh plant samples were ground in liquid nitrogen as one pooled sample. Four independent samples were taken from each treatment at each time point. Each sample had three technical replicates.

BA2H was extracted as described by Leon et al. [43]. About 100 mg powder was suspended in 500 µl of extraction buffer (20 mM Hepes, containing 10 mM sorbitol, 1% polyvinylpyrrolidone (PVP), 1 mM phenylmethylsulfonyl fluoride, 12.5 mM ß-mercaptoethanol, adjusted with NaOH to pH 7.0). The suspension was mixed, sonicated for 2 min and mixed again before being centrifuged for 10 min at 9,274 rpm and 4 °C. The supernatant was used as the enzyme extract. The reaction mixture (250 µl) contained 20 mM Hepes buffer, pH 7.0, 1 mM NADPH, 1 mM BA and 100 µl enzyme extract, and was incubated for 30 min at 30 °C. To stop the reaction, 125 µl of 15% (w/v) trichloroacetic acid was added. The mixture was centrifuged for 5 min at 10,000 × *g*, the supernatant was extracted twice with 250 µl of ethyl acetate:cyclopentane:isopropanol (100:99:1). The upper organic phase was evaporated to dryness for 1 h at 30 °C and the pellet was resuspended in 200 µl of HPLC grade methanol. The BA2H activity was determined as the rate of conversion of BA to SA. SA was quantified by HPLC as described above.

PAL was extracted according to Kamble et al. [44]. About 200 mg homogenized sample was suspended in 1 ml of 5 mM Tris-HCl buffer, pH 8.3 and centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was used as a crude enzyme extract. The activity of PAL was determined as the rate of conversion of L-phenylalanine to *t*CA. The reaction mixture (1 ml) contained 25 mM Tris HCl buffer, pH 8.8, 100 µM L-phenylalanine and 100 µl enzyme extract, and was incubated for 60 min at 30 °C. To terminate the reaction, 400 µl of 2 N HCl was added. For further extraction, 800 µl of toluene was added, and the samples were well mixed and then centrifuged at 1000 rpm for 5 min. The upper toluene layer was measured at 290 nm (HP845 × UV-Visible System) and pure toluene was set as blank. A series of *t*CA with concentrations of 5 µM to 80 µM in toluene was used as standards. Enzyme activity was expressed as change in *t*CA in µM/min/g FW.

C4H was extracted as described previously [45–47]. About 100 mg powder was suspended in 600 µl of extraction buffer (100 mM Tris-HCl buffer, pH 7.5 with 15 mM ß-mercaptoethanol). The suspension was well mixed, sonicated for 2 min, and mixed again before being centrifuged for 20 min at 15,000 rpm at 4 °C. The supernatant was used as the enzyme extract. The reaction mixture (1.5 ml) contained 100 mM Tris-HCl buffer, pH 7.5, 2 mM NADPH, 1.33 mM *t*CA and 250 µl enzyme extract, and was incubated for 35 min at 30 °C. To stop the reaction, 50 µl of 37% HCl was added and then adjusted to pH 11 with 2 M NaOH. The mixture was extracted twice with 1 ml of diethyl ether. After short centrifugation, the upper organic phase was evaporated to dryness for 65 min at 30 °C and the pellet was resuspended in 500 µl of 1 M NaOH, and measured at 330 nm (µQuant, Bio-Tek). The C4H activity was determined as the rate of conversion of *t*CA to *p*CA.

CAD was extracted as described by Chabannes et al. [48]. About 100 mg powder was suspended on ice in 500 µl of 100 mM Tris-HCl buffer, pH 7.5 with 2% PEG6000, 2% PVP and 5 mM freshly prepared dithiothreitol. The suspension was mixed, sonicated for 2 min, and mixed again before being centrifuged for 10 min at 9,274 rpm at 4 °C. The supernatant was used as the enzyme extract. The reaction mixture (500 µl) contained 100 mM Tris-HCl buffer, pH 8.8, 1 mM NADP, 1 mM coniferyl alcohol and 50 µl enzyme extract, and was incubated for 20 min at 30 °C. The formation of coniferaldehyde was monitored at 400 nm using the molar extinction coefficient of coniferaldehyde (2.1×10^4 M^{-1} cm^{-1}), and the activity of CAD was expressed as change in absorbance as $nkatal.g^{-1}$ FW (Sibout et al., 2003; Zhang et al., 2006).

POX measurement was carried out according to Mandal et al. [49]. About 200 mg of homogenized samples were resuspended in 2 ml of 0.1 M phosphate buffer, pH 7.5, containing 0.5 mM of Na-EDTA and 1% of PVP. The mixture was then centrifuged at 9,000 rpm for 30 min at 4 °C. The supernatant was used as enzyme extract. The activity of POX was determined as the rate of conversion of guaiacol to oxidized (dehydrogenated) guaiacol. The reaction mixture (1 ml) contained 81 mM phosphate buffer, pH 7.0, 4.5 mM guaiacol and 10 µl enzyme extract. After 30 µl of 10 mM, H_2O_2 was added to the

reaction mixture, the measurement was taken immediately at 470 nm for 7.5 min. The enzyme activity was calculated using the molar extinction coefficient of dehydrogenated guaiacol (26.6 mM^{-1} cm^{-1}) and expressed as change in absorbance as µkat/g FW.

Similar to the metabolite assays, enzyme activity data were related to fungal biomass measured in the plant tissue as ng fungal DNA per g plant FW.

5.11. Gene Expression

5.11.1. RNA Extraction and Synthesis of cDNA

About 100 mg of homogenized fresh hypocotyl tissue was suspended in 1 ml TRI-reagent (Invitrogen™, ThermoFisher) and incubated for 15 min at room temperature. Subsequently, 100 µl bromochloropropane was added, and the samples were shaken for 15 s. After incubation for 15 min at room temperature, the mixture was centrifuged for 15 min at 10,159 rpm at 4 °C. For RNA precipitation, 0.5 ml isopropanol was added to the upper aqueous phase (RNA), mixed for 10 s, incubated for 10 min at room temperature, and centrifuged for 10 min at 10,159 rpm at 4 °C. The supernatant was gently discarded, and the pellet was washed with 75% ethanol (freshly prepared in DEPC water). The dried RNA was dissolved in 30 µl DEPC treated ddH_2O. To check the quality of extracted total RNA, 4.5 µl of RNA was added to a mixture containing 1 × formaldehyde gel-running buffer, 50% formamide, 17.5% formaldehyde, and then incubated for 15 min at 65 °C and immediately chilled on ice to break down the secondary structure. Treated RNA was run on 1.5% agarose gel using TBE buffer.

The samples with good RNA quality were digested again with DNase to remove contaminant genomic DNA before being used for cDNA synthesis. The quality of the digested RNAs was confirmed again on 1.5% agarose gel. The RNA concentration was determined using a microplate spectrophotometer (Epoch, Bio-Tek) at 260 nm and controlled by the ratio OD_{260}/OD_{280}. For reversing mRNA to cDNA, a First Strand cDNA Synthesis Kit (ThermoFisher) with oligo dT_{18}, M-MLV reverse transcriptase and RiboLock RNase inhibitor (1 U/reaction) was used according to the manufacturer's protocol.

5.11.2. Reverse Transcription Quantitative PCR (RT-qPCR)

Primers for selected genes were constructed by using online primer tools such as Primer3 (Version 4.0), IDT OligoAnalyzer 3.1 with the help of sequence databases (http://www.ncbi.nlm.nih.gov and http://www.brassocadb.org). The sequences (5´ to 3´) of forward (F) and reverse (R) primers of each candidate gene used for RT-qPCR are listed in Table 4. The efficiency of each primer was tested by using standard cDNA copies. RT-qPCR was performed in a CFX384 real-time PCR detection system to determine relative gene expression levels with *actin-7* and *GAPDH* as endogenous reference genes with four independent biological replicates using SYBR Green for staining. PCR conditions were as described in Table 3. A no-template control was included in each experiment. Expression values considering the primer efficiency were normalized to the endogenous reference genes with the formula following Pfaffl [50], and the log2-fold change was related to fungal biomass measured in the plant tissue as ng fungal DNA per g plant FW.

Table 4. Primers used for reference and candidate genes in the biosynthetic pathway of salicylic acid and lignin.

Gene	NCBI Accession		Primer Sequence	PCR Efficiency [%]	Reference
4CL	XM_013895971.1	F R	ACGCCGAGATGAAAATCATC CCGTCTTTGTCAATGGTCTC	106.2	This study
ACT7	NM_001316079.1	F R	GCTGACCGTATGAGCAAAG AAGATGGATGGACCCGAC	73.6	Wang et al. 2014
C3H	XM_013879044.1	F R	AGACCAGAGAGGTTCTTGGA CGAGTCCAGGGTTTTCAGAC	119.4	This study
C4H	XM_013888134.1	F R	GTATGTGCCGTTTGGTGTTG GGACCTTGGCTTCATTACGA	73.8	This study
CAD	XM_013817405.1	F R	GGTGGCTTCGCTGACACTAT TCACACCCATGTGTCCAACT	70.2	This study
CCoAMT	XM_013799238.1	F R	TTCAAGGCAGCACACGATAG TGCCATACTTGTGGACCGTA	127.3	This study
CCR	XM_013836581.1	F R	TCCGCTAAGACTTACGCTAATC CCTCGTAGACCAGCACATGG	74.8	This study
COMT	XM_013793239.1	F R	CCGGAAAAAGGGAAAGTGATC TCACATCGAATAAAACCTGACC	123	This study
GAPDH	XM_013856115.1	F R	CGCTTCCTTCAACATCATTCCCA TCAGATTCCTCCTTGATAGCCTT	95.6	Alkooranee et al. 2015
PAL4	XM_013817346.1	F R	GGCACGGACAGTTATGGAGT GCCGACTTAGGTAGCGTGAG	96.8	This study
PDF1.2	XM_013862352.1	F R	ATCACCCTTCTCTTCGCTGCTCTC CATACTCCTGACCATGTCCCACTAG	109.4	Wu et al. 2016
POX	XM_013786965.1	F R	CTCTCTGGGGGTCACACATT TGTCGAAAACCGTAGGGGTA	82.3	This study
PR1	XM_013877950.1	F R	AAAGCTACGCCGACCGACTACGAG CCAGAAAAGTCGGCGCTACTCCA	108.8	Alkooranee et al. 2015
PR2	AF229403.1	F R	GTACGCTCTGTTCAAACCGACCC TTTCCAACGATCCTCCGCCTGA	109.1	Alkooranee et al. 2015

5.12. Statistical Analysis

The experimental data were analyzed as completely randomized designs with four replications using STATISTICA 13.2. All data were normal distributed and analyzed using factorial ANOVA. A multiple comparison was analyzed by Fisher LSD test. To analyze the relationship between disease severity and physiological parameters, Pearson's linear correlation was performed, and correlation coefficients were calculated. The experimental results were presented as means ± standard error at 5% significance level. A principal component analysis was performed using R package factoextra and FactoMineR.

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Chapter 6: Disease development of *Verticillium longisporum* on *Brassica napus* via artificial stem inoculation

6.1 Introduction

Verticillium longisporum (VL) is a soil-borne host-specialized vascular pathogen on Brassicaceae, of which the mycelium is normally hyaline, simple or branched, septate and multinucleate (Johansson et al. 2006; Depotter et al. 2016). Compared with other *Verticillium* species, *V. longisporum* has larger and longer conidia (Eynck et al. 2007; Inderbitzin et al. 2011a; Inderbitzin et al. 2011b). The morphological character of *Verticillium* spp. on PDA medium is diverse. *Verticillium longisporum* normally forms elongated, irregular and darkly pigmented microsclerotia. These microsclerotia on the colony show a typical 'web' of black radiating lines embedded in the medium (Karapapa et al. 1997; Inderbitzin et al. 2011a). As described by Inderbitzin et al. (2011b), *V. longisporum* is diploid and has three different lineages deriving from at least three independent hybridization of partly unknown species. Unknown species A1 as a common parent hybridized either with an unknown species D1, *V. dahliae* lineage D2 or *V. dahliae* lineage D3. Among these three lineages, lineage A1/D1 is the most virulent *V. longisporum* lineage on *Brassica napus*, which shows stem striping symptoms on pre-mature plants in the field and may cause significant reductions of yield (Dunker et al. 2008; Novakazi et al. 2015; Depotter et al. 2017b; Depotter et al. 2017c). *Verticillium longisporum* isolate VL43 originated from *B. napus* grown in Mecklenburg-Vorpommern, a federal state in the northeast of Germany, and belongs to lineage A1/D1, which is supposed to sub-cluster into an A1/D1 East population according to its relative localization in Europe and A1/D1 West which appeared to be genetically more diverse (Zeise and von Tiedemann 2001, 2002a, 2002b; Depotter et al. 2017a).

Germinated hyphae of *V. longisporum* colonize and grow along the surface of the fine roots in the root hair zone towards the root surface (Eynck et al. 2007; Zhou et al. 2006). After adhering to root hairs and entering the root by direct penetration, hyphae grow both intercellularly and intracellularly in the root cortex and further spread to the central vascular bundle (Fig. 6.1). Conidia may be produced in vessels and be transported upward by the transpiration stream. Artificially infected plants of *B. napus* in controlled growth conditions exhibit chlorosis, vascular discoloration and stunting in young plants (Eynck et al. 2007; Zeise and von Tiedemann 2002b). The microsclerotia formed during plant senescence and ripening beneath the stem epidermis and in the stem pith are released into the soil with plant debris following tissue decomposition (Heale and

Karapapa 1999). The density of microsclerotia of *V. longisporum* in the soil is lowest shortly after harvest, but it can accumulate in the soil or within the plant stubble and reaches its maximum six months later (Johansson et al. 2006; Depotter et al. 2016).

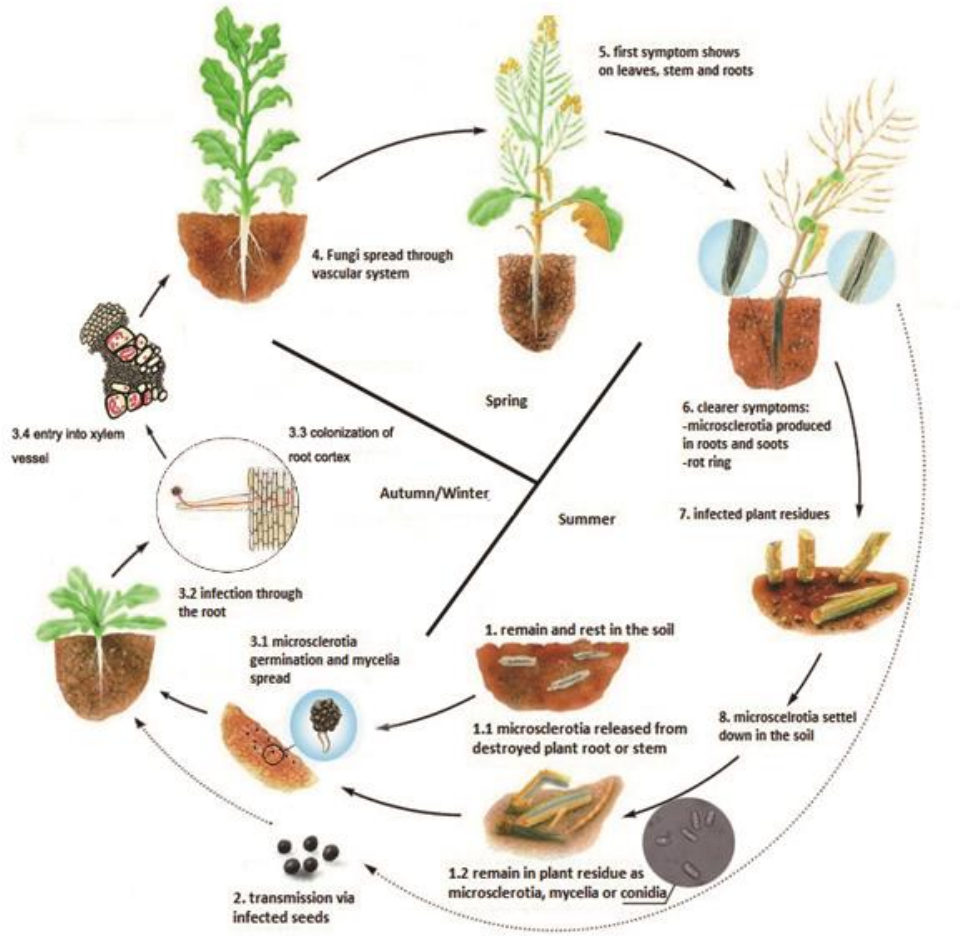


Figure 6.1 Life cycle of *Verticillium longisporum* on *Brassica napus* (modified from Paul 2003; Rowe and Powelson 2002; Depotter et al. 2016)

The hypocotyl of *B. napus* has been assumed to be a crucial tissue, expressing resistance and hindering *V. longisporum* to colonize the entire plant (Eynck et al. 2009a; Kamble et al. 2013). However, no investigation has been conducted so far to explore whether the stem tissue beyond the hypocotyl may also be involved in expression of susceptibility of resistance to *V. longisporum*. The aim of this study is to observe the disease development of *V. longisporum* on *B. napus* stems via artificial stem inoculation and to reveal the importance of the stem tissue in the interaction of *B. napus* and *V. longisporum*.

6.2 Materials and methods

6.2.1 Plant material

Two cultivars, *B. napus* L. cv. Falcon (Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, NPZ, Hohenlieth, Germany) and SEM 05-500256 (Syngenta, Germany) were used in

this study. Cultivar Falcon is a German commercial winter oilseed rape susceptible to *Verticillium* spp., *Sclerotinia* spp. and *Fusarium* spp.. SEM 05-500256 is resistant to *V. longisporum*. Seeds of these cultivars were surface sterilized with 70% ethanol for 1 min under constant shaking, and subsequently rinsed with sterilized ddH_2O. Seeds were then sown in multi-pot trays filled with a sterile soil-sand mixture (3:1) and kept in the greenhouse at 22±2˚C for 14 days. As soon as the first true leaf appeared, plants were transferred for ten weeks to 4°C for vernalization. After the vernalization, seedlings were transplanted in pots (7 x 7 x 8 cm) with a sterile soil-sand mixture (3:1) and kept in the greenhouse at 22±2˚C.

6.2.2 Fungal culture and inoculation

Verticillium longisporum isolate VL43 obtained from a diseased *B. napus* plant was used for inoculation. Conidial suspension, which had been kept in 25% glycerol at -80˚C, was used to initiate fresh cultures. For preparation of the fungal culture, 400 µl the stock conidial suspension was added into 250 ml autoclaved (121˚C, 20 min) potato dextrose broth (PDB), and then incubated on a rotary shaker at 80 rpm at 22˚C for 10 days. The resulting suspension was filtered through sterile gauze to remove mycelia, and the conidial concentration was adjusted with sterile water or sterile PDA medium (40°C) to 1 x 10^6 cfu/ml. The 3rd internode counted from the plant base at BBCH 34 was punctured with a sterile injection syringe (0.3 mm x 8 mm, Micro-Fine^{TM}+) or left without injury. Subsequently, 2 µl of conidia suspension or sterile water was pipetted and injected into the wound (Fig. 6.2). For plants without injury, an agar plug with 0.5 cm in diameter containing conidia (1 x 10^6 cfu/ml) was placed on the stem surface and fixed with Parafilm® M laboratory film (Bemis® Company Inc.).

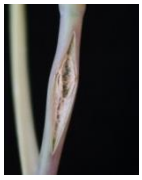
Figure 6.2 Stem inoculation of *Verticillium longisporum* by an injection syringe.

6.2.3 Disease assessment

The development of disease symptoms was observed and an assessment key (Tab. 6.1) was used to evaluate the effect of disease at 3, 7, 10, 14, 21 and 28 dpi. The length of

wounds on inoculated stems and plant height were measured at 28 dpi. Biomass of *V. longisporum* from 4 cm long part of inoculated stems was quantified by qPCR to verify the scores that derived from the assessment key for disease severity.

Table 6.1 Assessment key for *Verticillium longisporum* disease on *Brassica napus* via stem inoculation

Score	Description of Symptoms	Symptoms
1	No symptom, inoculation point is green and round.	
2	Brown or violet pigments visible around inoculation point, inoculation point is still in round form.	
3	Stem is swelling at the inoculation point or shows small cracks.	
4	Inoculation point is elongated, pointed at each end and slightly chapped.	
5	Stem is chapped emanating from the inoculation point, inside it is green.	
6	Stem is chapped and inside it is white.	
7	Typical symptoms of *V. longisporum* (yellowing, black veins) visible on leaves.	

6.2.4 Re-isolation of *V. longisporum* from diseased oilseed rape after stem inoculation

According to Koch's postulates, the fungus was re-isolated from diseased stems. About 4 cm of diseased stem tissue was collected and homogenized in 15 ml sterile ddH_2O by a mixer sterilized with 70% ethanol. The mixture was diluted 1:10 and 1:100, of which 200 µl was placed by triangle applicator on semi-*Verticillium* selection medium or SNA medium

(Tab. 6.2). The inoculated plates were kept in the dark at room temperature for 10 days. Single colonies were selected, transferred on PDA medium containing 200 ppm streptomycin and again kept in the dark at room temperature for 14 days.

Table 6.2 Semi-*Verticillium* selective medium (Menzies and Griebel 1967; Huisman and Ashworth 1974)

Material	Concentration
Soil extract	24 ml/l
KH_2PO_4	1.5 g/l
K_2HPO_4	4 g/l
Tergitol	1 ml/l
Sodium polypectate	2 g/l
Saline solution containing 100g/l KH_2PO_4, 50 g/l KCl, 50 g/l $MgSO_4$, 1 g/l $FeSO_4$, 200 g/l $NaNO_3$	2 ml/l
Agar	12 g/l
Chloramphenicol	3 mg/l
Chlortetracycline	3 mg/l
Streptomycin	3 mg/l
Biotin	0.3 mg/l

6.2.5 Morphological identification of fungal colonies

The morphology of colonies was observed under the stereo-microscope, and the form of hyphae and spores was identified by observation with light microscopy. Small amounts of mycelia were used for identification by multiplex qPCR.

6.2.5.1 Rapid mini-preparation of fungal DNA

According to rapid fungal DNA extraction method described by Liu et al. (2000), a lump of mycelia was add to 500 µl lysis buffer containing 400 mM Tris-HCl (pH 8.0), 60 mM EDTA, 150 mM NaCl and 1% sodium dodecyl sulfate (SDS) and incubated at room temperature for 10 min. After lysis, 150 µl 3 M potassium acetate (pH 4.8) was added, mixed briefly and centrifuged at 10,000 rpm for 1 min. The supernatant was transferred to a new tube and centrifuged again at 10,000 rpm for 1 min. Clear supernatant (550 µl) was transferred to a new tube and mixed by inversion with 480 µl isopropyl alcohol for extraction of fungal DNA. After centrifuging at 10,000 rpm for 2 min, the supernatant was discarded and the resultant DNA pellet was washed with 300 µl 70% ethanol. The supernatant was removed carefully with a pipette after centrifugation at 10,000 rpm for 1 min. The washed pellet was dried in a centrifugal concentrator for 10 min at 30°C and dissolved 10 µl TE buffer.

6.2.5.2 Multiplex PCR assay for fungal identification

A PCR system (Bio-Rad laboratories, Inc.) was used for the amplification and identification of *V. longisporum* DNA using three primer pairs (Tab. 6.3), as described by Inderbitzin et

al. (2013).

Table 6.3 Primers for *Verticillium dahliae-V. longisporum* lineages multiplex PCR assay.

Name	Sequence (*5'-3'*)
VL-D1f	CCCCGGCCTTGGTCTGAT
VL-ALfD1r	TGCCGGCATCGACCTTGG
VL-A1f	AAGTGGAGCCCCGTATCTTGAAT
VL-A1r	CAACTGGCAACAGGGCTTGAAT
VL-Df	CCGGTCCATCAGTCTCTCTG
VL-Dr	CTGTTGCCGCTTCACTCG

The amplification mix consisted of 12.5 µl GoTaq® G2 Hot Start Colorless Master Mix (PROMEGA, USA), 0.5 µM each of primers VL-D1f and VL-AlfD1r, 0.5 µM each of primers VL-A1f and VL-A1r, 0.25 µM each of primers VL-Df and VL-Dr and 1 µl of template DNA and filled up to a total volume of 25 µl with sterile ddH_2O. The PCR-program considers a 2 min initial denaturation step, followed by 41 cycles with a denaturation step (10 s at 95°C), annealing (20 s at 65°C) and subsequent extension (30 s at 72°C). The program was completed with a final elongation step of 7 min at 72°C. The PCR products were checked by 1% agarose gel with 100 bp marker and DNA of reference isolates 0120 (A1/D1), 1199 (A1/D2), 1194 (A1/D3) and 1189 (*V. dahliae*).

6.2.6 Aggressiveness of *Verticillium* isolates isolated from inoculated stems of *B. napus*

The aggressiveness of isolates was tested by direct root dipping of the susceptible cultivar *B. napus* cv. Falcon in 1 x 10^6 cfu/ml conidia spore suspension. The assessment key (Tab. 6.4) described by Eynck et al. (2009b) was used for quantification of the effect of disease. The plant height was measured at 28 dpi.

Table 6.4 Assessment key for scoring foliar symptoms induced by *Verticillium longisporum* on *Brassica* species inoculated with the root dip method.

Score	Symptom development
1	No symptoms
2	Weak symptoms on the oldest leaf (yellowing, black veins)
3	Weak symptoms on the next younger leaves
4	About 50 % of the leaves have symptoms
5	More than 50 % of the leaves have symptoms
6	Up to 50 % of the leaves are dead
7	More than 50 % of the leaves are dead
8	Only apex is still alive
9	The plant is dead

6.2.7 Re-inoculation of collected isolates via stem inoculation on *B. napus*

To confirm the symptoms caused by stem inoculation of *V. longisporum*, the identified isolates, which were tested as most aggressive, were used for re-inoculation of stems of *B. napus* plants. The inoculation was performed as described in 6.2.2 and 6.2.3.

6.2.8 Statistical analysis

The experimental data was analyzed as completely randomized designs with four replications using STATISTICA 13.2. Before testing for statistical significance, a probability plot with the Shapiro-Wilk test was used to test for the normal distribution of data. Suitable data were analyzed using factorial ANOVA. A multiple comparison was analyzed by Fisher LSD test. A nonparametric Kruskal-Wallis ANOVA and multiple comparison of mean were applied to compare independent multiple samples. The experimental results were presented as means ± standard error at 5% significance level.

6.3 Results

6.3.1 *V. longisporum* disease development in *B. napus* via stem inoculation

No symptoms were observed on plants without injury after *V. longisporum* inoculation with superficially attached agar plugs. However, plants with injury after injection of *V. longisporum* showed symptoms of infection and were scored from 1 to 7 as described above. Four to twelve plants with corresponding score were collected.

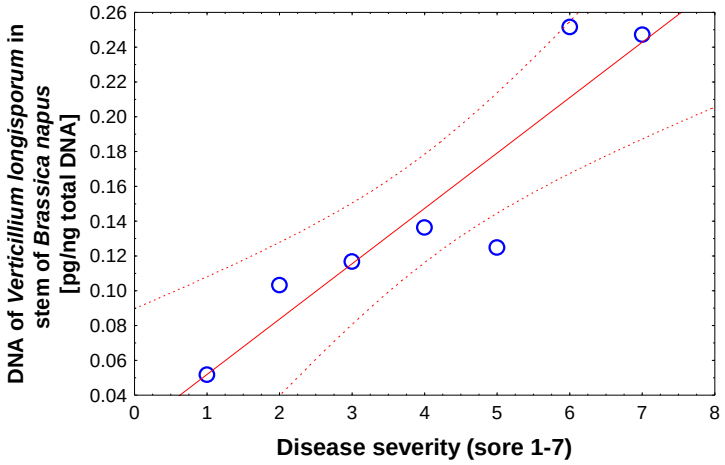


Figure 6.3 Correlation of disease scores with biomass of *Verticillium longisporum* in stems of *Brassica napus* cv. Falcon and SEM. Samples for DNA analysis were taken according to disease scores at different time points.

From a stem section excised from two centimeters above and two centimeters below the inoculation point, plant tissue was used for quantification of biomass of *V. longisporum*. The means of fungal biomass were positively correlated with assessment scores ($y = 0.0318x + 0.0201$, $R^2 = 0.8468$, P=0.03) (Fig. 6.3).

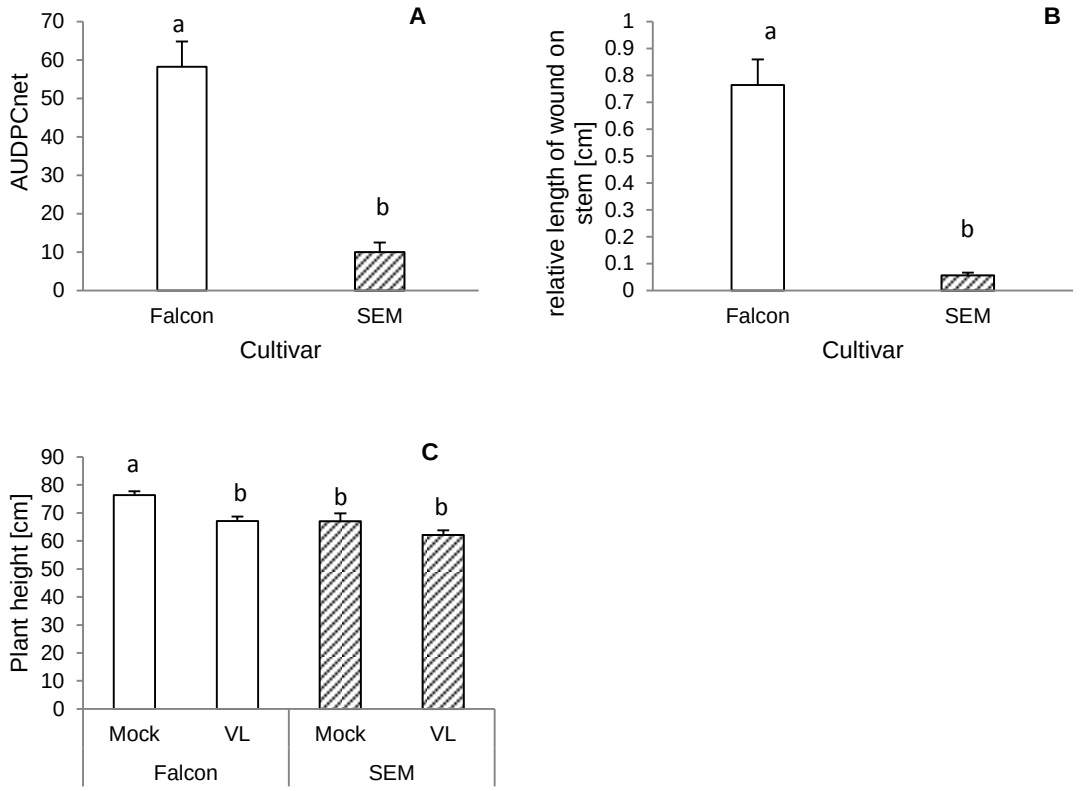


Figure 6.4 Development of *Verticillium longisporum* on *Brassica napus* cv. Falcon and SEM after stem inoculation determined by disease severity (A), relative length of wound on stem (B) and plant height at 28 dpi (C). Bars indicate standard errors. Different letters indicate significant differences among the treatments (Kruskal-Wallis test for AUDPCnet and relative length of wound on stem, LSD test for plant height, $P<0.05$).

Both disease severity (Fig. 6.4A) and length of wound on stem (Fig. 6.4B) were more severe in the susceptible cultivar Falcon. A clear opening of the inoculation points on the susceptible cultivar was observed already at 7 dpi. In contrast, the wounds in the resistant cultivar SEM were very small, and stems did not chap after inoculation by *V. longisporum*. However, a small portion of plants showed abnormal leaf yellowing at 28 dpi. Moreover, a significant reduction of plant height due to infection was observed in the susceptible but not in the resistant cultivar (Fig. 6.4C).

6.3.2 Re-isolation and identification of *V. longisporum* from diseased *B. napus*

Twenty *V. longisporum*-like isolates grown as single colonies on PDA were found from the semi-*Verticillium* selection medium or SNA and identified by stereo-microscopy. Although the conidiophores (Fig. 6.3A) and conidia (Fig. 6.5B) of the selected fungus were the same as described by Enyck et al. (2007), the characters of colonies were variable and did not show the typical 'web' of black radiating lines embedded in the medium.

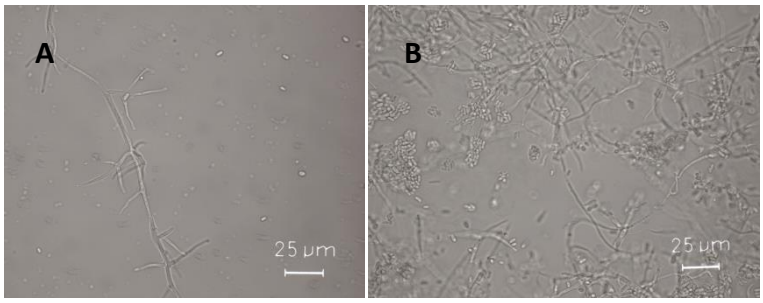


Figure 6.5 Microscopic appearance of *Verticillium longisporum in vitro.* A, verticillate conidiophores; B, conidia.

Therefore, colonies were grouped according to four different patterns (Fig. 6.6): I, white in the center and strongly black pigmented microsclerotia formed on an outer ring of the colony; II, black pigmented, microsclerotia formed strongly in the entire colony; III, white colony without any pigmented microsclerotium; IV, white in the center and small amount of black pigmented microsclerotia formed on an outer ring of the colony.

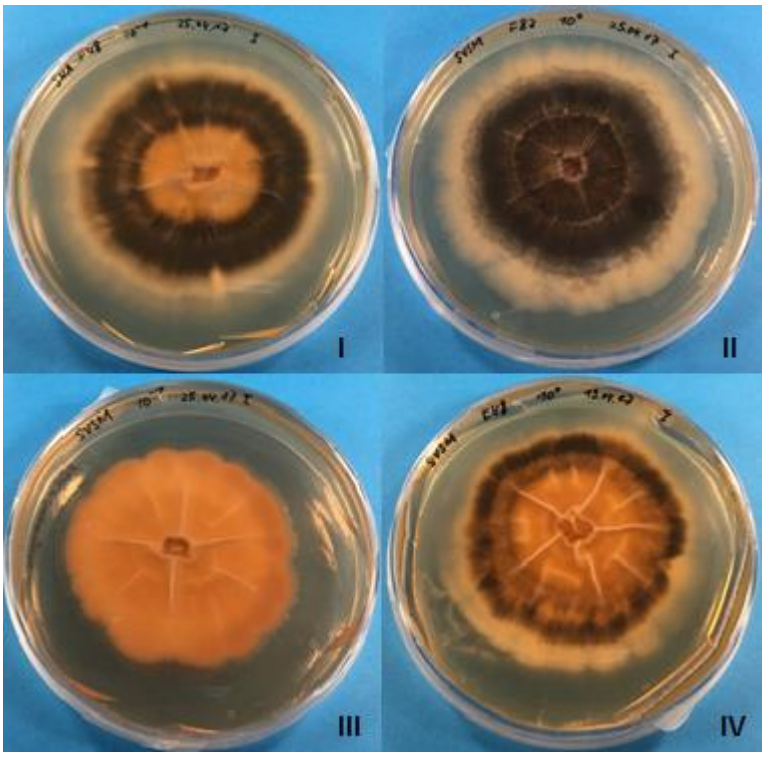


Figure 6.6 Cultural characteristics of four pure *Verticillium longisporum*-like isolates (I-IV) grown on PDA after isolation from stems of *Brassica napus* plants inoculated with the pathogen.

Due to the various morphological characters of these four selected colonies, a Multiplex PCR assay was used to further identify the lineage of the isolates. All four isolates showed two clear bands indicating products with a size of 1020 bp and 310 bp (Fig. 6.7), which are identical with the reference isolate 0120 (A1/D1). The original *V. longisporum* isolate VL43 belongs to A1/D1 lineage. Therefore, all four isolates collected from diseased stems were isolates derived from the original inoculation isolate.

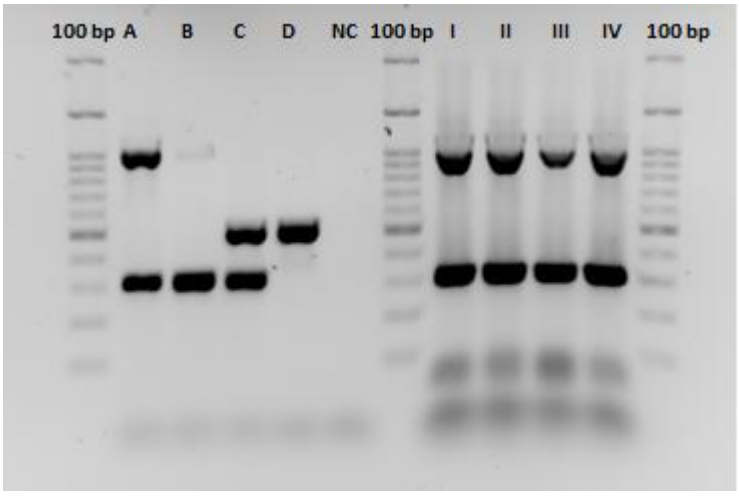


Figure 6.7 Identification of *Verticillium longisporum* by Multiplex PCR. A, isolate 0120 (A1/D1); B, isolate 1199 (A1/D2); C, isolate 1194 (A1/D3); D, isolate 1189 (*V. dahliae*); NC, negative control; 100bp, ladder DNA; I-IV, isolates from stem inoculated diseased plants.

Besides the morphological observation and molecular identification used to characterize the re-isolated strains of *V. longisporum*, the aggressiveness of these four isolates was tested using the root dipping method (Enyck et al. 2007) on the susceptible cultivar Falcon.

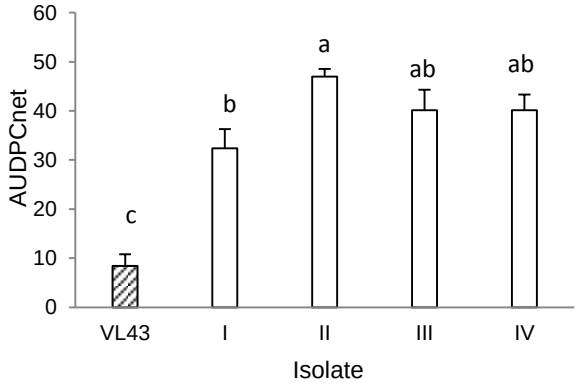


Figure 6.8 Aggressiveness of four *Verticillium longisporum* isolates (I-IV) collected from stem inoculation. VL43 was the reference isolate. Bars indicate standard errors. Different letters indicate significant differences among the treatments (LSD test, $P<0.05$).

All isolates collected from the symptomatic stems caused disease symptoms as described by Eynck et al. 2007) including abnormal half-side leaf yellowing and stunting. Interestingly, these isolates displayed a significantly higher aggressiveness than VL43, which had been reactivated from storage at -80°C (Fig. 6.8). Isolate II, which has the same morphological character as the type culture of VL43, was the most aggressive one on *B. napus* cv. Falcon. Therefore, this isolate was used for re-inoculation on *B. napus* by stem inoculation.

6.3.3 Disease development in *B. napus* via stem inoculation by *V. longisporum* isolate II

Both cultivars inoculated by stem inoculation with *V. longisporum* reisolate II showed the same disease symptoms as described above. However, in contrast to VL43 revived from storage at -80°C, *V. longisporum* isolate II caused more severe symptoms, even in the resistant cultivar: Some stems were clapped emanating from the inoculation point as well and more plants showed abnormal leaf yellowing. A significant difference of net AUDPC (Fig. 6.9A) and relative length of wound (Fig. 6.9B) between susceptible and resistant cultivars remained as in the previous experiment. However, the *V. longisporum* reisolate II caused a significant reduction of plant height both in the susceptible and in the resistant cultivar (Fig. 6.9C).

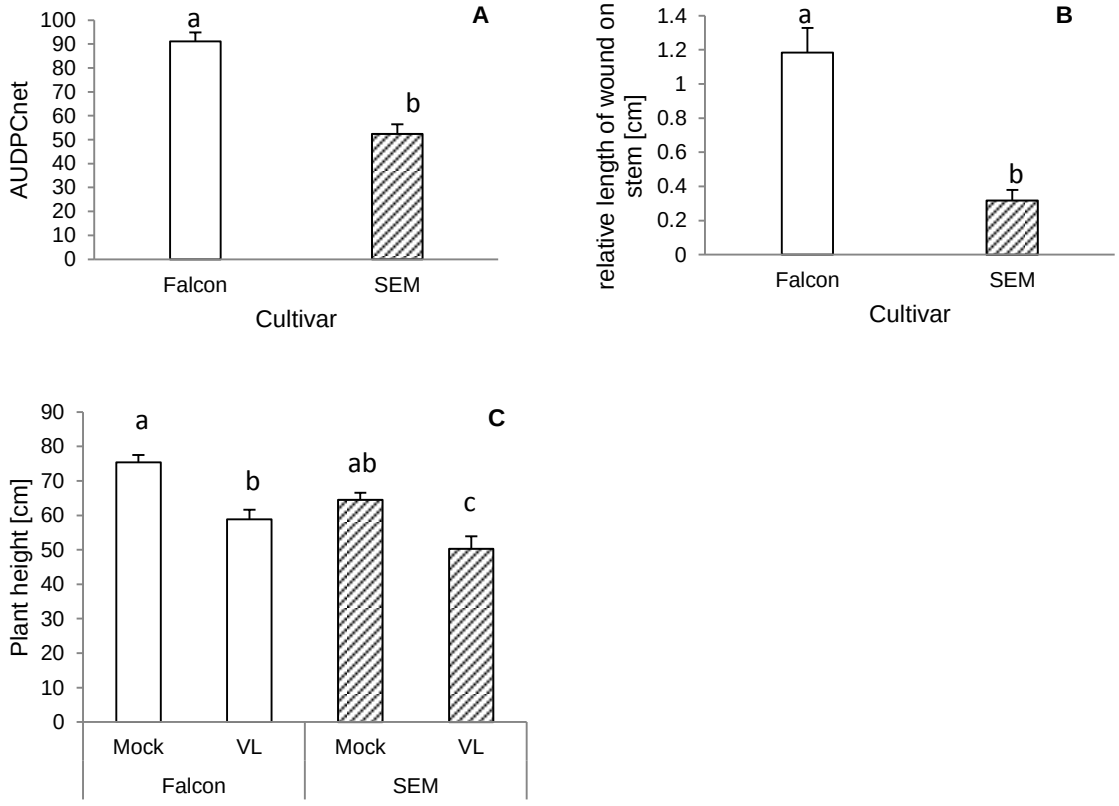


Figure 6.9 Development of re-isolated *Verticillium longisporum* in *Brassica napus* cv. Falcon and SEM determined by disease severity (A), relative length of wound on stem (B) and plant height at 28 dpi (C). Bars indicate standard errors. Different letters indicate significant differences among the treatments (Kruskal-Wallis test for AUDPCnet and relative length of wound on stem, LSD test for plant height, $P<0.05$).

6.4 Discussion

Under field conditions, hyphae from germinated melanized microsclerotia of *V. longisporum* in soil invade *B. napus* through direct penetration of the root (Eynck et al.

2007). At the final stages of infection, microsclerotia accumulate in stem tissues and are released to the soil when plant debris decomposes in the soil. Therefore, the resistance of stem tissue may be important to restrict the accumulation of microsclerotia of *V. longisporum* at late infection stages or after harvest. So far, researches into resistance against *V. longisporum* mainly focused on roots and hypocotyls (Ralhan et al. 2012; Debode et al. 2005; Eynck et al. 2009a; Kamble et al. 2013). Whether stem tissue is resistant or susceptible to *V. longisporum* remained unclear. Compared to root tissue, stem tissue is more compact and complex. The stem has a cuticle on the epidermis and hypodermis, while these two structures are not present in root. Furthermore, the cortex of the stem is relatively narrow and differentiated into collenchyma, chlorenchyma and parenchyma. However, the endodermis is not as conspicuous in stems as in roots (Setia et al. 1997; Tüzün et al. 2011). Any pre-penetration structure, such as an appressorium, is absent during penetration of *V. longisporum* on *B. napus* (Eynck et al. 2007).

This study showed an unsuccessful infection of *V. longisporum* without pre-injury of stems indicating that the cuticle on the epidermis may act as non-host physical barrier preventing *V. longisporum* from invading into stem. The cuticle is structurally diverse among plant species, however, it exhibits the organization of the composite material cutin polyester and is interspersed with waxes. As described by Samuels et al. (2008), secondary metabolites are also found among these wax components on *Arabidopsis thaliana*. The components of the cuticle may either be recognized by the fungus to process pathogenesis or by plants to activate defense signaling (Nielsen et al. 2000; Dickman et al. 2003; Chassot et al. 2007; Hansjakob et al. 2012; Serrano et al. 2014). The adhesion or penetration of *V. longisporum* may be perceived by *B. napus*, hence, the development of the fungus may be suppressed. However, as syringe injection causes a destruction of the epidermal structure, *V. longisporum* was able to advance into the stem. The symptom development caused by *V. longisporum* on *B. napus* was positively correlated to the fungal biomass in stems. The fungus can proceed through the xylem to upper parts of the plant. As one mechanism against *V. longisporum, B. napus* is able to accumulate vascular occlusions to prevent the spread of the fungus. The partial occupation of the xylem by *V. longisporum* (Eynck et al. 2007) may block the water transfer at the inoculation side. Therefore, stems were chapped along the inoculation point with wounds drying out. Furthermore, a contrasting symptom was observed in Falcon and SEM, which also showed contrasting susceptibility after infection of *V. longisporum* via root dipping inoculation. This result indicates that not only roots or hypocotyls play an important role in the expression of cultivar-related resistance against *V. longisporum*, but also the susceptibility of stem tissues can similarly affect the development and spread of *V. longisporum* in the plant. As

conclusion, cultivar-related resistance is not only expressed in hypocotyl but also in the stem tissues above. This finding implies that besides the conspicuous resistance responses identified in the hypocotyl, stem tissue resistance contributes to the limitation of *V. longisporum* to the plant base of resistant cultivars. Further study needs to be investigated to find out which mechanisms stem tissue resistance is exhibited and whether similar defense reactions as in the hypocotyl can also be found in the stem.

From previous research, dark pigmented microsclerotia are considered typical for *V. longisporum* (Karapapa et al. 1997), however, this study shows that this is not a necessary trait for *V. longisporum*. Presence or absence of microsclerotia does not affect the pathogenesis of *V. longisporum* on *B. napus*. However, the lack of formation of microsclerotia may lead to failure of overwintering as only mycelium remains in the field.

Another important finding of this study is that the accomplishment of its life cycle on a susceptible host plant apparently increases the aggressiveness of pathogenesis, which has been found also in *Phytophthora infestans* on potato previously (Pariaud et al. 2009). The course of the disease in the next generation of host plants was faster and a higher disease severity was observed. These results suggest that *V. longisporum* has the potential to increase its aggressiveness due to host plant passages which strongly emphasizes the need to effectively cut the life cycle and propagation of this pathogen in contemporary cropping systems with oilseed rape.

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Chapter 7: Identification of *Brassica napus* accessions with enhanced resistance to *Verticillium longisporum* under greenhouse and field conditions

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7.1 Introduction

Brassica spp. belongs to the mustard family (Brassicaceae), which has some 3,700 species including many economically important crops. *Brassica* plants are nowadays well established in Europe and temperate Asian region. *Brassica nigra, B. carinata, B. oleracea, B. napus, B. rapa and B. juncea* are the six species, which are used as oilseed crops, condiments or vegetable (Cheng et al. 2014). The U's triangle model describes their genetic relationships (Fig. 7.1).

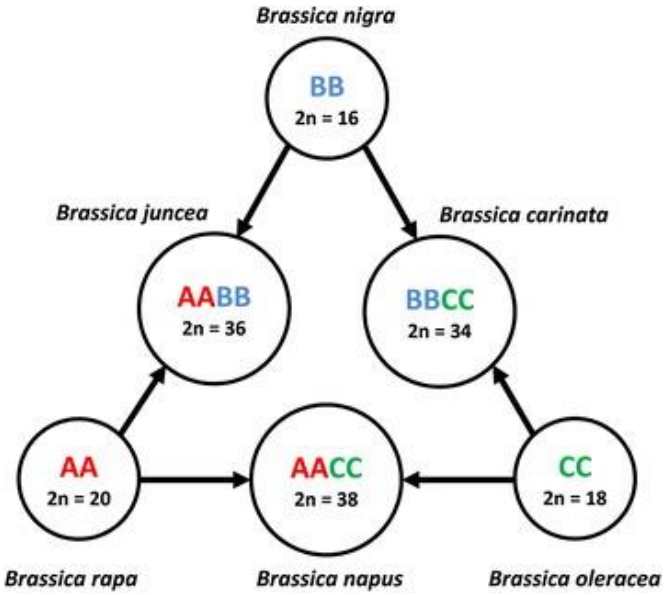

Figure 7.1 U's triangle model of *Brassica* spp. (Koh et al. 2017)

Among these species, *B. napus* (oilseed rape, OSR) is a relatively young crop species. However, due to intensive breeding for good quality of seed and oil, oilseed rape represents one of the most important crops for vegetable oil in the world. It is an interspecific hybrid from *B. rapa* and *B. oleracea*, besides, within *B. napus*, different types are used around the world. Winter oilseed rape, the most cultivated type, was likely to be grown in Europe 400 years ago in the early 17th century. Based on the winter type and the local climate, spring oilseed rape was developed in the late 17th or early 18th century and

is currently grown in Canada, northern Europe and Australia (Prakash et al. 2012). These types of oilseed rape were introduced from Europe, Canada and Japan to China in the 1930-1940s and crossed with Chinese *B. rapa* for adaptation to local environments and formed an intermediate type called semi-winter oilseed rape (Chen et al. 2008; Wu et al. 2014). Hence, the genetic diversity of semi-winter oilseed rape has been reported to be lower than those of winter and spring oilseed rape (Bus et al. 2011; Qian et al. 2006; Wu et al. 2014).

Due to the high demands of rapeseed oil, cultivation of oilseed rape was increased dramatically by mono-cultivation or shorter crop rotation cycles. As a consequence, *Verticillium longisporum* (VL) has become a threat to oilseed rape production, causing 'Verticillium stem striping'. *Verticillium longisporum* is a soil-borne vascular pathogen, which is host-specialized on Brassicaceae, especially oilseed rape (Depotter et al. 2016). This pathogen invades the host plants by direct penetration into roots and further grows through the root cortex towards the xylem vessels (Eynck et al. 2007). No visible symptoms can be observed in early growing stages under field conditions. There is no efficient chemical treatment available to prevent from potential yield losses caused by *V. longisporum*. Shortly before harvest, microsclerotia develop under the stem epidermis and in the stem pith, which are released into the soil during decomposition of plant debris. The microsclerotia in soil are estimated to survive for several years (Heale and Karapapa 1999). *Verticillium longisporum* is widely distributed in all main oilseed rape production regions in Europe and Canada (Steventon et al. 2002; Gladders et al. 2011; CFIA 2017). In Asia, this pathogen was reported to cause disease only on Chinese cabbage but not on oilseed rape (Yu et al. 2015). Good agronomic practice with long crop rotation cycles using non-host plants and resistant cultivars are the major strategies to manage this disease. Resistant cultivars of *Brassica* spp. were found in previous research (Eynck et al. 2009). However, the mechanisms of plant defense responses are not yet clear.

Resistance screening under greenhouse conditions may be an efficient tool to evaluate resistance of plant genotypes within a short time. However, due to the effect of various environmental factors under field conditions, accessions performing resistant under greenhouse conditions may show different responses in the field (Eynck et al. 2009).

The aim of this study was to screen different *Brassica* genotypes for resistance against *V. longisporum* under greenhouse conditions in order to identify and select resistant or partially resistant varieties for future breeding. Due to the different performance under greenhouse and field conditions, a field trial was conducted to validate the results obtained from the greenhouse resistance phenotyping.

7.2 Materials and methods

7.2.1 Plant material

159 founder *Brassica napus* accessions (Tab 7.1) from the *Brassica napus* Diversity Fixed Foundation Set (BnaDFFS) were used for screening. *Verticillium longisporum* susceptible cultivar Falcon (Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, NPZ, Hohenlieth, Germany) and resistant cultivar SEM 05-500256 (Syngenta, Germany) were used as references.

Table 7.1 Summary of *Brassica napus* accessions screened for resistance against *Verticillium longisporum* in greenhouse and field experiments.

Type	Number of accessions screened for *Verticillium longisporum* resistance	
	Green house screening	Field trial 2017
Winter oilseed rape	99	103
Spring oilseed rape	38	23
Semi-winter oilseed rape	12	8
Kale and swede	8	12
Leafy vegetable	1	2
Synthetic *Brassica napus*	1	1
Total	159	149

Seeds were sown in quartz sand and grown in a climate chamber with a 16 h photoperiod and a temperature of 22±2˚C for 10 days before root inoculation with *V. longisporum*.

7.2.2 Fungal culture and inoculation

Verticillium longisporum isolate VL43 obtained from a diseased *B. napus* plant was used for inoculation. Conidial suspension, which had been kept in 25% glycerol at -80˚C, was used to initiate fresh culture. For preparation of the fungal inoculum, 400 µl of the stock conidial suspension was added to 250 ml autoclaved (121˚C, 20 min) potato dextrose broth (PDB), and then incubated on a rotary shaker at 80 rpm at 22˚C for 10 days. The resulting suspension was filtered through sterile gauze to remove mycelia.

Ten-day-old seedlings, with cotyledons completely unfolded, were inoculated or mock-inoculated using the root-dip method. The roots were rinsed with tap water and dipped in a conidial suspension (1 x 10^6 cfu/ml) or water for 50 min, and two plants were replanted in one pot (9 x 9 x 11 cm) filled with a fresh soil-sand mixture (3:1). In order to avoid cross contamination due to runoff irrigation water, mock treated and *V. longisporum* inoculated plants were kept on separate trays. Plants were kept in a climate chamber with a 16 h photoperiod and a temperature of 22±2˚C (Fig. 7.2).

Figure 7.2 Partial view of screening experiment with *Brassica napus* lines for resistance against *Verticillium longisporum* at 7 dpi.

7.2.3 Screening procedure in the greenhouse

The disease progress in the greenhouse was quantified weekly from 7dpi to 28 dpi following the assessment key (Tab. 7.2) described by Eynck et al. (2009).

Table 7.2 Assessment key for scoring of foliar symptoms induced by *Verticillium longisporum* on *Brassica* species inoculated with the root dip method.

Score	Symptom development
1	No symptoms
2	Weak symptoms on the oldest leaf (yellowing, black veins)
3	Weak symptoms on the next younger leaves
4	About 50 % of the leaves have symptoms
5	More than 50 % of the leaves have symptoms
6	Up to 50 % of the leaves are dead
7	More than 50 % of the leaves are dead
8	Only apex is still alive
9	The plant is dead

The area under the disease progress curve (AUDPC) was calculated from the disease severity scoring according to the following formula:

$$\mathrm{AUDPC} = \sum_{i=1}^{n} \left(\frac{y_i + y_{i+1}}{2}\right) \times (t_{i+1} - t_i)$$

Where *yi* is the disease severity score for observation number *i*, *ti* is the corresponding number of days post inoculation of an observation, and *n* is the number of observations.

In order to compensate for fluctuating infection levels between different screening runs, a normalization factor was introduced for each accession on the basis of the internal reference standards Falcon and SEM. The value was calculated using the following formula (Eynck et al. 2009).

$$\text{normalisation factor} = \frac{(\text{AUDPC}_{\text{Falcon,VL}} - \text{AUDPC}_{\text{Falcon,control}}) + (\text{AUDPC}_{\text{SEM,VL}} - \text{AUDPC}_{\text{SEM,control}})}{2}$$

$$\text{normalized AUDPCnet} = \frac{(\text{AUDPC}_{\text{Falcon,VL}} - \text{AUDPC}_{\text{Falcon,control}})}{\text{normalization factor}}$$

Infection of *V. longisporum* results in plant height reduction, therefore, plant height was measured at 28 dpi and the relative stunting effect was calculated according to the following formula.

$$\text{relative stunting effect (\%)} = \frac{\text{height of non inoculated plant - height of inoculated plant}}{\text{height of noninoculated plant}}$$

7.2.4 Design of field trial and visual scoring

In total, 149 accessions were tested in the field located in Teschendorf, Fehmarn, Germany (54°26'8.87'' N, 11°6'58.74'' E) in the season 2016/17. The trial was conducted by the breeding company KWS Saat SE. Due to the high natural infection pressure of *V. longisporum* in Fehmarn, no supplemental application of pathogens was conducted in this experiment. The mean temperature and sum of precipitation during the season of 2016/17 were 9.9°C and 612.8 mm (weather station Fehmarn-Wulfener Hals). The exact weather data during the experiment are shown in table 7.3.

Table 7.3 Weather situation in Fehmarn (monthly means, WetterKontor 2016, 2017).

	Temperature [°C]	Precipitation [mm]	Sunshine duration [h]
Sep. 2016	17.7	25.3	39.8
Oct. 2016	10.1	72.3	61.4
Nov. 2016	5.3	35.9	65.5
Dec. 2016	5.1	45.8	209.8
Jan. 2017	1.6	31.2	49.8
Feb. 2017	2.3	36.5	38.4
Mar. 2017	5.6	43.0	117.4
Apr. 2017	7.3	35.0	204.1
May 2017	12.5	42.6	251.6
Jun. 2017	16.2	90.2	243.9
Jul. 2017	16.8	90.7	216.5
Aug. 2017	17.7	64.3	216.4

The total experimental area was 1,728 m^2. Each plot was 4 by 0.9 m in size. Seeds were sown on the 2nd September 2016. Fertilization (90 kg/ha nitrogen, 3 l/ha Profi® Bor and 5 kg/ha magnesium sulfate) and pesticides against weeds (2 l/ha Fuego®, 0.3 l/ha Centium® 36 CS and 1.8 l/ha Kerb Flo), insects (0.15 l/ha Karate® Zeon) and snails (5 kg/ha Schneckenkorn) were applied. Additionally, 0.5 l/ha Cantus® Gold was applied against *Sclerotinia* spp., *Alternaria* spp. and *Leptosphaeria* spp.. All agronomic measures were performed according to good agricultural practice.

Seven to twelve stubbles from each accession were collected from field experiment after harvest on the 10th August 2017 for disease assessment. However, due to the low germination rates of some accessions, only accessions, of which more than seven stubbles were scored, were used for further analysis.

Visual and microscopic investigation for the presence and intensity of microsclerotia formation under the epidermal and in the pith tissue of the stems as well as in the roots were used to determine disease severity according to the following assessment key with four scores (Tab. 7.4).

Table 7.4 Assessment key for evaluation of *Verticillium longisporum* disease severity on stubbles of *Brassica* spp. (Knüfer et al. 2017).

Score	Description
1	Healthy tissue: no microsclerotia visible in tissue
2	Slight infection: low abundance of microsclerotia
3	Advanced infection: high abundance of microsclerotia
4	Severe infection: tissue heavily infested with microsclerotia, epidermis is peeling off from the stubble

Based on these scores and the number of plants, a disease index (DI) was calculated for each accession as follows:

$$\mathrm{DI} = \frac{(1 \times \mathrm{N1}) + (2 \times \mathrm{N2}) + (3 \times \mathrm{N3}) + (4 \times \mathrm{N4})}{(\mathrm{N1} + \mathrm{N2} + \mathrm{N3} + \mathrm{N4})}$$

where *Ni* is the number of plants in the respective score.

7.2.5 Statistical analysis

Sixty-three accessions with efficient data of common lines obtained from greenhouse and field experiments were used for analysis by XLstat2017. The relationship between results from greenhouse and field experiments was performed by Pearson's correlation. Among these accessions, 27 lines, which had a higher or lower normalized net AUDPC than the reference lines were subjected to a Kruskal-Wallis test (two-tailed). Conover-Iman procedure at P=0.05 was applied for multiple pairwise comparisons among genotypes. The experimental results were presented as means ± standard deviation at 5% significance level.

7.3 Results

7.3.1 Greenhouse screening

In total, 159 *B. napus* accessions belonging to winter, spring, semi-winter oilseed rape, swede and kale were screened for resistance to *V. longisporum* in three runs of independent screening experiments with internal reference controls. In general, a wide

range of variation in response to infection with *V. longisporum* occurred among the screened accessions. Especially kale and swede, which is represented in purple in Figure 7.3, showed a broad range of resistance both in normalized net AUDPC and reduction of plant height. Detailed information and results from disease assessment are found in Appendix 2.

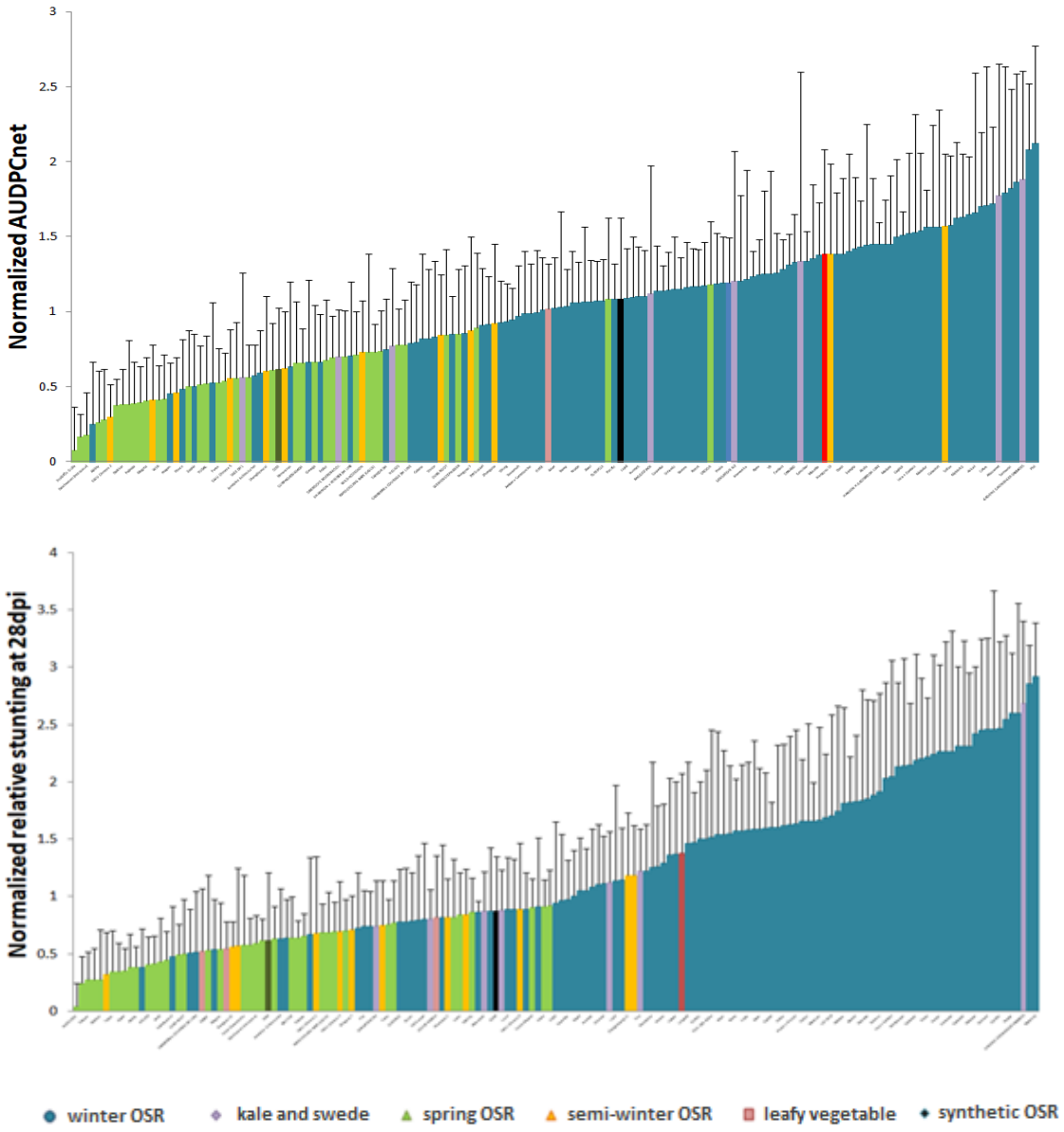

Figure 7.3 Response of *Brassica* founder accessions to *Verticillium longisporum* infection under greenhouse conditions. Normalized mean net AUDPC (top) and relative stunting (bottom) data are shown. For both parameters data shown are the means of 20 plants. Dark green and red bars show mean values derived from three independent experiments for the reference variety SEM (resistant) and Falcon (susceptible), respectively. Error bars indicate standard deviations. OSR, oilseed rape.

All screened 38 accessions of spring oilseed rape showed a lower normalized net AUDPC and relative stunting than the susceptible reference line Falcon. In contrast to the comparatively high level of resistance among the spring types, among total screened

winter oilseed rape, 31 accessions representing 31% had higher normalized net AUDPC and 58 accessions representing 59% showed more severe stunting than susceptible reference line Falcon. There were only five accessions (Tapidor DH, Sobotkowski, Dimension, Canberra x Courage DH line and Taisetsu) that showed less stunting compared to the resistant reference line SEM. Among these five accessions, two (Taisetsu and Sobotkowski) showed less normalized net AUDPC than the resistant reference line SEM as well.

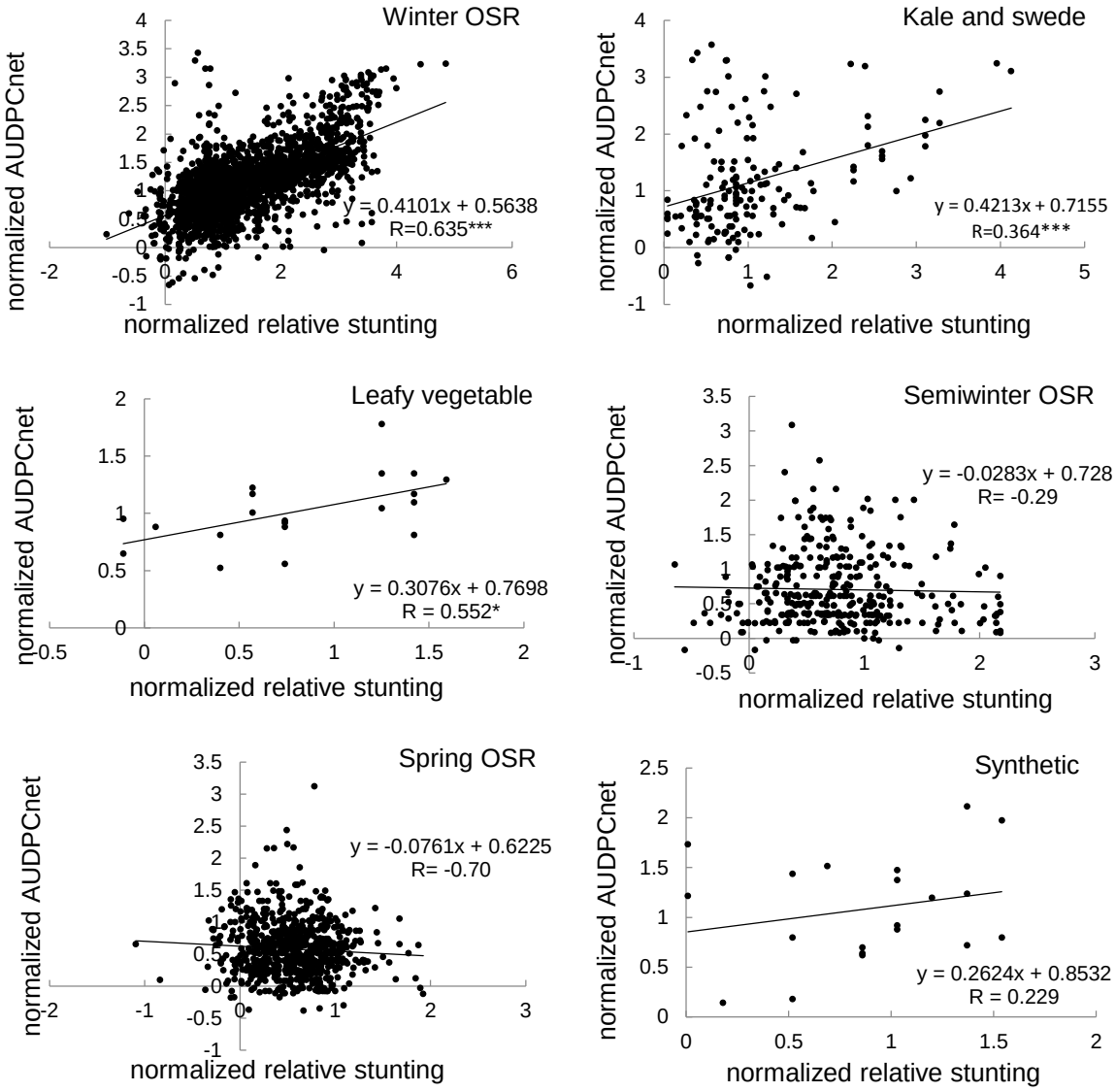

Figure 7.4 Correlation of mean normalized net AUDPC and normalized relative stunting data from 159 founder accessions. Pearson correlation was used for analysis. ***,** significant at *P*=0.05; ****,** significant at *P*=0.01; *****,** significant at *P*=0.001.

Normalized net AUDPC of semi-winter oilseed rape was similar to the level of spring oilseed rape, except for two accessions (Chuangyou2 and Xiangyou2) while the other accessions were moderately to strongly resistant against *V. longisporum*. However, all of

them had a smaller stunting effect than the susceptible reference line. Synthetics and *Brassicas* for vegetable use lay between the values of resistant and susceptible reference lines.

Correlation analysis between normalized net AUDPC and normalized relative stunting showed a significant, strong and positive correlation in winter oilseed rape, kale and swede significant but moderate correlation in leafy vegetables (Fig 7.4). However, these two parameters were not correlated in the responses of spring oilseed rape, semi-winter oilseed rape and synthetic rape after infection of *V. longisporum*.

7.3.2 Field resistance test

In the season 2016/2017, 149 *B. napus* accessions were sown for a field resistance test. However, due to the poor germination of seeds, only 56% of accessions were suitable for visual assessment of stubbles (n>7) after harvest. Most of these accessions were winter oilseed rape and swede. Similar to screening data obtained from the greenhouse, a large variation in responses to infection with *V. longisporum* among the screened accessions was observed (Fig. 7.5).

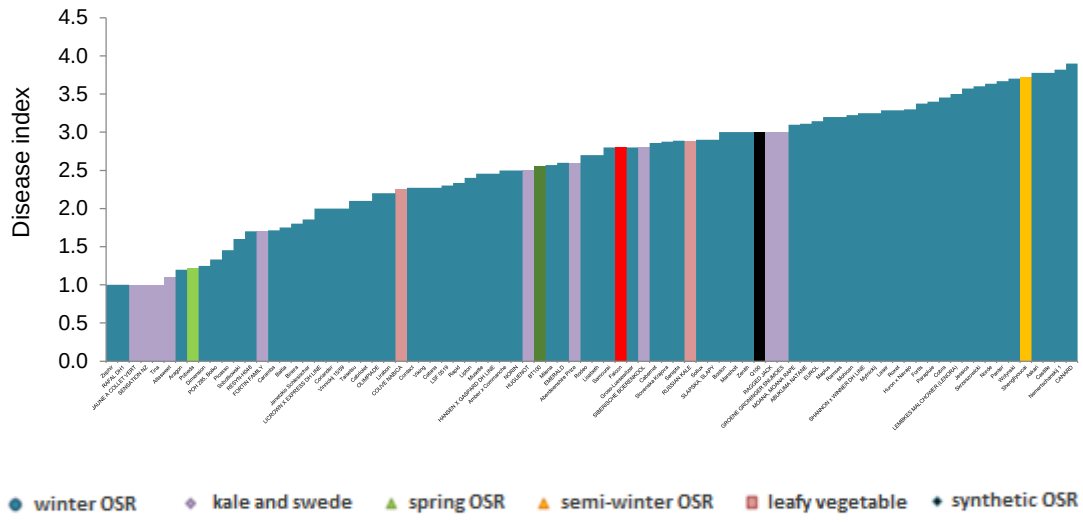

Figure 7.5 Response of 83 accessions to *Verticillium longisporum* infection in the field trial 2016/17. Disease index is shown. Dark green and red bars show the reference varieties BT100 (moderately susceptible) and Falcon (susceptible), respectively. OSR, oilseed rape.

Natural disease pressure of *V. longisporum* was fairly high in Fehmarn, more than 59% of accessions had a disease incidence higher than 80%. Under field conditions, kale and swede showed again a broad range of resistance response to *V. longisporum* infection. Five accessions (Zephir, Rafal DH 1, Janune a collet vert, Sensation NZ, Tina) were completely free from establishment of microsclerotia of *V. longisporum* in the stubbles, while 23 accessions (Nemertschanskij1, Castille, Askari and Shengliyoucai etc.) had

100% disease incidence with a relatively high disease index (2.56 to 3.90).

Table 7.5 Response of *Brassica napus* assessions to *Verticillium longisporum* infection under green house and field conditions. RR, resistant reference line; SR, susceptible reference line; Norm. ,normalized; DI, disease index. Different letters indicate significant differences among the treatments (Kruskal-Wallis test, Conover-Iman procedure for multiple pairwise comparisons, $P<0.05$).

Accessions No.	Variety	Field 2017	Green house	
		DI	Norm. AUDPCnet	Norm. rel. Stunting
BnASSYST-203	Taisetsu	2.10	0.25±0.41 c	0.54±0.44 i
BnASSYST-367	Pobeda	1.22	0.39±0.27 c	0.66±0.19 i
BnASSYST-061	Aragon	1.20	0.46±0.20 c	0.73±0.48 ghi
BnASSYST-068	Zephir	1.00	0.50±0.35 c	1.06±0.36 efghi
BnASSYST-173	Sobotkowski	1.60	0.52±0.54 c	0.48±0.44 i
BnASSYST-107	Janetzkis Schlesischer	1.86	0.57±0.21 c	0.64±0.43 hi
BnASSYST-105	Licrown X Express DH line	2.00	0.60±0.28 c	0.88±0.35 fghi
BnASSYST-526	Shengliyoucai	3.71	0.60±0.50 c	1.19±0.43 efgh
RR	SEM	-	0.62±0.41 c	0.62±0.59 i
SR	Falcon	-	1.38±0.70 b	1.38±0.69 efg
BnASSYST-213	Abukuma natane	3.11	1.38±0.41 b	2.31±0.64 abcd
BnASSYST-169	Sarepta	2.89	1.42±0.47 ab	0.97±0.58 fghi
BnASSYST-096	Hansen X Gaspard DH line	2.45	1.45±0.15 ab	2.86±0.34 ab
BnASSYST-124	Mohican	3.22	1.50±0.52 ab	1.67±0.81 def
BnASSYST-168	Ramses	3.20	1.53±0.53 ab	1.91±0.86 bcde
BnASSYST-119	Lirabon	2.20	1.57±0.68 ab	1.84±0.96 cde
BnASSYST-509	Cabernet	2.86	1.57±0.78 ab	2.19±0.93 abcd
BnASSYST-114	Sollux	2.90	1.58±0.46 ab	1.63±0.71 def
BnASSYST-137	Coriander	2.00	1.63±0.50 ab	1.89±0.82 cde
BnASSYST-163	Mytnickij	3.25	1.63±0.42 ab	2.92±0.47 a
BnASSYST-118	Askari	3.78	1.66±0.93 ab	2.26±0.96 abcd
BnASSYST-166	Panter	3.67	1.70±0.49 ab	2.26±0.75 dcba
BnASSYST-122	Cobra	3.45	1.71±0.92 ab	2.60±0.96 abcd
BnASSYST-510	Cabriolet	2.10	1.72±0.51 ab	2.31±0.91 abcd
BnASSYST-418	Altasweet	1.10	1.77±0.88 ab	0.87±0.34 fghi
BnASSYST-117	Maplus	3.20	1.79±0.84 ab	2.46±1.21 abcd
BnASSYST-113	Samourai	2.80	1.82±0.66 ab	2.26±1.05 abcd
BnASSYST-218	Groene groninger snijmoes	3.00	1.88±0.72 ab	2.69±0.71 abc
BnASSYST-511	Castille	3.78	2.08±0.44 a	2.47±0.76 abcd

In contrast to data obtained from the greenhouse screening experiment, the synthetic line was more susceptible to *V. longisporum* than the susceptible reference line Falcon. Taking into account data from the greenhouse and field trials, twenty-seven accessions, which had higher or lower normalized net AUDPC than the susceptible and resistant reference lines were selected for further statistical analysis. Among these 27 accessions, no accession, with regard to normalized net AUDPC, was found significantly more resistant to

V. longisporum as the resistant reference line SEM (Tab. 7.5). Accession Altasweet had higher normalized net AUDPC in the greenhouse; however, it showed less stunting and formed less microsclerotia in the field after infection of *V. longisporum*. Although the ranking of accessions did not remain the same among different parameters, resistant and susceptible clusters were observed. In order to find accessions that may be suitable for future research, top eight and last ten resistant accessions from common accession of greenhouse and field trials were sorted out from each parameter. Two accessions, Pobeda and Aragon, not only had less disease symptom and stunting effect in greenhouse, but also performed well in field experiment. Two other accessions, Castille and Cobra, were selected as susceptible line in response to infection of *V. longisporum* under greenhouse and field conditions.

7.3.3 Comparison of results from greenhouse screening and field experiment

In order to compare the data of resistance screening obtained from greenhouse and field experiments, 63 accessions, form which sufficient data had been obtained in both greenhouse and field trials, were selected for a correlation test. A significant, strong and positive correlation with a coefficient of 0.759 was found between two parameters used for greenhouse screening (Tab. 7.6). Data of normalized net AUDPC and normalized relative stunting obtained from greenhouse screening was also correlated to the field data, respectively.

Table 7.6 Correlation between field performance and greenhouse screening results of 63 *Brassica napus* accessions evaluated for *Verticillium longisporum* resistance with Pearson correlation test. DI, disease index; ***,** significant at *P*=0.05; ****,** significant at *P*=0.01; *****,** significant at *P*=0.001.

Trial	Parameter	Green house		Field 2017
		Norm. AUDPCnet	Norm. rel. stunting	DI
Green house	Norm. AUDPCnet	1		
	Norm. rel. stunting	0.759***	1	
Field 2017	DI	0.415**	0.580*	1

The selected 27 accessions at the extreme ends of the range of responses showed significantly stronger positive correlations between screening data obtained from greenhouse experiments and field trials (Tab. 7.7).

Table 7.7 Correlation between field performance and greenhouse screening results of 27 selected *Brassica napus* accessions evaluated for *Verticillium longisporum* resistance with Pearson correlation test. DI, disease index; ***,** significant at *P*=0.05; ****,** significant at *P*=0.01; *****,** significant at *P*=0.001.

Trial	Parameter	Green house		Field 2017
		Norm. AUDPCnet	Norm. rel. stunting	DI
Green house	Norm. AUDPCnet	1		
	Norm. rel. stunting	0.8113***	1	
Field 2017	DI	0.580**	0.665***	1

7.4 Discussion

The root-dip method is a well-established and efficient method used to evaluate a large number of accessions for resistance against *V. longisporum* within a short period of time under greenhouse conditions (Zeise and Buchmüller 1997; Eynck et al. 2009). The two estimation parameters, normalized net AUDPC and normalized relative stunting, were strongly positive correlated. However, disease symptoms of *B. napus* in the greenhouse are strongly differing from those under field conditions. As the performance of infected *B. napus* accessions in the greenhouse is not followed further than four weeks, this may lead to misevaluation of some accessions that may express disease symptoms later than four weeks after inoculation (Happstadius et al. 2003; Debode et al. 2005). Lack of vernalization of winter or semi-winter type oilseed rape in the greenhouse compared to the field may also have an impact on the interaction of *B. napus* and *V. longisporum* due to a different physiological status of plants (Rapacz 2002; Zanewich and Rood 1995). Moreover, *V. longisporum* is estimated to be more aggressive, on plants in flowering than juvenile stages (Zhou et al. 2006; Dunker et al. 2008). In addition, transfer of results from greenhouse screening to the field is hampered by various effects from the environment or different isolates used in the greenhouse or occurring with natural infection (Eynck et al. 2009). Nevertheless, a significant correlation of data from the greenhouse screening and field trial as demonstrated in the present study indicated similar responses of different accessions of *B. napus*. Stronger significantly positive correlations of disease severity can be achieved when using accessions at the extreme ends of the range of responses. Although no stunting symptom could be observed under field conditions, a higher correlation coefficient was found between disease index from field trial and normalized relative stunting from greenhouse screening. However, individual accessions may show a different ranking in the greenhouse screening as in the field trial.

Verticillium longisporum has evolved at least in three lineages, A1/D1, A1/D2 and A1/D3, among which A1/D1 is mainly responsible for ‘Verticillium stem striping’ (Depotter et al. 2016). According to a recent study (Depotter et al. 2017), based on 15 polymorphic simple sequence repeat markers, the population of *V. longisporum* in Germany belongs to a subgroup A1/D1 East, while the subgroup in Japan, USA and UK are A1/D1 West 2 and A1/D1 West 1, respectively. Isolate VL43 used in this study was originally isolated from *B. napus* (winter type) grown in northern Germany by Zeise and von Tiedemann (2001), which may be different to isolates from spring and semi-winter oilseed rape growing regions and has a better ability to adapt to local climate and the host type, winter oilseed rape. Therefore, a generally higher susceptibility of winter oilseed rape to *V. longisporum* isolate VL43 than spring and semi-winter oilseed rape was found in the present study. The

stunting parameter seems to be more suitable and efficient to use on winter oilseed rape swede and kale than spring and semi-winter oilseed, on which less response of plant height to *V. longisporum* infection was found. Previous research showed that *B. oleracea* and *B. carinata* sharing the C genome were comparatively more resistant to infection with *V. longisporum*, while *B. rapa* (A genome) was more susceptible (Happstadius et al. 2003; Rygulla et al. 2007; Eynck et al. 2009). The semi-winter type, commonly grown in China, was crossed with Chinese *B. rapa* and inherited unique alleles from this interspecific crossing, which assumingly resulted in higher susceptibility to *V. longisporum* similar to *B. rapa* (Chen et al. 2008). However, semi-winter types of oilseed rape in this study were relatively resistant to *V. longsiporum*. Accessions, which performed well not only in the greenhouse screening but also in the field trial, such as Pobeda and Aragon, are recommended to be considered in future breeding work.

The relatively large variability of responses of different groupe of *B. napus*. to infection with *V. longsiporum* in this study provides a valuable data base for further breeding research aiming at increased quantitative resistance in oilseed rape. In order to understand the reasons causing differences in responses, a powerful tool, such as a genome wide association study with SNPs and gene expression markers of screened accessions, needs to be investigated in future studies.

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Chapter 8: General discussion

8.1 Development of *V. longisporum* on *B. napus*

The present study demonstrates the course of colonization of *V. longisporum* on *B. napus* under field conditions, which has not yet been described before. As described by Johansson et al. (2006a), the threshold density of microsclerotia needed to cause disease by *V. longisporum* on oilseed rape is 1 cfu/g soil. Because no symptoms were observed in the early growing stage and damage caused by insects made it more complicated to identify diseased plants in late growing stages, the qPCR assay is an efficient tool for monitoring the development of *V. longisporum* on oilseed rape under field conditions. This tool is also useful to quantify the biomass of *V. longisporum*, thus, the exact fungal development within the plant can be recorded. The *ß*-tubulin primer pair used for this assay is specific to distinguish *V. longisporum* from other fungi including *V. dahliae* (Knüfer et al. 2017). The hyphae germinated from microsclerotia were able to invade the roots of oilseed rape plants about a month after seeds were sown in late summer. Possibly due to the low soil temperature (average daily soil temperature below 10°C) and although the fungus had already colonized the roots, it did not accumulate or spread further into the upper parts of the plants until late spring. An experiment conducted with different temperatures during infection showed the effect on fungal growth: (1) no fungal growth at all was detected in plants kept at 6 and 9°C and (2) in plants kept under medium low temperature of 12°C, small amounts of *V. longisporum* were detected 42 days after inoculation (Pfordt 2017). Maybe due to the higher average temperatures, only one month after sowing was needed for *V. longisporum* to colonize the roots of winter oilseed rape, while the first detection of fungal DNA in the roots of spring oilseed rape was two months after sowing and around beginning of flowering stage. Around one month with more than 15°C seems to be necessary for the colonization of *V. longisporum* in roots of oilseed rape. However, whether the low temperatures affect the germination of microsclerotia or growth and infectivity of *V. longisporum* hyphae is not clear so far. A further study is needed for clarification. After winter, the pathogen was observed to spread further in the hypocotyl. As in a previous study, in late spring to early summer during flowering stage, the amounts of *V. longisporum* strongly accumulated in the entire plants indicating that flowering stage may be essential for fungal development (Zhou et al. 2006). Until the end of flowering, the amount of nitrogen has been shown to increase in stems and is further allocated to flowers (Malagoli et al. 2005). Further internal physiological changes in the plant during

this stage may also contribute to the spread of *V. longisporum*. Disease symptoms with unilateral stem striping could be observed first at the end of pod enlargement.

Under greenhouse conditions, *V. longisporum* reached the pods before the beginning of ripening. Using qPCR, *V. longisporum* was detected in seeds of infected plants from field and greenhouse experiments. However, the levels of *V. longisporum* in seeds obtained from field grown diseased plants were substantially lower than in seeds collected from greenhouse grown plants which had been inoculated with spore suspension by root-dipping. It was very difficult to isolate *V. longisporum* from the field seed samples, as contamination with other fungi was hard to avoid using a combination of conventional seed surface sterilization methods and selective media. Therefore, so far, *V. longisporum* was not re-isolated from seeds from field experiments (Zhou et al. 2006). In contrast, 13.3% of the seeds from plants infected under greenhouse conditions were infected by *V. longisporum*. This percentage was much lower than the seed transmission incidence of *V. dahliae* on several host plants (Kadow 1934; Snyder and Wilhelm 1962; Vallad et al. 2005; Karajeh 2006; Göre et al. 2011). Similar to the winter oilseed rape, *V. longisporum* was able to transmit into the seeds of infected spring oilseed rape as well (Eseola 2017).

Regarding cultivar resistance, the development of *V. longisporum* showed differential colonization patterns in resistant and susceptible cultivars. Although *V. longisporum* colonized the entire plant of both resistant and susceptible cultivars, its biomass was significantly lower in the resistant cultivar. While *V. longisporum* spread faster in the susceptible cultivar from root to stem, high amounts of *V. longisporum* were constricted in the hypocotyl of the resistant cultivar. This observation indicates that the hypocotyl may be the battle field of oilseed rape to defend against *V. longisporum* (Kamble et al. 2013; Depotter et al. 2016). Moreover, not only hypocotyl tissue, but also stem tissue of contrasting cultivars showed different responses to infection with *V. longisporum*, which may be an additional reason for less colonization with *V. longisporum* in upper parts of the plants. Similar to the results of *V. longisporum* DNA, a higher proportion of severely infected plant stubbles was observed in the susceptible cultivar after harvest. Furthermore, greenhouse experiment also confirmed that susceptible plants, which were more severely infected by *V. longisporum*, had also higher biomass of *V. longisporum* in the seeds. As soon as *V. longisporum* established in the plants, it accumulated remarkably in both cultivars during the flowering stage.

Besides, due to the shorter season, the development period of spring oilseed rape is in general shorter. The slowly growing pathogen *V. longisporum* (Depotter et al. 2016) may have not enough time for spreading to the upper parts of plants, therefore, high amounts of fungal biomass were found in the hypocotyl and root but not in the stem of spring

oilseed rape. On account of the origin of the tested isolate, *V. longisporum* VL 43 collected from a winter oilseed rape field may have a different preference and virulence to winter and spring oilseed rape. As shown in the screening results, spring oilseed rape displayed less disease symptoms than most of the winter types with artificial root inoculation with conidia of *V. longisporum* under greenhouse conditions.

With supplemental application of inoculum, more than 80% disease incidence was observed with moderate to high disease severity, which is similar to the result obtained by Dunker et al. (2008). The yield loss caused by *V. longisporum* infection was estimated to range from 10% to 50% according to the assessment data of individual plants under greenhouse conditions (Dunker et al. 2008). In the present study, about 15% of yield loss was observed in the susceptible winter oilseed rape cultivar but not in the resistant one. In contrast to Gladders et al. (2013), who reported 12% to 24% reduction in thousand seed weight, no reduction of thousand seed weight was observed both in the field and greenhouse experiments. Like in the previous study, no effect was observed on seed quality, including oil content and fatty acid composition. In *Arabidopsis thaliana*, colonization with *V. longisporum* resulted in an increase in glucosinolates in the roots (Witzel et al. 2015). The breakdown products have been shown to have antifungal effects, and the induction of glucosinolates in xylem sap may be driven by the presence of breakdown products (Down et al. 2004; Andersen et al. 2013; Witzel et al. 2013; Witzel et al. 2015). However, in the present study no change of glucosinolate contents was observed in seeds infected by *V. longisporum*.

Although ‘Verticillium stem striping’ disease has not yet been proved to cause remarkable yield losses or reduction in oilseed quality under field conditions, the nature of seed transmission of this pathogen may be a potential threat to Brassicaceae production areas, where no disease has been reported so far. The international oilseed rape seed import and export market is a potential pathway to introduce or disseminate *V. longisporum* by long-distance seed transport. About 1% of second generation plants showed disease symptoms in roots and stems which seems to be a low risk. But the aggressiveness of pathogenesis of *V. longisporum* could increase, when it accomplishes its life cycle on a susceptible host plant. In addition, the formed microsclerotia may remain dormant and viable in the soil for more than ten years (Wilhelm 1955) and may thus be an initial inoculum source in the soil.

8.2 Role of salicylic acid in the response of *B. napus* to *V. longisporum*

Plant derived phenylpropanoids are the most common secondary metabolic compounds in plants, bacteria and fungi, many of which are used in the protection against biotic or

abiotic stresses or play essential roles in plant physiology (Seigler 1999; Korkina et al. 2011). Phenols bear a 3-carbon chain attached to a 6-carbon aromatic ring and commonly are formed from *t*-cinnamic or *p*-coumaric acids. Among these components, simple phenolic acids and esters, flavonoids and lignin have been reported to be functional in the resistance of various host plants against *Verticillium* pathogens or have direct effects on fungal growth (Howell et al. 1976; Picman et al. 1995; Debode et al. 2005; Eynck et al. 2009a; Xu et al. 2011; König et al. 2014). In cotton, enzyme activities of PAL and POX were increased after inoculation with *V. dahliae* (Xu et al. 2011). Furthermore, lignin is believed to contribute to the resistance of cotton to *V. dahliae* according to the observation of more intensive lignification in the resistant cultivar. Besides, salicylic acid (SA) has been reported to be involved in disease defense as well.

Similar to tomato and tobacco, a remarkable increase of susceptibility to *V. longisporum* was observed in the present study when using SA-deficient transgenic *Nah*G oilseed rape, indicating an essential role of SA in basal resistance of oilseed rape against *V. longisporum* (Achuo et al. 2004). As described in previous studies, SA can be transported through phloem to all plant parts as a mobile signal triggering local and systemic acquired resistance against viruses or biotrophic pathogens. In addition, it may be accumulated in xylem vessels by root colonized soil-borne or seed-borne pathogens and may be long-distance transported toward distal parts of the plants for response to disease (Ben-Tal and Cleland 1982; Rocher et al. 2006; Ratzinger et al. 2009; Rivas-San Vicente and Plasencia 2011). The level of conjugated SA found in wild type oilseed rape is at least 4-fold higher than free SA, which is believed to be actively transported from the cytosol into the vacuole for storage and released as free SA when necessary to trigger systemic acquired resistance during infection (Rivas-San Vicente and Plasencia 2011). Infection of *V. longisporum* caused induction of both conjugated and free SA in oilseed rape, but only conjugated SA and no free SA was accumulated after exogenous application of SA. This result proved that oilseed rape prefers to convert free SA into the conjugated form preventing phytotoxic effects similar to *A. thaliana* (Wildermuth 2006; Nobuta et al. 2007), since an exogenously applied higher concentration of SA was confirmed in the present study to be phytotoxic to host plants as well. In contrast to enhancing the resistance of plants against other pathogens (Chen et al. 2016), an exogenous application of 0.5 mM SA is not efficient to reduce infection of *V. longisporum* on oilseed rape. In the present study, no effect was observed on the growth of *V. longisporum* up to a concentration of 1.5 mM exogenously applied SA, which is substantially higher than the endogenous SA levels in diseased plants which have been reported to be in a range from 4 to 80 µg/g fresh weight in previous studies (Ratzinger et

al. 2009; Kamble et al. 2013). Since the reported concentration of SA in *V. longisporum* infected plant tissues did not inhibit fungal growth *in vitro* but showed a weak to moderate promoting effect, SA is unlikely to suppress the growth of pathogens in plants as a antifungal compound but plays a role in signaling as trigger or modulator (Rüffer et al. 1995).

The first penetration of roots by *V. longisporum* has been reported to appear after 60 hpi in plants root-dip inoculated with a conidia suspension (Eynck et al. 2007). In the early response of *B. napus* to *V. longisporum*, induction of SA was faster in the resistant cultivar than in the susceptible cultivar. However, in contrast to *A. thaliana*, the quantitative resistance of oilseed rape to *V. longisporum* seems to be dependent on neither SA- nor JA- associated plant resistant proteins PR1, PR2 and PDF1.2 (Johansson et al. 2006b; Kamble et al. 2013). By infection with *V. longisporum*, *PR1* and *PR2* were up-regulated. However, higher expression of *PR1* and *PR2* were unexpectedly found in susceptible cultivar, which may indicate that the induction of these proteins did not enhance cultivar-related resistance of oilseed rape against *V. longisporum*. Similar results have been obtained by Coquoz et al. (1995) on potato: the exogenous application of SA on potato led to an increase in *PR* gene expression but not the resistance to *Phytophthora infestans* and *Alternaria solani*. However, the gene expression to unit of pathogen amount showed another image, that stronger upregulation towards fungus was found in resistant cultivar. The responses relative to fungal biomass can more relevant to accurately describe plant responses tp a certain unit of pathogen biomas. In contrast to *A. thaliana*, in which the accumulation of SA induced by pathogen or elicitor is synthesized through the isochorismate pathway, a similar tendency of enhanced enzyme activity of benzoic acid 2-hydroxylase (BA2H) and content of SA was observed in the present study indicating that *V. longisporum*-induced accumulation of SA possibly involves the synthesis via phenylalanine. Interestingly, an identical induction of SA has not only been observed in the resistant but also in the susceptible cultivar. However, the induction in the susceptible cultivar was delayed and first observed only at 14 dpi, when the pathogen assumingly had already colonized the cortex of roots and spread into the vascular vessels (Eynck et al. 2007). A higher biomass of *V. longisporum* was detected as well in the hypocotyl of the susceptible cultivar at this time point. As well known, the biosynthesis of SA and lignin share the same precursors, such as *t*-cinnamic acid and phenylalanine. Therefore, these two biosynthetic pathways may have a competitive relationship. Phenolic acids and lignin have been shown to be important for resistance of *B. napus* to *V. longisporum* by producing occlusions in the xylem vessels for inhibiting the spread of the fungus (Eynck et al. 2009a; Kamble et al. 2013). Both resistant and susceptible cultivars tested in the

present study showed increases of phenolic acids after infection with *V. longisporum*. The accumulation of phenolic acids happened at 14 dpi, while increase of SA was induced by *V. longisporum* earlier at 7 dpi in the resistant cultivar. However, higher contents of SA were found in a susceptible than resistant cultivar in field-grown plants, which was at the growing stage of stem elongation and much later than the tested plants from greenhouse experiments. Induction of most of the tested phenolic acids was quicker and higher in the resistant cultivar. Results obtained from the present study strongly suggest that SA plays an important role in the early response of oilseed rape to *V. longisporum* infection probably during the early penetration stages of the pathogen. The induction of SA may be a key resistance mechanism for preventing *V. longisporum* from penetrating the root of oilseed rape. However, once *V. longisporum* is established in the plant, the increase in SA is no more related to disease resistance but becomes a negative compound, as in the later stages of colonization, SA synthesis may be at the expense of synthesis of *p*-coumaric acid, ferulic acid and sinapic acid, which are early precursors of lignin and may weaken physical plant defense resulting in a better development of *V. longisporum* in host plant tissues. Accordingly, pre-inoculation with a weakened, killed or avirulent pathogen, which induces the accumulation of SA (Glazebrook 2005; Huang et al. 2003), may be a potential control strategy to reduce the initial infection with *V. longisporum*.

In addition to resistance, the increase of SA level may also be associated with the symptoms caused by *V. longisporum*. As described in previous studies, SA is involved in the regulation of senescence by reducing chlorophyll contents and increasing ROS levels (Rao et al. 1997; Moharekar et al. 2003; Rivas-San Vicente and Plasencia 2011; Vogelmann et al. 2012). Therefore, a strong increase of SA levels after *V. longisporum* infection may lead to early yellowing of leaves. Moreover, in *A. thaliana*, inhibition of the gibberellin and auxin signaling pathway by SA was discovered (Wang et al. 2007; Gallego-Giraldo et al. 2011). Gibberellin and auxin are plant hormones stimulating shoot elongation. Thus, the growth-reducing effects observed on diseased plants may be mediated by the induction of SA. Nevertheless, the SA-deficient transgenic *Nah*G oilseed rape mutants also showed strong yellowing and stunting symptoms indicating that increase of SA is not the only reason for causing *Verticillium* disease symptom.

8.3 Identification of *B. napus* tissue and genotypes with enhanced resistance to *V. longisporum*

Due to intensive breeding, modern oilseed rape has a relatively narrow genetic base (Hasan et al. 2008; Girke et al. 2012), based on the hybridization between *B. rapa* and *B. oleracea*. Therefore, searching for sources of resistance in the parental species of *B. rapa* and *B. oleracea*, or in the wider gene pool of Brassicaceae, appears a promising

approach to expand genetic diversity in the germplasm of oilseed rape. Several studies with tomato, cotton and *Arabidopsis* illustrated the resistance mechanisms to *Verticillium* diseases (Fradin et al. 2009; Gayoso et al. 2010; Zhang et al. 2013; König et al. 2014). Although intensive research has been conducted, only one resistant gene, *Ve1* from tomato, encoding cell surface receptor proteins of the extracellular leucin-rich repeat receptor-like protein class, has been so far characterized in detail as resistance mechanism to race 1 strains of *V. dahliae* and *V. albo-atrum* (Fradin et al. 2009). However, *Ve1* did not confer resistance to *V. longisporum* in *A. thaliana* (Fradin et al. 2011).

Resistance of *B. napus* is believed to be quantitative in nature; hence, several studies aimed at identifying resistance to *V. longisporum* using quantitative trait loci (QTL) mapping (Rygulla et al. 2007; Obermeier et al. 2013). Besides QTL mapping, a new powerful tool, genome-wide association study (GWAS), has been introduced for investigating associations between responses and genome information (gene expression levels or single nucleotide polymorphisms), which has shown great potential for detecting quantitative resistance markers with high resolution (Korte and Farlow 2013; Chen et al. 2016). A sufficiently large set of phenotyping data is the basic requirement of GWAS analysis. The 159 accessions from the *B. napus* Diversity Fixed Foundation Set (BnaDFFS) showed high diversity in responses to infection with *V. longisporum* under greenhouse conditions. Among these accessions, spring and semi-winter oilseed rape types were relatively less susceptible to infection of *V. longisporum*. Previous research showed that winter and spring oilseed rape could be clearly differentiated by using RFLP or SSR markers (Diers et al. 1996; Charters et al. 1996; Plieske and Struss 2001). Therefore, the genetic differences may serve as potential information to search for resistance genes. Although results from greenhouse screening were hard to be transferred directly to the field because of various effects from the environment or different isolates used in greenhouse and under natural infection (Eynck et al. 2009b), a significant correlation of greenhouse screening data and results from field trials was found in the present study. Several interesting accessions that showed high susceptibility and resistance both under greenhouse and field conditions were identified. These accessions may be quite useful in further investigations which should use to reveal in more detail of the nature of quantitative resistance of oilseed rape to *V. longisporum*.

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Summary

Oilseed rape (*Brassica napus*) is the second most important source of vegetable plant oil. Thanks to the successful variety canola with low erucic acid and low glucosinolate contents, oilseed rape provides healthier and better quality oil for human consumption and meal for animal fodder production. Products from oilseed rape are nowadays used as edible oil for humans, as meal for animal feeding and as biodiesel for industry. With the growing demands, the global oilseed rape production is rapidly increasing since the last 40 years. Due to the intensive production of oilseed rape, several biotic and abiotic stresses threaten the growth and productivity of this crop. Among the economically important diseases, *Verticillium longisporum*, a soil-borne vascular pathogen causing 'Verticillium stem striping' on oilseed rape, has become a potential threat in major oilseed rape production areas. This Brassicaceae host-specific pathogen leads to abnormal foliar chlorosis, reduction of plant height under greenhouse conditions, and unilateral stem striping in the field. It has been estimated to cause 10% to 50% of yield losses in the field. No registered fungicide is available on the market to control this pathogen. In spite of intensive research on *V. longisporum* in the last decades, some basic knowledge on epidemiology, seed transmission and resistance mechanisms are still lacking. Therefore, the present study has focused on exploring the time course of infection and disease development in the field including the potential of seed transmission. Besides, the role of salicylic acid (SA) in resistance of oilseed rape to *V. longisporum* was investigated. Furthermore, tests to identify oilseed rape genotypes and accessions with enhanced resistance to *V. longisporum* were conducted.

Chapter 2 describes the development of *V. longisporum* on resistant and susceptible cultivars under field conditions. Field experiments with microsclerotia inoculated plots were conducted in the season 2015/16. A qPCR assay with a species specific *ß*-tubulin primer pair was used for detecting and monitoring the growth of *V. longisporum* in different plant parts during the whole production period. Endogenous levels of SA were measured in different plant tissues and growth stages and related to the development of *V. longisporum*. Besides, the disease effects on seed yield and quality were measured. The first detection of *V. longisporum* in roots of winter oilseed rape was before winter at BBCH 14. Low amounts of endogenous SA were recorded at flowering stage (BBCH 61-65). Possibly due to the increased temperature and low levels of SA, growth of *V. longisporum* increased rapidly during the flowering stage. In contrast to the susceptible cultivar of winter oilseed rape, in which *V. longisporum* easily spreads into the entire plants, resistant cultivar can restrict the growth of *V. longisporum* to the hypocotyl and prevent *V. longisporum* from further spreading into the stem.

Chapter 3 focuses on revealing the nature of seed transmission of *V. longisporum* on *B. napus*. Three contrasting cultivars were artificially inoculated with wild type *V. longisporum* and a DsRed labeled strain of *V. longisporum* in order to verify seed transmission of *V. longisporum* in greenhouse conditions. A qPCR assay using *ß*-tubulin primers was applied to quantify the amount of *V. longisporum* biomass in plant samples from greenhouse and field experiments. Seeds obtained from diseased plants under greenhouse condition had an incidence of *V. longisporum* infestation ranging from 0.08% to 13.3% in *in vitro* tests, among which the highest level of seed transmission incidence was recorded for the susceptible cultivar Falcon. The presence of *V. longisporum* in seeds was also confirmed by qPCR. In order to confirm whether *V. longisporum* transmitted into the seeds causes disease in the second generation or not, seeds from these lots were surface sterilized and sown for symptom observation. All cultivars had a portion of plants showing disease symptoms, which however was quite low at about 1% of plants being colonized by microsclerotia on the stem. Although seeds harvested from diseased field-grown plants did not yield isolates in the *in vitro* test, small amounts of *V. longisporum* were detectable by qPCR in these seeds.

Chapter 4 demonstrates the effect of SA on *B. napus* response to *V. longisporum*. In order to measure the direct effect of SA on *V. longisporum*, a series of PDA plates containing different concentrations of SA ranging from 0 to 5mM was applied. The same series of SA solutions was used for root-dipping of oilseed rape plants. Subsequently, after 24 hours, treated plants were inoculated with *V. longisporum* or mock-inoculated with water. No inhibition of *in vitro* growth of *V. longisporum* was found at concentrations up to 1.5 mM of SA. The inhibition of growth of *V. longisporum* on PDA containing more than 2 mM SA was not reversible. Exogenously applied SA not only had an antifungal effect on *V. longisporum* but also had a phytotoxic effect on *B. napus*. The concentration of SA equivalent to previously reported concentrations in *V. longisporum* infected plant tissues did not inhibit the growth of *V. longisporum in vitro*, in contrast, it showed a weak to moderate promotion. Enhanced endogenous SA levels in oilseed rape were induced by *V. longisporum*, except for plants pretreated with 2.5 mM SA. However, exogenous pretreatment of SA did not affect the development of *V. longisporum* on plants during the flowering period.

Chapter 5 focuses on discovering a differential role of SA in basal and cultivar resistance of *B. napus* to *V. longisporum*. Responses to *V. longisporum* infection of SA-deficient *Nah*G transgenic plants derived from spring oilseed rape cultivar Drakkar as well as the two contrasting winter type cultivars Falcon and SEM were studied. A remarkable increase of susceptibility to *V. longisporum* was observed on SA-deficient *Nah*G

transgenic plants that indicated an important role of SA in basal resistance of oilseed rape against *V. longisporum*. To discover the role of SA in cultivar specific resistance, metabolites, enzyme activities and gene expression of key enzymes within the lignin and SA biosynthesis pathways were measured by HPLC-DAD/Fluor, photometer and RT-qPCR with two reference genes, respectively. A faster increase of SA was observed in the resistant cultivar at 7 dpi, which allowed poorer growth of *V. longisporum* in the hypocotyl, indicating that elevated SA is important for disease defense in the asymptomatic phase of colonization. At later stages of infection at 14 and 21 dpi (symptomatic stages), the increased SA levels were similar in the susceptible and resistant cultivar. However, a remarkable increase in the levels of several phenolic acids was observed in resistance cultivar but not in susceptible cultivar. These results indicated that SA and phenolic acid synthesis are important in cultivar-related resistance during asymptomatic and symptomatic stages of infection, respectively.

Chapter 6 aimed at characterizing of oilseed rape stem reactions during infection with *V. longisporum*. The responses of injured and non-injured stems to *V. longisporum* infection were observed. In accordance with Koch's principle, new disease symptoms caused by *V. longisporum* were verified. Results showed that plants without stem injury were free from penetration of *V. longisporum*, while with injury, *V. longisporum* was capable to cause disease on the plants. These results indicated that the stem cuticle may be an effective physical barrier to prevent *V. longisporum* from penetrating into the stem.

The last chapter described resistance identification by screening under greenhouse and field conditions. A large number of the *Brassica* accessions from *Brassica napus* Diversity Fixed Foundation Set (BnaDFFS) were screened for resistance against *V. longisporum* in multiple greenhouse experiments with reference cultivars and under field conditions. Leaf chlorosis and reduction of plant height were the parameters to evaluate the response of plants to the pathogen in the greenhouse. An assessment key considering the formation of microsclerotia in the stem was used to estimate disease severity on mature plants after harvest. Large variation of responses to infection with *V. longisporum* occurred among the screened accessions. In the present study, a significant correlation of greenhouse screening data and disease scoring data from the field trial was found. Therefore, several interesting accessions that showed high susceptibility or resistance both under greenhouse and field conditions were identified and selected for further research.

Appendix

Appendix 1 Phenological growth stages and BBCH-identification keys of oilseed rape (*Brassica napus* L.) (Weber and Bleiholder, 1990; Lancashire et al., 1991)

Code	Description
Principal growth stage 0: Germination	
00	Dry seed
01	Beginning of seed imbibition
03	Seed imbibition complete
05	Radicle emerged from seed
07	Hypocotyl with cotyledons emerged from seed
08	Hypocotyl with cotyledons growing towards soil surface
09	Emergence: cotyledons emerge through soil surface
Principal growth stage 1: Leaf development[1]	
10	Cotyledons completely unfolded
11	First leaf unfolded
12	2 leaves unfolded
13	3 leaves unfolded
1 .	Stages continuous till . . .
19	9 or more leaves unfolded
Principal growth stage 2: Formation of side shoots	
20	No side shoots
21	Beginning of side shoot development: first side shoot detectable
22	2 side shoots detectable
23	3 side shoots detectable
2 .	Stages continuous till . . .
29	End of side shoot development: 9 or more side shoots detectable
Principal growth stage 3: Stem elongation[2]	
30	Beginning of stem elongation: no internodes (“rosette”)
31	1 visibly extended internode
32	2 visibly extended internodes
33	3 visibly extended internodes
3 .	Stages continuous till . . .
39	9 or more visibly extended internodes
Principal growth stage 5: Inflorescence emergence	
50	Flower buds present, still enclosed by leaves
51	Flower buds visible from above (“green bud”)
52	Flower buds free, level with the youngest leaves
53	Flower buds raised above the youngest leaves
55	Individual flower buds (main inflorescence) visible but still closed
57	Individual flower buds (secondary inflorescences) visible but still closed
59	First petals visible, flower buds still closed (“yellow bud”)

[1] Stem elongation may occur earlier than stage 19; in this case continue with stage 20

[2] Visibly extended internode n develops between leaf n and leaf n+1

Appendix 1 continued

Code	Description
Principal growth stage 6: Flowering	
60	First flowers open
61	10% of flowers on main raceme open, main raceme elongating
62	20% of flowers on main raceme open
63	30% of flowers on main raceme open
64	40% of flowers on main raceme open
65	Full flowering: 50% flowers on main raceme open, older petals falling
67	Flowering declining: majority of petals fallen
69	End of flowering
Principal growth stage 7: Development of fruit	
71	10% of pods have reached final size
72	20% of pods have reached final size
73	30% of pods have reached final size
74	40% of pods have reached final size
75	50% of pods have reached final size
76	60% of pods have reached final size
77	70% of pods have reached final size
78	80% of pods have reached final size
79	Nearly all pods have reached final size
Principal growth stage 8: Ripening	
80	Beginning of ripening: seed green, filling pod cavity
81	10% of pods ripe, seeds dark and hard
82	20% of pods ripe, seeds dark and hard
83	30% of pods ripe, seeds dark and hard
84	40% of pods ripe, seeds dark and hard
85	50% of pods ripe, seeds dark and hard
86	60% of pods ripe, seeds dark and hard
87	70% of pods ripe, seeds dark and hard
88	80% of pods ripe, seeds dark and hard
89	Fully ripe: nearly all pods ripe, seeds dark and hard
Principal growth stage 9: Senescence	
97	Plant dead and dry
99	Harvested product

Appendix 2 Response of Brassicaceae accessions to *Verticillium longisporum* under greenhouse conditions as measured by AUDPC and relative stunting. OSR: oilseed rape.

Accessions No.	Variety	Type	Normalized AUDPCnet	Relative stunting [%]
BnASSYST-001	Alesi	Winter OSR	1.07±0.28	0.67±0.67
BnASSYST-002	Remy	Winter OSR	1.04±0.24	1.57±0.45
BnASSYST-005	Pirola	Winter OSR	1.20±0.30	2.22±0.51
BnASSYST-009	Agalon	Winter OSR	1.18±0.29	1.83±0.58
BnASSYST-014	Rodeo	Winter OSR	0.86±0.45	1.63±0.77
BnASSYST-015	Rapid	Winter OSR	0.79±0.39	1.06±0.46
BnASSYST-016	Boston	Winter OSR	1.31±0.20	1.69±0.55
BnASSYST-020	Pacific	Winter OSR	1.08±0.24	1.14±0.46
BnASSYST-021	Savannah	Winter OSR	0.97±0.34	1.61±0.71
BnASSYST-028	Capitol	Winter OSR	1.51±0.16	1.60±0.22
BnASSYST-029	Idol	Winter OSR	1.15±0.25	0.82±0.63
BnASSYST-033	Lisabeth	Winter OSR	1.11±0.30	1.55±0.59
BnASSYST-034	Lipid	Winter OSR	1.09±0.33	1.14±0.84
BnASSYST-035	Lipton	Winter OSR	1.14±0.30	1.37±0.64
BnASSYST-037	Contact	Winter OSR	1.28±0.20	1.48±0.43
BnASSYST-039	Oase	Winter OSR	1.02±0.34	0.95±0.70
BnASSYST-040	Apex	Winter OSR	1.25±0.24	1.59±0.52
BnASSYST-044	Laser	Winter OSR	0.67±0.32	0.89±0.45
BnASSYST-045	Fortis	Winter OSR	1.17±0.26	1.58±0.59
BnASSYST-046	Smart	Winter OSR	1.19±0.34	1.66±0.54
BnASSYST-047	Roxet	Winter OSR	1.17±0.25	1.60±0.48
BnASSYST-048	NK Bravour	Winter OSR	1.45±0.30	2.14±0.94
BnASSYST-051	Sansibar	Winter OSR	1.34±0.20	2.15±0.54
BnASSYST-052	SWGospel	Winter OSR	0.91±0.38	0.80±0.66
BnASSYST-053	Verona	Winter OSR	1.16±0.30	1.30±0.51
BnASSYST-056	Musette	Winter OSR	1.38±0.35	1.85±0.86
BnASSYST-057	Kvintett	Winter OSR	1.10±0.33	1.08±0.51
BnASSYST-058	Falstaff	Winter OSR	0.95±0.21	1.01±0.40
BnASSYST-060	Viking	Winter OSR	0.94±0.25	0.89±0.43
BnASSYST-061	Aragon	Winter OSR	0.46±0.20	0.73±0.48
BnASSYST-068	Zephir	Winter OSR	0.50±0.35	1.06±0.36
BnASSYST-069	SLM 0413	Winter OSR	0.85±0.25	0.78±0.46
BnASSYST-070	SLM 0512	Winter OSR	1.07±0.28	0.78±0.47
BnASSYST-071	LSF 0519	Winter OSR	1.07±0.50	1.71±0.88
BnASSYST-073	Amor	Winter OSR	1.02±0.34	1.54±0.73
BnASSYST-076	Caramba	Winter OSR	1.14±0.17	0.98±0.34
BnASSYST-078	Express 617	Winter OSR	1.44±0.30	2.25±0.86
BnASSYST-080	Orlando	Winter OSR	1.15±0.34	1.12±0.41
BnASSYST-081	Pollen	Winter OSR	1.06±0.34	1.66±0.85
BnASSYST-082	Prince	Winter OSR	0.48±0.33	0.91±0.60

Appendix 2 continued

Accessions No.	Variety	Type	Normalized AUDPCnet	Relative stunting [%]
BnASSYST-089	Amber x Commanche	Winter OSR	0.99±0.33	1.51±0.60
BnASSYST-093	Canberra x Courage DH Line	Winter OSR	0.79±0.41	0.51±0.54
BnASSYST-096	Hansen X Gaspard DH Line	Winter OSR	1.45±0.15	2.86±0.34
BnASSYST-097	Madrigal x Recital DH Line	Winter OSR	1.07±0.26	1.82±0.40
BnASSYST-098	Rafal DH1	Winter OSR	0.93±0.28	0.80±0.56
BnASSYST-099	Tapidor DH	Winter OSR	0.75±0.34	0.38±0.34
BnASSYST-101	Eurol	Winter OSR	1.26±0.26	1.50±0.50
BnASSYST-105	Licrown X Express DH Line	Winter OSR	0.60±0.28	0.88±0.35
BnASSYST-106	Shannon x Winner DH Line	Winter OSR	0.70±0.49	1.26±0.53
BnASSYST-107	Janetzkis Schlesischer	Winter OSR	0.57±0.21	0.64±0.43
BnASSYST-108	Lembkes malchower (Lenora)	Winter OSR	0.91±0.32	1.58±0.58
BnASSYST-109	Norin	Winter OSR	1.24±0.17	1.66±0.33
BnASSYST-112	Mansholt	Winter OSR	1.15±0.21	1.23±0.41
BnASSYST-113	Samourai	Winter OSR	1.82±0.66	2.26±1.05
BnASSYST-114	Sollux	Winter OSR	1.58±0.46	1.63±0.71
BnASSYST-115	Akela	Winter OSR	1.45±0.80	1.74±0.92
BnASSYST-117	Maplus	Winter OSR	1.79±0.84	2.46±1.21
BnASSYST-118	Askari	Winter OSR	1.66±0.93	2.26±0.96
BnASSYST-119	Lirabon	Winter OSR	1.57±0.68	1.84±0.96
BnASSYST-122	Cobra	Winter OSR	1.71±0.92	2.60±0.96
BnASSYST-124	Mohican	Winter OSR	1.50±0.52	1.67±0.81
BnASSYST-125	Flip	Winter OSR	0.99±0.42	0.74±0.32
BnASSYST-126	Zenith	Winter OSR	1.35±0.49	1.59±0.77
BnASSYST-127	Phil	Winter OSR	2.12±0.65	2.54±0.74
BnASSYST-128	Leopard	Winter OSR	1.45±0.44	1.46±0.71
BnASSYST-129	Resyn-H048	Winter OSR	1.20±0.30	0.87±0.09
BnASSYST-137	Coriander	Winter OSR	1.63±0.50	1.89±0.82
BnASSYST-138	Diamant	Winter OSR	1.65±0.38	2.46±0.80
BnASSYST-139	Dippes	Winter OSR	1.54±0.52	2.14±0.73
BnASSYST-140	Doral	Winter OSR	1.38±0.51	1.54±0.90
BnASSYST-149	Krapphauser	Winter OSR	1.10±0.41	0.89±0.31
BnASSYST-150	Kromerska	Winter OSR	1.22±0.73	1.26±0.91
BnASSYST-157	Madora	Winter OSR	1.45±0.45	1.81±0.83
BnASSYST-160	Matador	Winter OSR	1.56±0.25	2.43±0.59
BnASSYST-163	Mytnickij	Winter OSR	1.63±0.42	2.92±0.47
BnASSYST-165	Norde	Winter OSR	1.06±0.27	2.60±0.52
BnASSYST-166	Panter	Winter OSR	1.70±0.49	2.26±0.75
BnASSYST-168	Ramses	Winter OSR	1.53±0.53	1.91±0.86
BnASSYST-169	Sarepta	Winter OSR	1.42±0.47	0.97±0.58
BnASSYST-173	Sobotkowski	Winter OSR	0.52±0.54	0.48±0.44
BnASSYST-178	Vinnickij 15/59	Winter OSR	0.82±0.46	0.88±0.55

Appendix 2 continued

Accessions No.	Variety	Type	Normalized AUDPCnet	Relative stunting [%]
BnASSYST-180	V8	Winter OSR	1.25±0.68	2.45±0.80
BnASSYST-185	Canard	Winter OSR	1.33±0.32	2.20±0.70
BnASSYST-203	Taisetsu	Winter OSR	0.25±0.41	0.54±0.44
BnASSYST-204	Q100	Synthetic OSR	1.08±0.54	0.88±0.47
BnASSYST-208	Couve nabica	Leafy vegetable	1.02±0.30	0.82±0.54
BnASSYST-209	Ragged jack	Rape kale	1.12±0.85	1.12±0.44
BnASSYST-211	Siberische boerenkool	Siberian kale	0.70±0.32	0.53±0.54
BnASSYST-212	Slapska, Slapy	Winter OSR	1.03±0.64	1.11±0.52
BnASSYST-213	Abukuma natane	Winter OSR	1.38±0.41	2.31±0.64
BnASSYST-218	Groene groninger snujmoes	Siberian kale	1.88±0.72	2.69±0.71
BnASSYST-221	Rapid cycling rape (CrGC5)	Spring OSR	0.73±0.18	0.69±0.35
BnASSYST-224	Wild accession	Semi-winter OSR	0.73±0.34	0.32±0.37
BnASSYST-229	SWU Chinese 1	Semi-winter OSR	0.41±0.37	0.68±0.67
BnASSYST-230	SWU Chinese 2	Semi-winter OSR	0.30±0.21	0.57±0.67
BnASSYST-231	SWU Chinese 3	Semi-winter OSR	0.84±0.41	0.70±0.43
BnASSYST-232	SWU Chinese 5	Semi-winter OSR	0.55±0.33	0.89±0.58
BnASSYST-236	SWU Chinese 9	Semi-winter OSR	0.62±0.38	0.85±0.40
Reference for SS	Zhongyou281	Semi-winter OSR	0.92±0.52	0.75±0.39
BnASSYST-238	Drakkar	Spring OSR	0.38±0.23	0.28±0.43
BnASSYST-239	Stellar DH	Spring OSR	0.40±0.24	0.92±0.31
BnASSYST-240	Westar DH	Spring OSR	1.08±0.55	0.42±0.24
BnASSYST-241	Yudal	Spring OSR	0.52±0.32	0.24±0.23
BnASSYST-242	Brutor	Spring OSR	0.50±0.38	0.64±0.36
BnASSYST-245	Cresor	Spring OSR	1.18±0.42	0.38±0.29
BnASSYST-251	Karat	Spring OSR	0.28±0.34	0.53±0.65
BnASSYST-257	Monty-028DH	Spring OSR	0.42±0.29	0.64±0.14
BnASSYST-258	N01D-1330	Spring OSR	0.89±0.50	0.63±0.28
BnASSYST-259	N02D-1952	Spring OSR	0.74±0.27	0.04±0.20
BnASSYST-260	Surpass400-024DH	Spring OSR	0.66±0.23	0.82±0.50
BnASSYST-261	Cubs root	Spring OSR	0.85±0.57	0.50±0.47
BnASSYST-263	Erglu	Spring OSR	0.66±0.41	0.68±0.25
BnASSYST-264	Helios	Spring OSR	0.78±0.24	0.41±0.24
BnASSYST-268	Mazowiecki	Spring OSR	0.54±0.18	0.45±0.25
BnASSYST-269	Tantal	Spring OSR	0.78±0.30	0.87±0.30
BnASSYST-270	Weinhenstephaner	Spring OSR	0.85±0.44	0.58±0.23
BnASSYST-272	Alku	Spring OSR	0.38±0.17	0.77±0.36
BnASSYST-273	Bronowski	Spring OSR	0.71±0.29	0.49±0.27
BnASSYST-274	Ceska Krajova	Spring OSR	0.73±0.65	0.91±0.25
BnASSYST-275	Duplo	Spring OSR	0.55±0.38	0.35±0.20
BnASSYST-282	SvalšfÕs Gulle	Spring OSR	0.08±0.29	0.70±0.27
BnASSYST-283	Topas	Spring OSR	0.16±0.15	0.35±0.36

Appendix 2 continued

Accessions No.	Variety	Type	Normalized AUDPCnet	Relative stunting [%]
BnASSYST-284	Tribute	Spring OSR	0.39±0.42	0.28±0.28
BnASSYST-290	Ability	Spring OSR	0.26±0.34	0.38±0.18
BnASSYST-300	Magma	Spring OSR	0.40±0.29	0.54±0.41
BnASSYST-307	Tribune	Spring OSR	0.70±0.31	0.27±0.24
BnASSYST-323	Daichousen (mizuyasu)	Spring OSR	0.18±0.28	0.59±0.24
BnASSYST-325	Erake	Spring OSR	0.68±0.40	0.92±0.23
BnASSYST-326	Furax	Spring OSR	0.53±0.23	0.76±0.21
BnASSYST-349	Loras	Spring OSR	0.56±0.22	0.84±0.37
BnASSYST-359	Odin	Spring OSR	0.61±0.31	0.70±0.26
BnASSYST-361	Omega	Spring OSR	0.66±0.38	0.62±0.19
BnASSYST-367	Pobeda	Spring OSR	0.39±0.27	0.66±0.19
BnASSYST-373	Rucabo	Spring OSR	0.69±0.28	0.35±0.25
BnASSYST-394	Willi	Spring OSR	0.41±0.23	0.43±0.38
BnASSYST-399	Zairai Chousenshu	Spring OSR	0.51±0.26	0.58±0.61
BnASSYST-401	Vige DH1	Swede	0.56±0.69	0.81±0.26
BnASSYST-414	Sensation NZ	Swede	1.20±0.87	0.74±0.40
BnASSYST-418	Altasweet	Swede	1.77±0.88	0.87±0.34
BnASSYST-436	Tina	Swede	0.77±0.52	1.22±0.37
BnASSYST-438	York	Swede	1.34±1.26	0.55±0.24
BnASSYST-509	Cabernet	Winter OSR	1.57±0.78	2.19±0.93
BnASSYST-510	Cabriolet	Winter OSR	1.72±0.51	2.31±0.91
BnASSYST-511	Castille	Winter OSR	2.08±0.44	2.47±0.76
BnASSYST-512	Catana	Winter OSR	0.82±0.57	1.36±0.67
BnASSYST-513	Chuanyou 2	Semi-winter OSR	1.57±0.48	0.82±0.33
BnASSYST-514	Dimension	Winter OSR	0.63±0.57	0.51±0.38
BnASSYST-517	Huron x Navajo	Winter OSR	1.25±0.55	1.63±0.82
BnASSYST-518	Inca x Contact	Winter OSR	1.53±0.79	2.05±1.01
BnASSYST-520	Ningyou 7	Semi-winter OSR	0.88±0.62	0.71±0.29
BnASSYST-521	Palmedor	Winter OSR	0.99±0.42	0.74±0.30
BnASSYST-522	POH 285, Bolko	Winter OSR	1.21±0.57	1.52±0.93
BnASSYST-523	Quinta	Winter OSR	1.87±0.72	2.31±0.69
BnASSYST-524	Rocket	Winter OSR	1.41±0.64	2.03±0.83
BnASSYST-526	Shengliyoucai	Semi-winter OSR	0.60±0.50	1.19±0.43
BnASSYST-527	Temple	Winter OSR	0.66±0.55	0.64±0.34
BnASSYST-528	Vision	Winter OSR	0.83±0.51	0.79±0.42
BnASSYST-529	Xiangyou 15	Semi-winter OSR	1.38±0.60	0.56±0.22
BnASSYST-530	Zhongshuang 11	Semi-winter OSR	0.46±0.24	1.19±0.54
Susceptible reference	Falcon	Winter OSR	1.38±0.70	1.38±0.69
Resistant reference	SEM	Winter OSR	0.62±0.41	0.62±0.59

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Statutory declaration

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

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