Studies on Resistance of Oilseed Rape (*Brassica napus*) to *Verticillium longisporum* – Interaction with Drought Stress, Role of Xylem Sap Modulations and Phenotyping Under Controlled and Field Conditions

Dissertation

to obtain the Ph.D. degree

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

at the Faculty of Agricultural Sciences,

Georg-August-University Göttingen, Germany

Daniel Teshome Lopisso

Born in Addis Ababa, Ethiopia

Göttingen, November 2014

D7

1. Name of referee: Prof. Dr. Andreas von Tiedemann

2. Name of co-referee: Prof. Dr. Petr Karlovsky

Date of disputation: 19 November 2014

Table of contents

1. General Introduction 1

1.1 Oilseed rape 1

1.2 *Verticillium* diseases 3

1.3 *V. longisporum*: the youngest vascular pathogen of oilseed rape 3

1.3.1 Significance and geographical distribution of *V. longisporum* 5

1.3.2 *V. longisporum* disease cycle 6

1.3.3 Pathogenicity factors in *V. longisporum* 8

1.3.4 Management of *V. longisporum* in oilseed rape 8

1.4 Aims of the thesis 9

1.5 References 11

2. Phenotypic and molecular evaluation of *Brassica napus* lines for *V. longisporum* resistance under greenhouse, outdoor and field conditions 17

2.1 Introduction 17

2.2 Materials and methods 18

2.2.1 Greenhouse screening 18

2.2.1.1 Plant material 18

2.2.1.2 Experimental design 19

2.2.1.3 Inoculation procedures and disease scoring 19

2.2.2 Field trials 20

2.2.2.1 Plant materials 20

2.2.2.2 Description of experimental locations 20

2.2.2.3 Experimental design 20

2.2.2.4 Inoculation and agronomic practices 21

2.2.2.5 Disease assessment 22

2.2.3 Outdoor experiment 23

2.2.3.1 Plant materials and growing conditions 23

2.2.3.2 Inoculation and disease scoring 24

2.2.3.3 Experimental design and outdoor environmental conditions 24

2.2.3.4 Agronomic and disease data collection 25

2.2.4 Data analysis 25

2.3 Results and discussion 25

2.3.1 Greenhouse screening 25

2.3.2 Field trials 28

2.3.2.1 *B. napus* DH lines evaluated for *V. longisporum* resistance in multi-

location field trials for three consecutive seasons…………………………… 29
2.3.2.2 Different sets of *B. napus* lines screened for *V. longisporum* resistance under field conditions for one season……………………………………………… 37
2.3.2.3 Determination of the critical crop growth stage for molecular detection and differential quantification of *V. longisporum* disease severity in field grown winter oilseed rape plants ……………………………… 45
2.3.3 Outdoor experiment …………………………………………………………………………… 48
2.4 References ………………………………………………………………………………………… 52

3. Searching for cultivar-related resistance factors to *V. longisporum* in oilseed rape (*Brassica napus)* xylem sap ………………………………………………………………………… 54
3.1 Introduction ……………………………………………………………………………………… 54
3.2 Materials and methods ………………………………………………………………………… 56
3.2.1 Treatments and experimental design …………………………………………………… 56
3.2.2 Plant material and cultivation …………………………………………………………… 56
3.2.3 Fungal growth and inoculation technique ……………………………………………… 57
3.2.4 Xylem sap collection …………………………………………………………………………… 58
3.2.5 Disease evaluation ……………………………………………………………………………… 58
3.2.6 *In vitro* bioassay ………………………………………………………………………………… 59
3.2.7 Total protein assay ……………………………………………………………………………… 59
3.2.8 Quantification of xylem sap total carbohydrate content ……………………………… 59
3.2.9 Data analysis ……………………………………………………………………………………… 60
3.3 Results ………………………………………………………………………………………………… 60
3.3.1 Greenhouse experiments ……………………………………………………………………… 60
3.3.2 Analysis of *V. longisporum* growth on *B. napus* xylem sap ……………………………… 62
3.3.2.1 Xylem sap bioassay ……………………………………………………………………… 62
3.3.2.2 Xylem sap total protein content ……………………………………………………… 63
3.3.3 Analysis of *V. longisporum* growth in xylem sap extracted from different age plants ……………………………………………………………………………………………… 65
3.3.3.1 Xylem sap bioassay ……………………………………………………………………… 66
3.3.3.2 Xylem sap total carbohydrate content ……………………………………………… 66
3.3.4 Correlation analysis …………………………………………………………………………… 67
3.4 Discussion …………………………………………………………………………………………… 69
3.5 References …………………………………………………………………………………………… 73

4. Physiology, gene expression and agronomic performance of winter oilseed rape under drought stress and infection with *V. longisporum* 77

4.1 Introduction 77

4.2 Materials and methods 81

4.2.1 Treatments and experimental conditions 81

4.2.2 Determination of field capacity in the soil substrate 83

4.2.3 Plant material and growing conditions 83

4.2.4 Fungal isolate and inoculation procedures 84

4.2.5 Determination of soil water content and maintenance of watering regime 84

4.2.6 Plant sample collection and preservation 85

4.2.7 Disease assessment 85

4.2.8 Physiological data 87

4.2.9 Measurement of yield and agronomic variables 89

4.2.10 Expression of drought responsive genes 89

4.2.11 Data analysis 94

4.3 Results 94

4.3.1 *V. longisporum* disease development under drought stress conditions 94

4.3.2 Physiological responses to *V. longisporum* infection and drought stress 98

4.3.3 Effect of drought stress and *V. longisporum* infection on expression of drought responsive genes 104

4.3.4 Effect of *V. longisporum* and drought stress on yield and morphological traits.. 107

4.3.5 Correlation and regression analyses 116

4.3.6 Principal component analysis 120

4.4 Discussion 122

4.4.1 *B. napus* response to *V. longisporum* under drought stress 122

4.4.2 *B. napus* physiology during drought stress and infection with *V. longisporum* .. 125

4.4.3 Expression of drought responsive genes during drought stress and infection with V. *longisporum* 130

4.4.4 Single and combined effects of *V. longisporum* infection and drought stress on yield and agronomic traits 131

4.4.5 Correlation, regression and principal component analyses 138

4.5 References 140

5. General discussion 152

5.1 Identification of *B. napus* genotypes with enhanced resistance to *V. longisporum*...... 152

5.2 Searching for VL resistance factors in OSR xylem sap 159

5.3 *B. napus - V. longisporum* interaction under drought stress conditions 162

5.4 References 167

Summary 171

Appendices 175

Acknowledgments 205

Selected contributions in non-reviewed journal paper/conference proceedings 208

Curriculum vitae 209

Declaration 210

1. General Introduction

1.1 Oilseed rape (*Brassica napus* L.)

Oilseed rape (OSR) is one of the economically most important crops in the family Brassicaceae which consists of approximately 338 genera and 3709 plant species (Warwick and Al-Shehbaz, 2006). It is an allotetraploid species (AACC genome; 2n=38) derived from a spontaneous hybridization between turnip rape (*Brassica rapa* L.; 2n=20; AA genome) and cabbage (*Brassica oleracea* L.; 2n=18; CC genome). *B. napus* comprises two subspecies, *napobrassica* and *napus*. The latter includes the most widely cultivated winter and spring rapeseed forms (Song and Osborn, 1962; Snowdon *et al.,* 2007). The centre of origin for the two diploid parents of *B. napus* is located around the Mediterranean basin (*B. oleracea*) and Euro-Siberia and the Mediterranean basin (*B. rapa*) (Warwick et al., 2009). Regarding *B. napus* however, the exact period and place of origin is not known but its domestication is believed to have occurred in Europe in the early Middle Ages and later commercial plantings primarily for its use as oil for lamps were recorded in the Netherlands as early as the 16th century (OECD, 1997).

OSR also called rapeseed, swede rape or canola (Orlovius, 2013) is a winter or spring type annual plant. The spring types differ from winter varieties because they do not require vernalization (winter chilling) to flower, although vernalization speeds up flowering. The name 'canola' refers to the high quality varieties with significantly lower contents of two naturally occurring harmful metabolites, erucic acid (below 2% in oil) and glucosinolates (total glucosinolates of 30µmoles/g toasted oil free meal) (OGTR, 2012). The OSR plant, depending on the variety and environmental conditions, can grow up to 1.5m high. Its stem is well branched and it has two types of roots, a deep tap root and fibrous lateral roots near the surface. Leaves are smooth and dark bluish green with few scattered hairs near the margin. Although wind or insect-borne cross-pollination is possible, OSR is generally considered as a self-fertile species. Its flowers are bright yellow with four petals and borne in clusters in the form of elongate racemes. Seeds are round, small (1.8-2.8 mm in diameter) and brown-black coloured (OECD, 1997; Sattell *et al.,* 1998; Alford, *et al.,* 2003; Orlovius, 2013).

OSR provides diverse economic and ecological benefits. The oil extracted from its seed is primarily used in human nutrition. The oil is also used as a raw material in non-food products such as in biodiesel and oleochemical industries. A residue from oil production, the cake, contains proteins and is used in animal nutrition. Integration of OSR in different cropping systems also provides several advantages since it has rapid growth characteristics in fall, it is grown as catch crop to capture part of the available soil nitrogen, which otherwise might be lost due to leaching during winter. OSR also provides excellent erosion control and good ground cover over winter. It is known as a 'break crop' for it is beneficial for the following crop in crop rotation. The characteristic large biomass production

helps to suppress weed growth. Plant residues used as 'bio-fumigant' can significantly reduce weed and disease infestation in the following season. Furthermore, its deep tap root system can help in loosen plough pans and improve soil structure. Last but not least, OSR flowers serve as a source of nectar and pollen for honey bees (Sattell *et al.,* 1998; Orlovius, 2003; Haramoto and Gallandt, 2004; Haramoto and Gallandt, 2005).

World OSR production is increasing rapidly. Since 2000, global OSR production has surpassed cottonseed and became the world's second most important source of vegetable oil after soybean (Carré and Pouzet, 2014). In 2012, it was produced on more than 34 million hectares with an estimated total production of 65 million tonnes (FAOSTAT, 2014). At present, Canada, China, EU, India, Australia, and Ukraine are the world's top major production areas (Figure 1.1). In particular, due to the expansion of the biodiesel industry, OSR has become the major oilseed in European agriculture and its production is increasing from time to time (Carré and Pouzet, 2014).

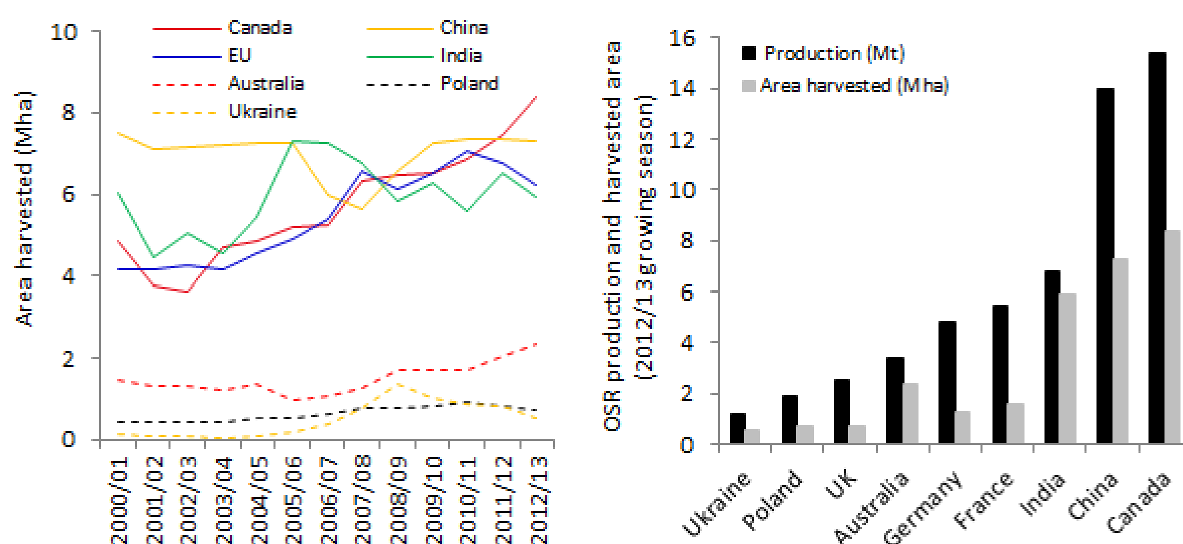


Figure 1.1 Proportion of OSR harvested area in major production regions of the world from 2000 to 2012 (left) and total rapeseed production in the nine top producing countries in 2012/13 growing season (right). **Mha**, million hectares. **Mt**, million tonnes. (Source: FAOSTAT, 2014).

The ever-increasing global OSR production is not without problems. Even though the degree of importance of the various production constraints varies depending on agro-ecological conditions, a number of biotic and abiotic stress factors can potentially affect growth and productivity of OSR. Cold stress is one of the primary abiotic stress factors. It causes a reduced number of seeds per plant and production of seeds with lightweight (Lardon and Triboi-Blondel, 1995). Similar effects, which ultimately lead to significant yield losses, can be induced by waterlogging occurring particularly at seedling and inflorescence emergence stages (Zhou and Lin, 1995). High temperature causes flower sterility and production of abnormal seeds (Polowick and Sawhney, 1988). On the other hand, water deficit stress interferes with plant development and several physiological processes that affect

quantity and quality of yield (Sadaqat *et al.,* 2003; Sinaki *et al.,* 2007; Din *et al,* 2011). Among the biotic factors, a wide range of invertebrate and vertebrate pests (Lane and Gladders, 2000; Alford, *et al.,* 2003) and different types of foliar and root pathogens (Lane and Gladders, 2000; Gabor *et al.,* 2013; Leino, 2006) can cause yield reduction in OSR and its relatives. In addition, the presence of weeds in OSR fields not only competes for shared resources and causes yield reduction, but also interferes with harvesting and may contaminate grain yield (Gianessi *et al.,* 2003). From the biotic factors, the fungal diseases caused by *Leptosphaeria maculans, anamorph: Phoma ligam* (Blackleg), *Sclerotinia sclerotiorum* (Sclerotinia stem rot), *Alternaria brassicae* (Alternaria black spot), *Verticillium longisporum, Pyrenopeziza brassicae*, anamorph: *Cylindrosporium concentricum* (Light leaf spot); the oomycete pathogens *Albugo candida* (White rust) and *Peronospora parasitica* (Downy mildew) and the protist pathogen *Plasmodiophora brassicae* (Clubroot) are among the economically most important production constraints in many OSR growing regions of the world (Gómez-Campo, 1999; Lane and Gladders, 2000; Alford *et al.,* 2003).

1.2 *Verticillium* diseases

Verticillium is a small genus consisting of Ascomycete phytopathogens that cause vascular wilt diseases in several economically important vegetable, ornamental, forest and industrial crop species (Agrios, 2005). Recently, morphological, DNA sequence and herbarium based phylogenetic studies recognized ten species belonging to this genus (*Verticillium* sensu stricto). According to this study, the genus Verticillium fell into two clades, Flavexudans (with yellow pigmented hyphae) and Flavnonexudans (without yellow pigmented haphae). *V. longisporum* is placed under the clade Flavnonexudans, which also consists of *V. alfalfae*, *V. dahliae*, *V. nonalfalfae*, *V. nubilum* and its two unknown ancestral species (species A1 and species D1). The remaining five species (*V. albo-atrum*, *V. tricorpus*, *V. zaregamsianum*, *V. isaacii* and *V. klebahnii*) belong to the clade Flavexudans (Inderbitzin *et al.,* 2011b).

1.3 *V. longisporum*: the youngest vascular pathogen of OSR

The existence of a distinct physiological strain of *V. dahliae* with restricted host range to the cruciferous plant Brussels sprout (*Brassica oleracea*) was first reported from UK in 1957 (Isaac, 1957). Later in 1961, *V. longisporum* (VL) as a long-spored isolate of *V. dahliae* was reported from horseradish in Germany. At that time, it was described as a variety of *V. dahliae* and named *V. dahliae* var. *longisporum* (Stark, 1961). A few years later, auxotrophic mutant analysis of the long- and short-spored isolates of *V. dahliae* demonstrated VL as a naturally occurring diploid species which is stable under laboratory conditions (Ingram, 1968). The first reliable molecular evidence for the presence of genetic variability in *V. dahliae* was shown by Messner *et al.* (1996), who based on RAPD-PCR and 18SrRNA sequencing analysis showed the separate clustering of *B. napus* derived

isolates from those collected from a wide range of other hosts. A year later, a more extensive study that involved several techniques (morphological characters, enzymatic activity, DNA profiles and pathogenicity testing) and consisted of several crucifer isolates from different geographical regions showed VL to be a near-diploid species whose host rage is restricted to crucifers. This study proposed the taxonomic position of VL to be raised from a variety level to a higher, namely species rank (Karapapa *et al.,* 1997).

Since then, several studies focusing on the taxonomy and phylogenetic relationship of VL have been conducted. Fahleson *et al.* (2004), based on nuclear and mitochondrial sequence analysis, have shown distinct features of VL and its close relation to *V. albo-atrum* and suggested VL to be regarded as a separate species. In support of this study, Pantou *et al.* (2005) by molecular and immunochemical analysis of different VL isolates reinforced recognition of VL as a separate species and argued that VL is closer to *V. albo-atrum* than to *V. dahliae*. Clewes et al. (2008) showed the presence of two or more β-tubulin genes and 5*S* rRNA associated sequences in VL but only one in most *V. dahliae* isolates and suggested the origin of VL as an interspecific hybridization between two or three parental species similar to *V. dahliae*. Recent genome sequencing of two Swedish VL isolates showed 86% similarity with the *V. dahliae* genome. These isolates have double the genome size (approximately 70 Mb) than that of *V. dahliae* and *V. albo-atrum* and harbour more than 20,000 protein coding genes (Roos, 2014). Several other studies have also shown morphological, physiological and DNA based evidences (Table 1.1) that explain the distinct differences between VL and the closely related species *V. dahliae* (Karapapa *et al.,* 1997; Zeise and Tiedemann, 2001; Steventon *et al.,* 2002; Inderbitzin *et al.,* 2011b). In addition, it is obvious that VL, unlike other Verticillium species including VD, does not induce wilting and its host range is restricted mainly to crucifers (Karapapa *et al.,* 1997; Bhat and Subbarao, 1999; Zeise and Tiedemann, 2002). Quite recently, an extensive phylogenetic analysis based on ITS and five protein coding genes sequence data from over 200 Verticillium isolates, which represent a wide range of hosts and geographic regions, also confirmed that VL is a diploid hybrid. This study identified four different alleles in VL, each representing four different lineages (lineage A1, D1, D2 and D3). Accordingly, it was demonstrated that the origin of VL involved three different parental species (*V. dahliae* and two other unknown parental species called species A1 and species D1). Species A1 was involved in each hybridization events and resulted in three VL lineages, VL lineage A1/D1, VL lineage A1/D2 and VL lineage A1/D3. VL lineage A1/D1 originated from hybridization events between the two unknown species A1 and D1. This lineage is known to be confined in Europe and is pathogenic to OSR and cauliflower. Lineage A1/D2 is the product of hybridization between species A1 and the other VD lineage D2. This lineage is confined to horseradish in Illinois (USA) and it is not known whether it causes disease in OSR. The third VL lineage, A1/D3, evolved from the hybridization of species A1 and

the VD lineage D3, is avirulent on OSR and it is confined in Europe and Japan (Inderbitzin *et al.,* 2011a).

Table 1.1 Evidences for a separate taxonomic grouping of *V. longisporum* from *V. dahliae*.

Parameters	*V. dahliae*	*V. longisporum*
MS Shape[1,3,7]	mostly rounded or spherical	mainly elongate
Conidia size[1,5,7]	Short (3.5 - 5.5µm)	Long (7.1 - 8.8µm)
DNA content [1,5]	Low (most isolates)	High (most isolates)
PPO activity[1,3,5]	Strong (most isolates)	None (most isolate)
Culture filtrate fluorescence[1]	Absent	Present
Host range[1,2,4]	Broad (Vegetables, trees, legumes, and ornamental crops)	Mainly restricted to Crucifers
Wilting symptom[6]	yes	no

MS, microsclerotia. **PPO,** extracellular polyphenol oxidase. [1]Karapapa *et al.,* 1997; [2]Bhat and Subbarao, 1999; [3]Zeise and Tiedemann, 2001; [4]Zeise and Tiedemann, 2002; [5]Steventon *et al.,* 2002; [6]Dunker *et al.,* 2008. [7]Inderbitzin *et al.,* 2011b.

According to the evidences described in the above paragraphs, the present taxonomic position of VL as a distinct independent species in the Verticillium genus seems not controversial any more. As mentioned earlier, a new classification of the genus with five new species (*V. alfalfae, V. isaacii, V. klebahnii, V. nonalfalfae and V. zaregamsianum*) has been recently demonstrated (Inderbitzin *et al.,* 2011b). Molecular methods that discriminate not only among the different Verticillium species, but also among VL lineages or between VL isolates that differ in virulence towards *B. napus* are available (Tran *et al.,* 2013; Inderbitzin *et al.,* 2013). Nevertheless, the debate regarding the species nomenclature of VL (*longisporum*) which refers to the possession of larger-sized asexual spores seems not yet resolved. For instance, based on the reports in Zeise and Tiedemann (2001), Collins *et al.* (2003) and Inderbitzin *et al.* (2011b) who observed rare production of medium to long sized conidia by few isolates of the haploid species *V. albo-atrum* and *V. dahliae*, there is an argument saying that the name *longisporum* is confusing (Fahleson *et al.,* 2004; Pantou *et al.,* 2005; Inderbitzin *et al.,* 2011b). Another somehow controversial issue, which might be related to the different VL lineages or correct identification of Verticillium species, is the host specificity of VL. Johansson *et al.* (2006) reported possible infections of highly susceptible plants outside the Brassicaceae. This contradicts with reports of earlier studies that showed Brassicaceae to be the restricted host range of this pathogen (Karapapa *et al.,* 1997; Zeise and Tiedemann, 2002).

1.3.1 Significance and geographical distribution of *V. longisporum*

Although evidences showed that recent evolution of VL, the precise geographic location where this pathogen first originated is unknown. Based on current distribution of the different VL lineages, Inderbitzin *et al.* (2011a) speculated one of the following three regions for the origin of VL. These are Illinois (where VL lineage A1/D2 is restricted), Europe (where VL lineage A1/D1 and A1/D3 are

confined) and Japan (where lineage A1/D3 is confined). Besides, analysis of the genetic variation among large collections of *V. longisporum* isolates showed significant geographic structuring of German, Swedish and Californian isolates, possibly due to long distance geographic separation of these locations by forest and water barriers (Fahleson et al., 2003). VL is currently known from Belgium, Czech Republic, France, Germany, Japan, the Netherlands, Poland, Southern Russia, Sweden, UK and USA (CA, IL) (CABI and EPPO, 2011). More importantly, field yield loss assessment studies in Germany (Dunker et al., 2008) and UK (Gladders, 2009) indicated significant yield loss potentials of this pathogen in OSR.

1.3.2 *V. longisporum* disease cycle

The life cycle of VL can be divided into three major phases: dormant, parasitic and saprophytic. During the dormancy period, the fungus survives in the soil or in plant debris via its microsclerotia (Fig. 1.3; Fig. 1.2). Microsclerotia are the major structures that enable the fungus to resist extreme environmental conditions and serve as a viable source of inoculum for several years. Contaminated or perhaps infected seeds can also serve as a source of inoculum. The parasitic phase starts when microsclerotia germinate and produce hyphae possibly in response to stimulation by root exudates (Leino, 2006; Berlanger and Powelson, 2000). Initial infection occurs during autumn primarily by direct penetration of epidermal cells of lateral roots and root hairs. Once the fungus has entered into the root cortex, it starts colonizing the root xylem vessels (Eynck *et al.,* 2007) and spends most part of its life cycle in this host tissue. Systemic spread to the shoot is rather slow and infection can be latent up to nine months. VL infection in OSR induces plugging of vascular vessels with polyphenolic and lignin materials. Initial typical symptoms of VL infection in OSR are leaf chlorosis (one-sided or irregular yellowing) which is followed by senescence. During later disease development, yellow or brown longitudinal lesions are formed on stems and branches (Fig. 1.3). As plants mature, the fungus bursts out of the xylem vessels and produces microsclerotia, first in the pith and then underneath the epidermis causing stem and root pith tissues to turn dark greyish or black. This marks the beginning of the saprophytic stage. In contrast to other Verticillium species, VL causes no wilting possibly because of the absence of wilting toxins and/or the presence of sufficient xylem vessels unblocked by occlusions in infected plants (Dunker et al., 2008). Premature ripening and senescence of leaves, stems or branches are the typical symptoms (Gladders, 2009; Leino, 2006). Plants inoculated under greenhouse conditions show additional symptoms such as stunting of shoots, reduction of root length (Dunker et al., 2008) and excessive production of branches (Fig. 4.2). Further plant aging towards harvest promotes intense formation of microsclerotia underneath the stem epidermis, in the stem pith and roots. As diseased plants senescence, microsclerotia are released into the soil together with dead plant material. At this point, the pathogen enters the dormant stage (Fig. 1.3).

Spreading of VL can occur via several mechanisms. Transport of non-symptomatic, but infected plant products and/or seeds can move the pathogen long distance. Once established in a field, spread of the pathogen occurs primarily by soil cultivation and movement of soil by wind, water or farm equipment (Gladders, 2009; Berlanger and Powelson, 2000).

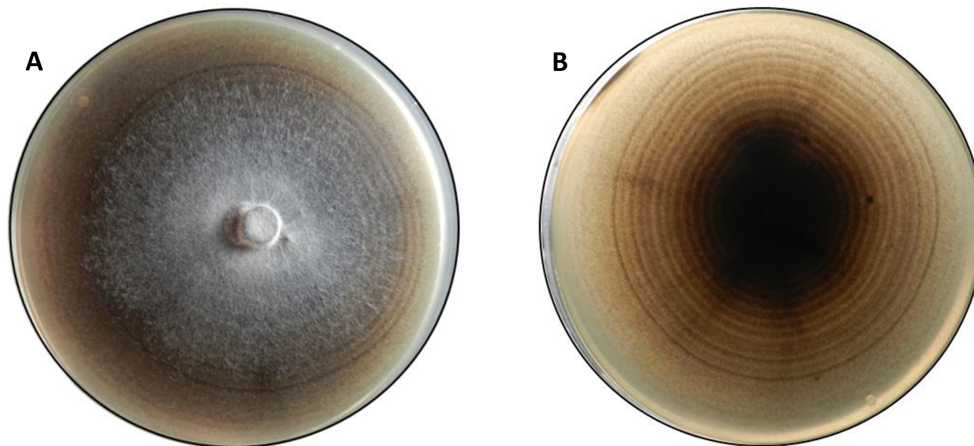


Figure 1.2 Growth of *Verticillium longisporum* isolate VL43 on potato dextrose agar plate four weeks after incubation at 23°C in the dark. **A.** Frontal view: flat white mycelial growth and balck microsclorotia produced over the whole plate. **B.** Bottom view: dark microsclerotia forming a ring pattern of growth.

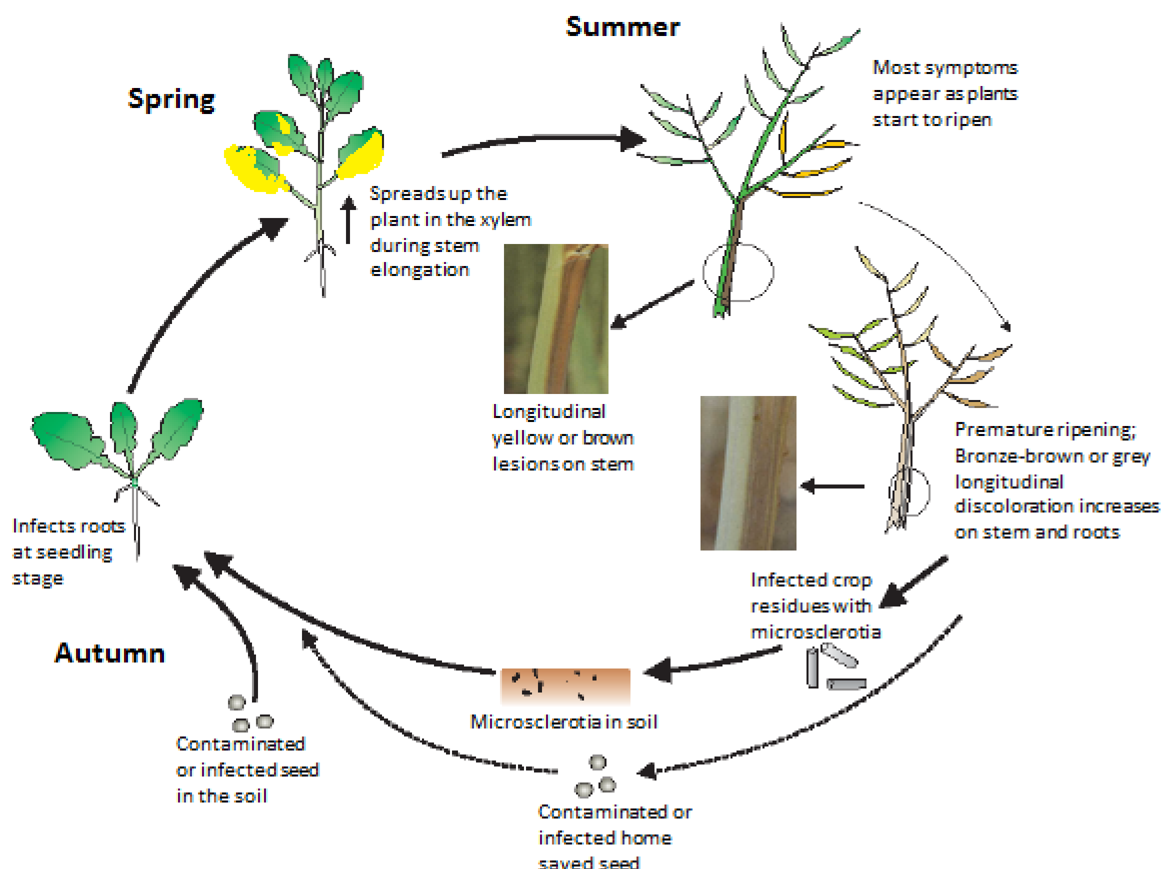


Figure 1.3 Disease cycle of *Verticillium longisporum* in winter oilseed rape (*B. napus* L.) (Adapted from Gladders, 2009 and Leino, 2006)

1.3.3 Pathogenicity factors in *V. longisporum*

Pathogenicity factors of VL are not yet exclusively known. Only few studies have shown the possible involvement of some genes or metabolites in infection of *B. napus* and *Arabidopsis thaliana*. For instance, Singh *et al.* (2010) have shown that silencing of a gene encoding chorismate synthase (Vlaro2), the first branch point intermediate of aromatic amino acid biosynthesis, caused a bradytrophic mutant that had reduced virulence in Arabidopsis and *B. napus*. Floerl *et al.* (2012) suggested rapid down-regulation and delayed induction of plant defence genes as possible mechanisms of enhanced virulence of VL in Arabidopsis. Singh *et al.* (2012) proposed increased expression of catalase peroxidase (VlCPEA gene) and other oxidative stress response proteins in VL to protect the fungus from oxidative stress generated by *B. napus*. Timpner *et al.* (2013) have shown the significant role of the amino acid synthesis regulatory cross-pathway control system gene CPC1 in pathogenicity and colonization VL in *B. napus*. Production of pathogenesis related cell death and wilt inducing toxins are known from the closely related species *V. dahliae* (Xie *et al.,* 2013) and *V. albo-atrum* (Mansoori and Smith, 2005). However, so far, there are no reports on production of pathogenesis related toxins by VL.

1.3.4 Management of *V. longisporum* in OSR

The characteristic systemic mode of infection and capability of long-term survival in soil makes Verticillium species difficult to control pathogens. As a result, despite the associated risks on the environment, control of Verticillium species in general has heavily relied on soil fumigation with chemicals (Klosterman *et al.,* 2009). For VL in particular, no registered fungicides are currently available. Studies suggest that soil amendment with organic products or biological agents has the potential to reduce soil inoculum and may provide an effective suppression of Verticillium diseases. Nevertheless, the efficacy of this method is dependent on soil, climatic and agronomic factors. Moreover, there are some economic and ecological risks associated with this method of disease control (Goicoechea, 2009; França *et al.,* 2013). The other possible alternative is crop rotation. Because of the existing host range specificity in the genus Verticillium, some crop rotation schemes that potentially minimize the risk of VL disease epidemics are suggested (Bhat and Subbarao, 1999; Zeise and Tiedemann, 2002). Interesting results from long-term field studies on the role of crop rotation in minimizing yield reduction in OSR due to fungal pathogen has been shown recently (Hilton *et al.,* 2013). However, since Verticillium inoculum can remain viable in the soil for more than a decade (Wilhelm, 1955), the effectiveness of this option as a sole means of VL control is questionable. Although VL transmission via seeds is not a likely scenario (Zhou *et al.,* 2006), seed treatment or the use of pathogen-free seed can minimize the risk of pathogen spread. For more effective quarantine however, accurate identification and knowledge on the identity of Verticillium species are essential (Inderbitzin and Subbarao, 2014).

In general, until present, the use of plant resistance is the only feasible means for the management of VL in OSR. Breeding and resistance study efforts made in the last decade identified genotypes with enhanced VL resistance in OSR (Rygulla *et al.,* 2007b; Eynck *et al.,* 2009a) and cauliflower (Debode *et al,* 2005). Moreover, some of the resistance mechanisms in OSR and Arabidopsis are known. Among these, the major mechanisms of VL-resistance known in OSR are physical barriers (such as occlusions and cell wall bound lignin and phenolics) and other soluble phenolic compounds (Eynck *et al.,* 2009b; Obermeier *et al.,* 2013). Similarly, the significance of soluble phenylpropanoids in defence response of Arabidopsis towards VL is known (König *et al.,* 2014). Another recent study on the Arabidopsis-VL interaction demonstrated the role of the Erecta gene (which encodes for a receptor-like kinase involved in plant development and disease resistance) in mediating resistance against VL-induced stunting in Arabidopsis (Häffner *et al.,* 2014). Floerl *et al.* (2008) identified VL-induced enhanced accumulation of antifungal proteins in *B. napus.* Regarding plant hormones, despite the fact that VL-infection causes increased accumulation of salicylic acid, several studies have shown no role of this hormone in signalling VL resistance in Arabidopsis and *B. napus* (Veronese *et al.,* 2003; Johansson *et al.,* 2006; Ratzinger *et al.,* 2009; Kamble et al., 2013).

1.4 Aims of the thesis

Even though much is known about the basics of VL resistance mechanisms in OSR, there is a lack of information regarding the nature of disease resistance under abiotic stress conditions. Siebold and Tiedemann (2013) recently demonstrated the potential effect of high soil temperature in causing early and severe VL infection in OSR. Besides this, a review on the impact of climate change on OSR diseases clearly showed a gap of knowledge on the influence of changing soil conditions on soil-borne diseases of OSR including VL (Evans *et al.,* 2009). This indicates the significance of understanding the nature of pathogen virulence, disease development and host resistance in the presence of prevailing abiotic stress conditions, particularly, drought and high temperature. With this background, the present thesis focused on a functional analysis of VL-resistance in OSR. Accordingly, several studies from the identification of VL resistant lines, towards further investigation of cultivar-related resistance mechanisms and the nature of plant resistance under drought stress conditions were conducted under various experimental conditions. The particular rationale behind each study is given in the different chapters. Here, the general objectives of the respective chapters are briefly described.

If plant resistance is to be used as one alternative means of disease management, the development or identification of plant genotypes with enhanced disease resistance is the first step. Accordingly, screening of *B. napus* lines for resistance against VL using molecular and phenotypic disease assessment tools was conducted in greenhouse, outdoor and field experiments. The major

objectives of these experiments were to identify *B. napus* double haploid lines and other accessions with high level of resistance against VL. A further objective of this part of the thesis (Chapter 2) was validating the applicability of qPCR (quantitative polymerase chain reaction) as an alternative method of disease evaluation in the field.

In order to make practical use of plant resistance, resistance traits found from whatever source need to be transferred to a desired crop variety such as to high yielding cultivars. Among other things, the pre-requisite for successful transfer of these traits is the in-depth understanding of the resistance mechanisms in the host plant. This helps not only the easy and selective transfer of traits, but also provides a space to address specific agro-ecological requirements. As mentioned earlier, the role of some basic physical and biochemical resistance factors that work against VL are known in OSR. However, nothing is known regarding the existence and role of soluble, antifungal and cultivar-related VL-resistance in the OSR xylem sap, an environment where the pathogen spends most part of its life cycle. To answer this important question, a study involving greenhouse experiments, *in vitro* bioassays and biochemical analyses was conducted using VL-susceptible and resistant genotypes. The general objective of this study (Chapter 3) was to find out whether xylem sap plays a major role in cultivar-related resistance of OSR against VL.

Since the effects of vascular pathogens (like VL) mimic the effects of other abiotic stress factors such as drought or high temperature, it is indispensable to understand what happens to host resistance to either of the stress factors particularly under conditions where both stresses occur simultaneously. This critical issue, with particular importance under conditions of changing global climate, was addressed in an extensive study with a general objective of investigating the main and interactive effects of VL infection and drought stress on VL and OSR. In this study (Chapter 4), the nature of pathogen development and host reaction towards both stress factors was investigated by analysing several phenotypic, physiological, molecular, agronomic and yield parameters.

1.5 References

Agrios G. N. 2005. Plant pathology (5th edition). Elsevier-Academic Press. San Diego, CA.

Alford D. V., Nilsson C. and Ulber B. 2003. Insect pests of oilseed rape crops. *In*: Alford, D. V. (ed.), Bio-control of oilseed rape pests, 9-39. Blackwell Science, Oxford.

Berlanger I. and Powelson M. L. 2000. Verticillium wilt. The plant health instructor. Available: http://www.apsnet.org/edcenter/intropp/lessons/fungi/ascomycetes/Pages/VerticilliumWilt.aspx. doi: 10.1094/PHI-I-PHI-1-2000-0801-01. Updated 2005.

Bhat R. G. and Subbarao, K. V. 1999. Host range specificity in *Verticillium dahliae*. *Phytopathology*, 89:1218-1225.

CABI and EPPO, 2011. *Verticillium longisporum*. Distribution maps of plant diseases. Map No. 1117 (Edition 1). ISSN 0012-396X. CAB International, Wallingford, UK.

Carré P. and Pouzet A. 2014. Rapeseed market, worldwide and in Europe. Oilseeds and fats crops Lipids, 21 (1) D102.

Clewes E., Edwards S. G., and Barbara D. J. 2008. Direct molecular evidence supports long-spored microsclerotial isolates of *Verticillium* from crucifers being interspecific hybrids. *Plant Pathology*, 57:1047-1057.

Collins A., Okoli C. A. N., Morton A., Parry D., Edwards S. G., and Barbara D. J. 2003. Isolates of *Verticillium dahliae* pathogenic to crucifers are of at least three distinct molecular types. *Phytopathology*, 93:364-376.

Debode J., Declercq B. and Höfte M. 2005. Identification of cauliflower cultivars that differ in susceptibility to *Verticillium longisporum* using different inoculation methods. *Journal of Phytopathology*, 153:25–263.

Din J., Khan S. U., Ali I. and Gurmani A. R. 2011. Physiological and agronomic response of canola varieties to drought stress. *The Journal of Animal & Plant Sciences*, 21(1):78-82.

Dunker S., Keunecke H., Steinbach P., von Tiedemann A. 2008. Impact of *Verticillium longisporum* on yield and morphology of winter oilseed rape (*Brassica napus*) in relation to systemic spread in the plant. *Journal of Phytopathology* 156: 698-707.

Evans N., Gladders P., Fitt B.D.L. and von Tiedemann V. 2009. Climate change in Europe : altered life cycles and spread of major pathogens in oilseed rape. In GCIRC technical meeting. GCIRC technical meeting, New Delhi, India, 2-4 February .

Eynck C., Koopmann B. and von Tiedemann A. 2009a. Identification of *Brassica* accessions with enhanced resistance to *Verticillium longisporum* under controlled and field conditions. *Journal of Plant Diseases and Protection*, 116(2), 63-72.

Eynck C., Koopmann B., Grunewaldt-Stoecker G., Karlovsky P. and von Tiedemann A. 2007. Differential interactions of *Verticillium longisporum* and *V. dahliae* with *Brassica napus*

detected with molecular and histological techniques. *European Journal* of *Plant Pathology*, 118:259-274.

Eynck C., Koopmann B., Karlovsky P. and von Tiedemann A. 2009b. Internal resistance in winter oilseed rape inhibits systemic spread of the vascular pathogen *Verticillium longisporum*. *Phytopathology*, 99:802-811.

Fahleson J., Hu Q. and Dixelius C. 2004. Phylogenetic analysis of *Verticillium* species based on nuclear and mitochondrial sequences. *Archives of Microbiology*, 181:435–442.

Fahleson J., Lagercrantz U., Hu Q., Steventon L. A. and Dixelius C. 2003. Estimation of genetic variation among *Verticillium* isolates using AFLP analysis. *European Journal of Plant Pathology*, 109:361-371.

Floerl S., Majcherczyk A., Possienke M., Feussner K., Tappe H, Gatz C., Feussner I., Kües U. and Polle A. 2012. *Verticillium longisporum* infection affects the leaf apoplastic proteome, metabolome, and cell wall properties in *Arabidopsis thaliana*. *PLoS ONE* 7(2): e31435.

França S. C., Spiessens K., Pollet S., Debode J., De Rooster L., Callens D. and Höfte M. 2013. Population dynamics of *Verticillium* species in cauliflower fields: Influence of crop rotation, debris removal and ryegrass incorporation. *Crop Protection*, 54:134-141.

Gabor B., John K. and Diane K. 2013. In Conn K. E. and Rosenberger S. A. (eds). Crucifer disease reference guide. A practical guide for seedsmen, growers and agricultural advisors. Seminis vegetable seeds. CA, USA.

Gianessi L., Sankula S. and Reigner N. 2003. Plant biotechnology: Potential impact for improving pest management in European agriculture. oilseed rape-herbicide-tolerant case study. The National Center for Food and Agricultural Policy. DC, USA.

Gladders P. 2009. Relevance of Verticillium wilt (*Verticillium longisporum*) in winter oilseed rape in the UK. HCGA Research Review, 72:1-39.

Goicoechea N. 2009. To what extent are soil amendments useful to control Verticillium wilt? *Pest Management Science*, 65:831-839.

Gómez-Campo C. 1999. Biology of Brassica coenospecies. Developments in plant genetics and breeding, 4. Elsevier, Amsterdam/New York.

Häffner E., Karlovsky P., Diederichsen E. 2010. Genetic and environmental control of the *Verticillium* syndrome in *Arabidopsis thaliana*. *BMC Plant Biology*,14.85:1-16.

Häffner E., Karlovsky P., Splivallo R., Traczewska A. and Diederichsen E. 2014. ERECTA, salicylic acid, abscisic acid, and jasmonicacid modulate quantitative disease resistance of *Arabidopsis thaliana* to *Verticillium longisporum*. *BMC Plant Biology*, 14:85.

Haramoto E. R. and Gallandt E. R. 2005. Brassica cover cropping: I. Effects on weed and crop establishment. *Weed Science*, 53(5):695-701.

Haramoto E. R. and Gallandt E. R. 2004. Brassica cover cropping for weed management: A review. *Renewable Agriculture and Food Systems*, 19: 187-198

Hilton S., Bennett A.J., Keane G., Bending G.D., Chandler D., Stobart R. and Mills P. 2013. Impact of shortened crop rotation of oilseed rape on soil and rhizosphere microbial diversity in relation to yield decline. *PLoS ONE*, 8(4): e59859.

Inderbitzin P. and Subbarao K. V. 2014. *Verticillium* systematics and evolution: How confusion impedes Verticillium wilt management and how to resolve it. *Phytopathology*, 104:564-574.

Inderbitzin P., Bostock R.M., Davis R. M., Usami T., Platt H. W. and Subbarao K. V. 2011b. Phylogenetics and taxonomy of the fungal vascular wilt pathogen Verticillium, with the descriptions of five new species. *PLoS ONE,* 6(12): e28341.

Inderbitzin P., Davis R. M., Bostock R. M. and Subbarao K. V. 2011a. The Ascomycete *Verticillium longisporum* is a hybrid and a plant pathogen with an expanded host range. *PLoS ONE*, 6(3): e18260.

Inderbitzin P., Davis R.M., Bostock R. M., Subbarao K.V . 2013. Identification and differentiation of *Verticillium* species and *V. longisporum* lineages by simplex and multiplex PCR assays. *PLoS ONE*, 8(6): e65990.

Ingram R. 1968. *Verticillium dahliae var. longisporum*, a stable diploid. *Transactions of the British Mycological S*ociety, 51: 339-341.

Isaac I. 1957. *Verticillium* wilt of Brussels sprout*. Annals of Applied Biology*, 45:276-283.

Johansson A., Goud Jan-K. C. and Dixelius C. 2006. Plant host range of *Verticillium longisporum* and microsclerotia density in Swedish soils. *European Journal of Plant Pathology*, 114:139-149.

Kamble A., Koopmann B. and von Tiedemann A. 2013. Induced resistance to *Verticillium longisporum* in *Brassica napus* by b-aminobutyric acid. *Plant Pathology*, 62:552-561.

Karapapa V. K., Bainbridge B. W. and Heale J. B. 1997. Morphological and molecular characterization of *Verticillium longisporum* comb. nov., pathogenic to oilseed rape. *Mycological Research*, 101:1281-1294.

Klosterman S. J., Atallah Z. K., Vallad G. E. and Subbarao K. V. 2009. Diversity, pathogenicity, and management of Verticillium Species. *Annual Review of Phytopathology*. 47:39-62.

König S., Feussner K., Kaever A., Landesfeind M., Thurow C., Karlovsky P., Gatz C., Polle A. and Feussner I. 2014. Soluble phenylpropanoids are involved in the defense response of Arabidopsis against *Verticillium longisporum*. *New Phytologist* , 202:823-837.

Lane A. and Gladders P. 2000. Pests and diseases of oilseeds, Brassica seed crops and field beans. In: Alford D. V. (ed): Pest and disease management handbook. Blackwell science: Oxford.

Lardon A., and Triboi-Blondel A.M. 1995. Cold and freeze stress at flowering Effects on seed yields in winter rapeseed. *Field Crops Research*, 44:95-101.

Leino M.2006. Fungal diseases on oilseed rape and turnip rape. Norrköping, Sweden.

Mansoori B. and Smith C. J. 2005. Verticillium-toxins: their role in pathogenesis. *Journal* of Agricultural *Science* and *Technology*, 7: 103-114.

Messner R., Schweigkofler W., Ibl M ., Berg G . and Prillinger H.. 1996. Molecular characterization of the plant pathogen *Verticillium dahliae* Kleb. using RAPD-PCR and sequencing of the 18SrRNA-gene. *Journal of Phytopathology*, 144: 347-354.

Obermeier C., Hossain M. A., Snowdon R., Knüfer J., von Tiedemann A. and Friedt W. 2013. Genetic analysis of phenylpropanoid metabolites associated with resistance against *Verticillium longisporum* in *Brassica napus*. *Molecular Breeding*, 31:347-361.

OECD. 1997. Consensus document on the biology of *Brassica napus* L. (Oilseed rape). Series on harmonization of regulatory oversight in biotechnology, No.7. Organization for economic co-operation and development (OECD), Paris.

OGTR. 2002. The biology and ecology of canola (*Brassica napus*). Australian government, office of the gene technology regulator, Canberra, Australia.

Orlovius K. 2003. Oilseed rape. In: Kirbky, E. A. (Ed.).Fertilizing for high yield and quality, Bulletin 16. International potash institute. Basel, Switzerland.

Pantou M. P., Strunnikova O. K., Shakhnazarova V. Yu., Vishnevskaya N. A., Papalouka V. G. and Typas M. A.. 2005. Molecular and immunochemical phylogeny of Verticillium species. *Mycological Research*, 109:889-902.

Polowick P. L. and Sawhney V. K. 1988. High temperature induced male and female sterility in canola *(Brassica napus* L). *Annals of Botany* 62, 83-86.

Ratzinger A., Riediger N., von Tiedemann A. and Karlovsky P. 2009. Salicylic acid and salicylic acid glucoside in xylem sap of *Brassica napus* infected with *Verticillium longisporum*. *Journal* of *Plant Research*, 122:571-579.

Roos J. 2014. *Verticillium longisporum* and plant immunity responses in Arabidopsis. PhD thesis. Swedish University of Agricultural Sciences. Uppsala, Sweden.

Rygulla W., Snowdon R. J., Eynck C., Koopmann B., von Tiedemann A., Lühs W. and Friedt W. 2007b. Broadening the genetic basis of *Verticillium longisporum* resistance in *Brassica napus* by interspecific hybridization. *Phytopathology*, 97:1391-1396.

Sadaqat H. A., Tahir M. H. N. and Hussain M. T. 2003. Physiogenetic aspects of drought tolerance in canola (*Brassica napus*).*International Journal of Agriculture and Biology*, 5:(4) 611-614.

Sattell R., Dick R., Ingham R., Karow R., Kaufman D. and D. McGrath. 1998. Rapeseed (*Brassica campestris*/*Brassica napus*). Oregon State University, USA.

Siebold M. and von Tiedemann A. 2013. Effects of experimental warming on fungal disease progress in oilseed rape. *Global Change Biology*, 19:1736-1747.

Sinaki J. M., Heravan E.M., Rad A.S.H., Noormohammadi G. and Zarei G. 2007. The effects of water deficit during growth stages of canola *(Brassica napus* L.). *American-Eurasian Journal of Agricultural & Environmental Sciences*, 2(4):417-422.

Singh S., Braus-Stromeyer S. A., Timpner C., Valerius O., von Tiedemann A., Karlovsky P., Druebert C., Polle A. and Braus G. H. 2012. The plant host *Brassica napus* induces in the pathogen *Verticillium longisporum* the expression of functional catalase peroxidase which is required for the late phase of disease. *Molecular Plant-Microbe Interactions*, 25(4):569-581.

Singh S., Braus-Stromeyer S. A., Timpner C., Tran Van T., Lohaus G., Reusche M., Knüfer J., Teichmann T., von Tiedemann A., Braus G. H. 2010. Silencing of Vlaro2 for chorismate synthase revealed that the phytopathogen *Verticillium longisporum* induces the cross-pathway control in the xylem. *Applied Microbiology and Biotechnology*, 85:1961-1976.

Snowdon R. J., Luhs W. and Friedt W. (2007) Oilseed rape. In: C. Kole. Genome mapping and molecular breeding in plants Volume 2. Springer, Heidelberg. pp. 55-114.

Song K. and Osborn T. C. 1992. Polyphyletic origins of *Brassica napus*: new evidence based on organelle and nuclear RFLP analyses. *Genome*, 35(6): 992-1001.

Steventon L. A., Fahleson J., Hu Q. and Dixelius C. 2002. Identifcation of the causal agent of Verticillium wilt of winter oilseed rape in Sweden, *V. longisporum*. *Mycological Research*, 106(5): 570-578.

Timpner C., Braus-Stromeyer S. A., Tran Van T. and G. H. Braus. 2013. The Cpc1 regulator of the cross-pathway control of amino acid biosynthesis is required for pathogenicity of the vascular pathogen *Verticillium longisporum*. *Molecular Plant-Microbe Interactions*, 26(11):1312-1324.

Tran Van T., Braus-Stromeyer S. A., Timpner C. and Braus G. H. 2013. Molecular diagnosis to discriminate pathogen and apathogen species of the hybrid *Verticillium longisporum* on the oilseed crop *Brassica napus*. *Applied* Microbiology and *Biotechnology*, 97:4467-4483.

Veronese P., Narasimhan M. L., Stevenson R.A., Zhu J. K., Weller S. C., Subbarao K. V., Bressan R. A. 2003. Identification of a locus controlling *Verticillium* disease symptom response in *Arabidopsis thaliana*. *The Plant Journal*, 35(5):574-87.

Warwick S. I. and Al-Shehbaz I. A. 2006. Brassicaceae: Chromosome number index and database on CD-Rom. *Plant Systematics and Evolution*, 259:237-248.

Warwick S. I., Francis A., Gugel R. K. 2009. Taxonomic checklist and life history, ecological, and geographical data. In: Warwick S.I., Francis A., Gugel R.K. (ed.). Guide to wild germplasm of Brassica and allied crop (tribe Brassiceae, Brassicaceae). 3rd Edition.

Wilhelm S. 1955. Longevity of the *Verticillium* wilt fungus in the laboratory and in the field. *Phytopathology*, 45:180-181.

Xie C., Wang C., Wang X. and Yang X. 2013. Proteomics-based analysis reveals that *Verticillium dahliae* toxin induces cell death by modifying the synthesis of host proteins. *Journal* of *General Plant Pathology*, 79:335-345.

Zeise K. and von Tiedemann A. 2001. Morphological and physiological differentiation among vegetative compatibility groups of *Verticillium dahliae* in relation to *V. longisporum*. *Journal of Phytopathology,* 149,469-475.

Zeise K. and von Tiedemann A. 2002. Host specialization among vegetative compatibility groups of *Verticillium dahliae* in Relation to *Verticillium longisporum*. *Journal of Phytopathology,* 150, 112-119.

Zhou L., Hu Q., Johansson A. and Dixelius C. 2006. *Verticillium longisporum* and *V. dahliae*: infection and disease in *Brassica napus. Plant Pathology*, 55:137-144.

Zhou W. and Lin X. 1995. Effects of waterlogging at different growth stages on physiological characteristics and seed yield of winter rape (*Brassica napus* L.). *Field Crops Research*, 44(2-3):103-110.

2. Phenotypic and molecular evaluation of *Brassica napus* lines for *Verticillium longisporum* resistance under greenhouse, outdoor and field conditions

Parts of this chapter have been published in: Knüfer, J.[1,3], Lopisso, D. T.[1], Koopmann, B.[1], Karlovsky P.[2] and von Tiedemann A[1]. 2016. **Assessment of latent infection with *Verticillium longisporum* in field-grown oilseed rape by qPCR**. *Eur J Plant Pathol*. doi:10.1007/s10658-016-1045-0.

[1]Department of Crop Sciences, Section of Plant Pathology and Crop Protection, Georg August University, Grisebachstr. 6, 37077 Göttingen, Germany; [2]Department of Crop Sciences, Section of Molecular Phytopathology and Mycotoxin Research, Georg August University, Grisebachstr. 6, 37077 Göttingen, Germany; [3]Current address: Strube Research GmbH & Co. KG, Hauptstraße 1, 38387 Söllingen, Germany.

2.1 Introduction

Oilseed rape (OSR) is the world's second most produced oilseed behind soybeans (Carré and Pouzet, 2014). It is a multipurpose crop which is predominantly grown for vegetable oil and biodiesel (Orlovius, 2003), but also has gained large importance as a protein source for feed. OSR is a key component in crop rotation serving as a "catch crop" and ground cover over winter. It suppresses weed growth and loosens plow pans to help improve soil tilth (Sattell *et al.,* 1998). Apart from this, OSR flowers provide an early spring period source of nectar and pollen for honeybees (Farkas and Zajácz, 2007). Due to the increasing importance of this crop, the global production has undergone sustained growth over the past two decades (Carré and Pouzet, 2014). Nevertheless, the increasing production of OSR is not without problems. Several diseases and insect pests are known to hamper its productivity. The recently evolved fungal vascular pathogen *Verticillium longisporum* (Inderbitzin et al., 2011) is one of the most important biotic constraints with high potential of threatening OSR production, particularly in areas with intense production of OSR (Carré and Pouzet, 2014), notably in Sweden (Johansson et al., 2006), Germany (Dunker et al., 2008), France and Poland (Karapapa et al., 1997) and quite recently in the UK (Gladders, 2009).

Verticillium longisporum (VL) is a host-specialized pathogen of crucifers (Karapapa et al., 1997; Zeise and von Tiedemann, 2002). In oilseed rape, it causes premature senescence and ripening which can cause up to 60% yield reduction (Dunker et al., 2008; Gladders, 2009). Inaccessibility of Verticillium species during infection and their long-term field persistence make them difficult to control. As a result, control of Verticillium diseases strongly relies on fumigation of soil with chemicals (Klosterman et al., 2009; Goicoechea, 2009). For *V. longisporum* in particular, no registered or effective fungicides are available. Because of its narrow host range characteristics, long-term crop rotations that likely decline the abundance of viable soil microsclerotia are suggested as an alternative measure for the control of VL (Rygulla et al., 2007; Gladders, 2009). Nevertheless, this

option seems to be largely ineffective (França et al., 2013) mainly because of the long survival (up to 15 years) of the pathogen with its microsclerotia in soil (Berg et al., 1999; Leno, 2006). As a result, for the control of *Verticillium* diseases, the use of host-plant resistance remains the preferred strategy (Klosterman et al., 2009). The integration of such an easily adopted and environmentally safe method of disease management in crop protection packages provide several economic and ecological advantages (Agrios, 2005).

Regarding host-plant resistance against *V. longisporum*, elevated levels of resistance to VL were identified in cabbage (*B. oleracea*) genotypes a decade ago (Happstadius et al., 2003). Later on, by crossing tolerant *B. oleracea* accessions with *B. rapa*, resynthesized *B. napus* lines with enhanced VL resistance were developed (Happstadius et al., 2003; Rygulla et al., 2007a; Rygulla et al., 2007b). Previous greenhouse and field evaluation also identified promising *B. napus* lines with significantly higher level of resistance against VL (Rygulla et al., 2007b; Eynck et al., 2009a). In the present study VL resistance of winter OSR double haploid (DH) lines generated from resistant resynthesized *B. napus* lines and other sets of *B. napus* accessions obtained from several private plant breeding companies were evaluated in greenhouse, outdoor and multi-site field trials. This study was conducted as part of a joint project on improving OSR resistance to *V. longisporum*. The project involved three partners, the University of Göttingen, Division of Plant Pathology and Crop Protection, the Justus-Liebig-University of Gießen, Department of Plant Breeding (JLU) and nine plant breeding companies (Table 2.1) through their association called GFP (an association for the promotion of private plant breeding in Germany). The major objectives of this part of the project was to identify best performing and highly VL-resistant lines for use in future breeding programs aiming at integration of VL-resistance in commercial high-yielding winter OSR varieties. Furthermore, the applicability of quantitative PCR (qPCR) as a method of VL disease assessment under field conditions was validated.

2.2 Materials and methods

2.2.1 Greenhouse screening

Greenhouse screening was carried out in four rounds of experiments (Appendix 2.1). The experiments were conducted during the periods from February 2011 to January 2014 in the Division of Plant Pathology and Crop Protection, Göttingen University, Germany.

2.2.1.1 Plant material

More than 230 winter oilseed rape lines obtained from different breeding companies (Table 2.1) were screened for *Verticillium longisporum* resistance under greenhouse conditions over three

years. The descriptions and sources for all lines are indicated in Appendix 2.1. During each screening run, two reference varieties, Falcon (susceptible) and Express (resistant) were used as a check.

2.2.1.2 Experimental design

The experiment was arranged in a completely randomized design (CRD). After inoculation, two seedlings were grown in one pot (200ml) and considered as one replication. Each treatment was replicated 10 times resulting in 40 plants (20 mock inoculated and 20 VL-inoculated).

Table 2.1 Summary of *Brassica napus* lines screened for resistance against *Verticillium longisporum* in greenhouse and field experiments.

Seed company	Number of lines screened for VL resistance	
	Field trial	GH screening
DM	2	-
DSV	25	38
JLU	22	-
KWS	3	30
NICK	3	18
NPZ	-	23
SRG	-	22
SW	22	36
SYN	3	39
WVB	-	30
Total	80	236

DM, Dieckmann GmbH & Co. KG. **DSV**, Deutsche Saatveredelung AG. **JLU**, Justus-Liebig-University of Gießen, Department of Plant Breeding. **KWS**, KWS SAAT AG. **NICK**, Limagrain GmbH. **NPZ**, NorddeutschePflanzenzucht Hans-Georg Lembke KG. **SRG**, Raps GbR Saatzucht Lundsgaard. **SW**, Lantmännen SW Seed. **SYN**, Syngenta Seeds GmbH. **WVB**, W. von Borries-Eckendorf GmbH & Co. **VL**, *Verticillium longisporum*.

2.2.1.3 Inoculation procedures and disease scoring

Seeds of *B. napus* lines were double surface sterilized with 70% ethanol and grown on sterile silica sand for 10 days under optimum conditions (20°C temperature, 16 h light and an average relative humidity of 60%). Spore suspension (10^{6}conidia/ml) was prepared from ten days old cultures of the highly virulent *V. longisporum* isolates VL43 and VL40 mixed in equal ratio (See section 4.2.4 for details). Inoculation was performed following the cut root-dip inoculation method as described in section 4.2.4. Inoculated seedlings were transferred to 200ml plastic pots (two plants per pot) filled with a substrate composed of sand, peat and compost (1:1:2) and grown under optimum conditions as described above. Two disease assessment methods (disease severity and stunting effects) were used. Evaluation of disease severity was performed on a weekly basis for four consecutive weeks (7-28 days post inoculation, DPI). Disease severity was scored using a standard VL disease index (Table

4.2). From these disease index recordings, AUDPC and net AUDPC values were calculated as described in section 4.2.7.1. To evaluate stunting effects, plant height of VL and mock-inoculated plants was measured at 28 DPI and the percentage of relative stunting due to VL was calculated as $[(h_{Mock} - h_{VL}) \times (100)]/ [(h_{Mock})]$, where h_{Mock} and h_{VL} are the plant heights of mock and VL inoculated treatments, respectively. In order to standardize fluctuating levels of infection during different screenings, normalization of net AUDPC was done by dividing the net AUDPC values of each line to that of the average values of the reference cultivars. That is, normalized AUDPC ($AUDPC_{norm}$) = [Net $AUDPC_{lines}$]/[(Net $AUDPC_{Falcon}$+ Net $AUDPC_{Express}$)/2]. Normalized percent relative stunting was calculated in the same way as for net AUDPC values (Eynck et al, 2009a).

2.2.2 Field trials

Field studies were conducted with the aim of verifying resistance of *B. napus* accessions under natural conditions, with diversity in terms of soil, climate and disease factors. For this trial, *B. napus* lines were used that showed a reasonably better resistance than the reference standard varieties under greenhouse conditions. The field experiments were conducted in three locations (Table 2.2) and for three seasons (2010/11, 2011/12 and 2012/13).

2.2.2.1 Plant materials

A total of 80 lines (Table 2.1) selected on the basis of previous greenhouse screening results were evaluated under field conditions. Of these, 61 were double haploid (DH) lines derived from resynthesized VL-resistant *B. napus* lines. The remaining 19 lines with undisclosed genetic backgrounds were obtained from different breeding companies. Fourteen lines (thirteen DH lines and one accession) were repeatedly tested for three consecutive growing seasons across all locations (Appendix 2.2). During each trial, 40 lines (including the reference varieties) were tested. Cultivars Falcon, Laser, Oase, and Express were used as a standard check. Detailed descriptions and sources of all plant materials are found in (Appendix 2.2). Similar seed lots of all lines tested in the field were used in the parallel evaluation in the greenhouse.

2.2.2.2 Description of experimental locations

The three locations with field trials were Göttingen (Northern Central Germany), Fehmarn (an island in the Baltic Sea, Northern Germany) and Svalöv (Southern Sweden). These locations were selected on the basis of variation in the level of natural disease infestation, climatic factors and soil conditions (Table 2.2).

2.2.2.3 Experimental design

All experiments were laid down in a randomized complete block design (RCBD) with two (Fehmarn and Svalöv) or four (Göttingen) replications. In all locations, 40 accessions including the reference

varieties were tested during each growing season. Experimental plot size was 8m x 1.5m with eight rows. Distance between blocks was kept 0.3 meter apart. Plant samples harvested from the central two rows were used for data recordings.

Table 2.2 Field characteristics of the three research locations where field studies were conducted from 2011 to 2013.

Location	Site name	Latitude	Alt (mas)	T (^{o}C)	RF (mm)	RH (%)	ST	VL-NI
Göttingen								
2010/11	Große Breite	51°33’N 9°54’E	138	7.8	463.2	78.0	Loam	None/Low[1&2]
2011/12	Große Breite	51°33’N 9°54’E	138	9.3	567.8	73.0	Loam	None/Low[1&2]
2012/13	Rosdorf	51°30’N 9°54’E	169	8.3	438.8	85.7	Loam	None/Low[1&2]
Fehmarn								
2010/11	Niendorf	54°27'N 11°12'E	ND	8.6	687.2	ND	ND	High [1&2]
2011/12	Niendorf	54°27'N 11°12'E	ND	9.3	671.1	ND	ND	High [1&2]
2012/13	Niendorf	54°27'N 11°12'E	ND	8.6	481.7	ND	ND	High [1&2]
Svalöv								
2010/11	Svalöv	55°54'N 13°6'E	ND	6.9	830.4	86.7	ND	Very high[2&3]
2011/12	Svalöv	55°54'N 13°6'E	ND	8.1	703.2	81.1	ND	Very high[2&3]
2012/13	Svalöv	55°54'N 13°6'E	ND	7.2	594.4	80.0	ND	Very high[2&3]

Key: **Alt**, Altitude. **mas**, meters above sea level. **T,** mean annual temperature. **RF**, Total annual rainfall. **RH**, Average annual relative humidity. **ST**, soil type. **ND**, data not available. **VL-NI**, level of natural *Verticillium longisporum* infestation. For Göttingen and Svalöv sites the mean weather data from August to July (time period between sowing and harvest of oilseed rape) was obtained from data logger instruments installed inside the trial sites. For Fehmarn regional weather data was obtained from http://www.wetterkontor.de. [1]Knüffer, 2013; [2]Eynck, 2008; [3]Johansson et al., 2006.

2.2.2.4 Inoculation and agronomic practices

At Svalöv and Fehmarn, where there is a reasonably high level of inoculum in the soil (Eynck, 2008; Johansson, 2006), experiments were carried out under natural infestation conditions. In Göttingen, artificial inoculation was performed using chopped VL-infected stubbles collected from previous year experiments. This was done by evenly spreading the inoculum on a prepared seedbed at a rate of 15g microsclerotia infested stubbles/m^2. The inoculum was applied just before sowing and it was thoroughly incorporated into the soil using a disk plough. Seed rate was adjusted to local recommendations i.e. 50, 60 and 80 seeds/m^2 in Fehmarn, Göttingen and Svalöv, respectively. Early season insect pests (such as aphids and flea beetles) and seed borne pathogens (*Phoma lingam, Alternaria spp.* and *Pythium spp.*), were controlled by seed treatment with ‘CRUISER OSR’ (280 g/l thiamethoxam, 8 g/l fludioxonil and 32.3 g/l metalaxyl-M; Syngenta Crop Protection UK Limited) at a rate of 0.02ml per gram of seed. All other agronomic practices including fertilization and spraying

against weeds, blackleg (*Phoma lingam)* and stem rot (*Sclerotinia sclerotiorum)* were applied following standard recommendations.

2.2.2.5 Disease assessment

To measure VL-resistance of selected *B. napus* lines under field conditions, molecular and phenotypic disease assessment tools were used. For molecular disease measurements, stem samples were collected at the beginning of the ripening stage (BBCH 80) and from these samples, fungal biomass was quantified by qPCR. For this protocol, sampling was done by taking 10cm long stem sections cut approximately 5cm above the base of the plant (Knüfer, 2013). For each accession, 10 stem samples were collected from each replication. Genomic DNA was extracted from 1 gram of lyophilized and ground stem sample taken from a pool of 10 stem samples. All other subsequent qPCR analysis steps including extraction of genomic DNA, DNA quality check, preparation of VL DNA standards, primers and qPCR conditions were similarly performed as described in section 4.2.7.3.

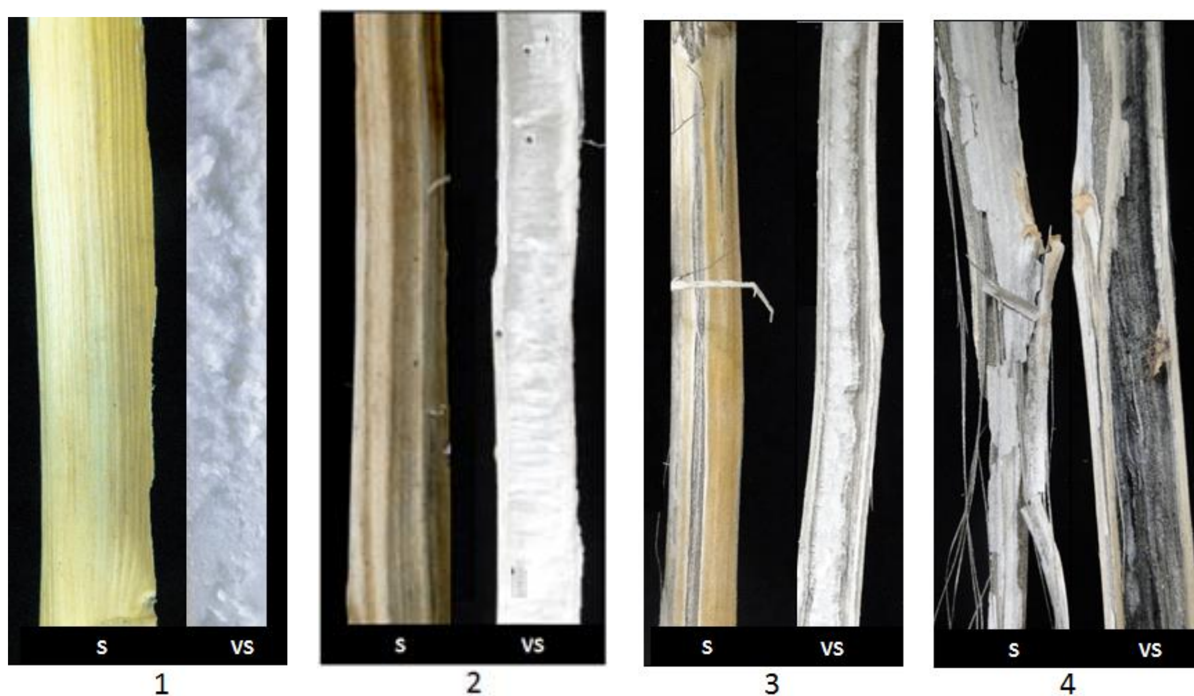


Figure 2.1 Assessment key (1-4) for evaluation of *Verticillium longisporum* disease severity on stubble and root of oilseed rape (*Brassica napus*). **1. Healthy tissue:** Surface free of any symptoms and fungal structures; pith white and with cottony appearance; no microsclerotia visible. **2. Slight infestation:** Narrow and short longitudinal lesions on the surface; pith still whitish but a few patches of microsclerotia visible. **3. Advanced infestation:** Very long lesions and abundant microsclerotia visible under the epidermis; epidermis peeling off; long patches of black microsclerotia visible in the pith. **4. Severe infestation:** Most part of the stem epidermis peeling off and disintegrated; the surface black and covered with microsclerotia; pith becomes dark, disintegrated and filled with microsclerotia. **S**, stem surface. **VS**, vertical section.

In order to identify the most suitable stage of plant growth at which VL can be detected (quantified) in stem tissue of field grown plants and to precisely know the plant stage at which field grown susceptible and resistant plants can be best differentiated using this method, VL biomass from the

four reference varieties was quantified at three different growth stages. This study was conducted in 2012/13. For this study, stem samples from the central four rows of each plot were collected at BBCH 65, 70, and 80 representing 50% flowering, end of flowering, and beginning of ripening stages, respectively. For each variety, 40 stem samples representing four biological replicates (10 plant samples per replication) were collected. In Göttingen, since there were four replications, sampling was done by collecting 10 stem samples per plot. In Fehmarn and Svalöv sites, since there were two replications, sampling was done by dividing each plot into two halves and collecting 10 stem samples from each part.

For phenotypic disease assessment, stubble sample collected after harvest was evaluated for stem and root disease severity. For every season study, a total of 50 (Fehmarn and Svalöv) or 100 (Göttingen) stubble samples were collected from each line (i.e 25 samples per replication). Disease severity evaluation was carried out by visual assessment of VL symptoms as well as microscopic estimation of the amount of microsclerotia both on the stem surface as well as in the pith tissue of stem and root samples using a four scale disease index (Fig. 2.1; Appendix 2.6).

2.2.3 Outdoor experiment

Unlike field conditions, infection of OSR with *V. longisporum* in greenhouse conditions typically induces clear symptoms of stunting and excessive production of side branches (see 4.4.1). Previous studies revealed weak or no correlation between results of field disease index and greenhouse AUDPC or stunting parameters (Knüfer, 2013; Eynck et al, 2009a). The possible reasons for such discrepancies in symptom development and resistance reactions between field and greenhouse experiments might arise from climatic variations, the type or amount of inoculum used, or even from the method of inoculation. In order to verify this hypothesis, *B. napus* lines used in the field trial 2011/12 were simultaneously tested under outdoor conditions with potted plants, which represent an intermediate situation between greenhouse and field conditions. Accordingly, inoculum source and method of inoculation were used in the same way as in the greenhouse screening experiments. To represent field conditions, vernalized seedlings infected with VL were transplanted to pots and grown outdoor (open air) where they experienced similar climatic conditions as field grown plants. The duration of this experiment was 12 weeks, until most genotypes reached the fruit development stage (BBCH 80).

2.2.3.1 Plant materials and growing conditions

For this study, forty *B. napus* genotypes (thirty six lines and four reference varieties) evaluated in the 2011/12 field experiment were used (Appendix 2.2). Surface sterilized seeds (70% ethanol for two min) of all genotypes were sown in a multipot tray filled with sterile silica sand and soil (1:1).

Seedlings were grown in a climate chamber with 16 h light, 20°C temperature and 60% relative humidity. Two weeks after sowing (BBCH12), seedlings were subjected to vernalization by gradual lowering of light duration and temperature conditions to 10 h and 4°C, respectively. After 13 weeks of vernalization, seedlings were acclimatized for three days by daily gradual increasing of light duration and temperature conditions up to 16 h and 20°C, respectively.

2.2.3.2 Inoculation and disease scoring

Vernalized seedlings were inoculated with mixed spore suspensions of the highly aggressive VL isolates VL40 and VL43. Inoculum preparation and its density adjustment, method and procedure of inoculation and all other procedures were done exactly the same way as in the greenhouse screening experiments (section 2.2.1.3). Control plants were similarly inoculated with sterile water. In order to avoid cross contaminations due to runoff during rain or irrigation, pots with control plants were kept on trays (Fig. 2.2). Measurement and analysis of AUDPC and stunting effects were done as described in section 4.2.7. Net branching and net growth stage values were calculated by subtracting the average values of mock-inoculated plants from that of the respective VL-inoculated treatments.

Figure 2.2 Partial view of outdoor experiment: Screening of *Brassica napus* lines for resistance against *Verticillium longisporum*. Vernalized plants at 36 DPI are shown. Pot plants placed on trays are mock-inoculated plants and those on the surface of the outdoor ground are VL-inoculated treatments.

2.2.3.3 Experimental design and outdoor environmental conditions

The outdoor (open air) pot experiment was conducted at the Department of Plant Pathology and Crop Protection, University of Göttingen. The trial was arranged in a completely randomized design (CRD) with three replications. For each line, 9 VL-inoculated and 9 mock-inoculated plants, each representing three biological replicates (three plants per replication), were used. Immediately after

inoculation, seedlings were transplanted into 1.5liter plastic pots filled with river sand and twelve weeks (3rd of April 2012 until 26th of July 2012). Average climatic conditions of the outdoor area during the experimental period were 14.2°C mean temperature, 74.6% mean relative humidity and 298.4 mm total rainfall (Appendix 2.4). All other agronomic practices including fertilization (*Hakaphos* COMPO, Germany) and insecticide application against flower beetles were applied following standard recommendations. Whenever required, plants were appropriately irrigated with tap water.

2.2.3.4 Agronomic and disease data collection

Disease severity, plant height, number of branches per plant, and phenological growth stage were measured and analyzed according to the procedures described in chapter 4. Disease index was assessed weekly from 7 to 56 DPI. Number of primary branches per plant in control and infected plants was counted on a weekly basis from 35 to 56 DPI. To obtain net branching values, number of branches in mock-inoculated plants of each line was counted and this value was subtracted from corresponding VL-inoculated treatments. Similarly, assessment of phenological stages was assessed from 35 to 56 DPI using the BBCH scale (Appendix 4.13). Measurement of plant height was done at the end of the experiment (84 DPI).

2.2.3.5 Data analysis

For all lines screened in field experiments, mean data obtained from field, outdoor and greenhouse screening experiments were subjected to one-way ANOVA using the STATISTICA package for windows (version 10, StatSoft, inc. 2011). As a post-hoc comparison among genotypes, Fisher's least significant difference (LSD) test at $P<0.05$ was applied. For the rest of all data obtained from greenhouse and outdoor experiments, mean values of the different disease parameters derived from each line were compared with the respective values of the reference varieties. To analyze the relationship among the results of greenhouse, outdoor and/or field experiments as well as field results across locations, Pearson's correlation analysis was performed.

2.3 Results and discussion

2.3.1 Greenhouse screening

For the *B. napus* - *V.longisporum* pathosystem, a well-established and efficient greenhouse screening technique that enables screening of large numbers of plants within short period of time is available (Eynck, 2008). Using this method, a total of 235 *B. napus* lines were screened for VL resistance in four rounds of independent experiments. Disease development was monitored by measuring disease severity and stunting. In general, a wide range of variation in disease reaction among the

different *B. napus* lines was observed. There were large numbers of genotypes that showed better performance than the resistant reference variety. Some others had extremely high disease levels even when compared to the susceptible reference control. Figure 2.3 shows the results of some lines screened in different experiments that represent the range of responses from extremely susceptible to highly resistant phenotypes. Detailed information and results of all lines screened in the greenhouse experiments are found in Appendix 2.1 and Appendix 2.3.

According to the results of disease severity assessments, from the total 235 lines screened in all greenhouse experiments, 90 had lower normalized net AUDPC (NNA) values than the mean value of the resistant reference variety Express (0.58). Eight lines showed extremely high levels of NNA which were greater than the values recorded from the susceptible reference variety Falcon (NNA=1.42). The remaining 137 lines had NNA values ranging between the resistant and susceptible reference varieties (Appendix 2.3). Comparable results have also been found from the analysis of relative stunting data. With some exceptions, the extent of stunting due to VL infection closely correlates with net AUDPC values of most lines. That means in most cases genotypes with severe stunting also had high NNA values and *vice versa*. Nevertheless, some lines with very low NNA were found to be severely stunted.

For instance, from 90 lines with less NNA values than the resistance reference variety Express, 35 had greater normalized relative stunting (NRS) values than the one recorded from the same resistant reference variety (NRS=0.53). Of these lines, six of them,SW08-309035, WVB226, WVB225, WVB227, WVB232 and SYNVER256 (Appendix 2.3), showed extreme stunting with NRS values greater than twice the value recorded in Express (i.e NRS > 1.06). In general, considering plant height as a disease parameter, 73 lines representing 31% of the total number had lower NRS value than the resistant reference cultivar Express (NRS =0.53). There were only six lines (SYNVER258, DSV10-42, DSV10-41, KWS204, WVB223, and SYNVER256) that showed more severe stunting compared to the resistant cultivar Falcon (NRS=1.47). The NRS value of the remaining 156 lines representing more than 66% of the total number lay between the values of the resistant and susceptible controls (Appendix 2.3). This is a more or less similar proportion compared to the one observed in disease severity (NNA) results.

Correlation analysis between NNA and NRS measurements showed a significant, strong and positive correlation (Fig. 2.4) indicating that these parameters are most appropriate and reliable phenotypic traits for evaluation of VL resistance in oilseed rape under controlled conditions. Overall, from the results of the four independent greenhouse experiments, 23 lines (NICK191,NICK189, DSV10-33, NICK186, DSV10-30, NICK190, SW08-309026, DSV10-32, SW08-309029, NICK187, SRG228, NICK184,

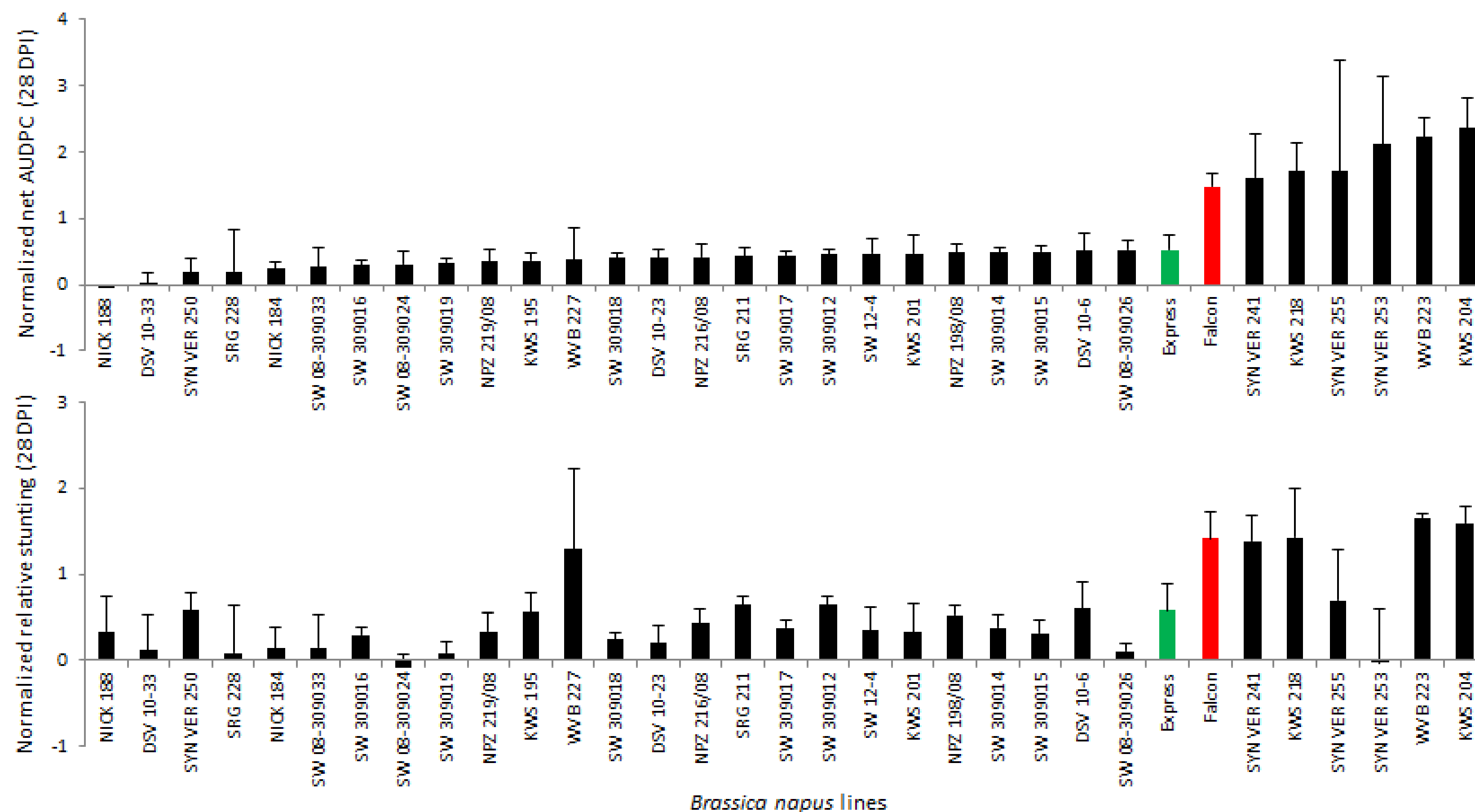


Figure 2.3 Response of *Brassica napus* lines to *Verticillium longisporum* infection under greenhouse conditions. Normalized mean net AUDPC (top) and relative stunting (bottom) data of 33 representative *Brassica napus* lines from four different screenings are shown. For both parameters data shown are the means of 20 plants. Genotypes are sorted according to increasing level of net AUDPC values. Green and red bars show mean values derived from four independent experiments (GHS42, GHS43, GHS44 and GHS46) for the reference variety Express (resistant) and Falcon (susceptible). Errors bars indicate standard deviations. **DPI**, days after inoculation with *Verticillium longisporum*.

WVB231, SW08-309033, NICK192, SW309016, SW08-309024, SW08-309030, DSV10-29, DSV10-28, SW309019, NPZ219/08, and KWS200) that had NNA and NRS values less than 0.40 and showed very strong resistance against VL were identified. These lines are recommended for further test under field condition and to be considered in future breeding works.

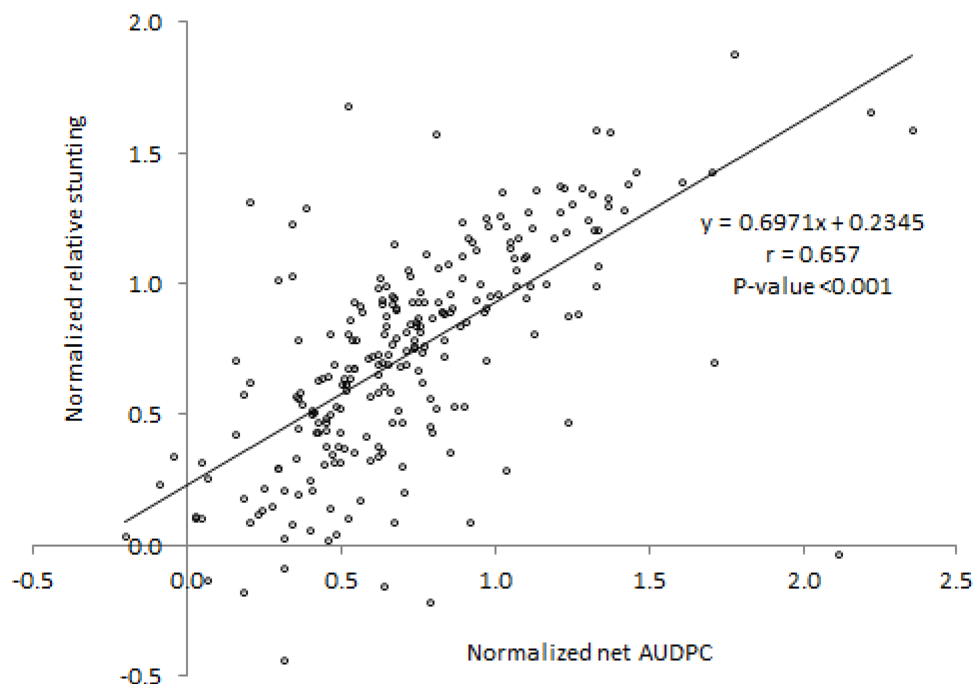


Figure 2.4 Correlation analysis describing the relationship between disease assessment parameters recorded in the greenhouse experiment. Analysis was performed using mean normalized net AUDPC and relative stunting data from 235 *Brassica napus* lines and two reference varieties (n=237). Pearson correlation analysis was done using STATISTICA (Version 10 StatSoft, inc. 2011).

2.3.2 Field trials

To better understand the results of the different field experiments and perform correlation analyses among the results of different locations, years and types of experiments, data analysis for the results of field trials was divided into three parts. In the first part (2.4.2.1), ANOVA and correlation analysis was conducted for 14 DH lines which were tested for three seasons and across all locations. The second part (2.4.2.2) shows the results of 22 lines which were tested only once in the field (i.e. in one season trial only). For both the first and the second part, mean data obtained from phenotypic and molecular disease evaluation of each *B. napus* line was compared with the corresponding values of the resistant and susceptible reference varieties. The third part (2.4.2.3), focused on the molecular detection and quantification of *V. longisporum* in the stems at different growth stages of field grown resistant and susceptible reference OSR varieties.

2.3.2.1 *B. napus* DH lines evaluated for *V. longisporum* resistance in multi-locational field trials for three consecutive seasons (years)

Fourteen best performing *B. napus* lines (13 DH lines and one accession) which were selected based on previous greenhouse screening results were evaluated for three consecutive growing seasons (2010/11, 2011/12 and 2012/13) across three locations differing in climatic and disease conditions (Table 2.2). Source and descriptions of all plant materials can be found in Appendix 2.2. Results of the experiments conducted in Fehmarn, Svalöv and Göttingen are discussed as follows.

Analysis of data obtained from **Fehmarn** showed that only in a few cases field disease parameters have shown significant differences (Table 2.3). In **2010/11**, disease evaluation data was obtained from assessment of disease severity from shoots and roots of stubbles samples. One-way ANOVA showed significant differences in the shoot disease index (SDI) but not in the root disease index (RDI). Interestingly, there were significant differences in SDI between the two susceptible varieties (Falcon and Laser) and the resistant variety Oase. Four lines (SEM05-500256, SW08-190001-7, SW08-190002-8 and SW08-190002-9) had also significantly lower SDI than both susceptible controls. Of these three lines SW08-190002-8 was the one that had the lowest SDI which is even significantly lower than the one recorded from both resistant reference varieties. SDI and RDI evaluations in **2011/12** showed no significant differences among all genotypes. From the same experiment, molecular quantification of VL biomass by qPCR, however, revealed significant differences. Considering this parameter, the difference between the resistant and susceptible controls was significant. Accordingly, the amount of VL DNA quantified from the susceptible cultivars Falcon and Laser was 9.8 and 11.4ng per gram of stem dry matter, respectively. In the resistant varieties Express and Oase, the amount was 0.8 and 1.8ng/g, respectively. Regarding the DH lines, except SW08-190001-11 and SW08-190001-1, all other lines had significantly lower amounts of VL DNA than both susceptible controls. The least amount of VL DNA, which is significantly lower than both resistant controls, was detected from accession SEM05-500256. In **2012/13**, again data only on SDI and RDI measurements was available and both measurements showed no significant differences among all genotypes. Unlike field conditions however, analysis of the phenotypic disease evaluation parameters used in outdoor and greenhouse experiments (NNA and NRS) showed significant differences between the reference controls and among the tested genotypes as well (Table 2.3).

When we compare field performance (in Fehmarn) with outdoor and greenhouse screening results, it was found that VL DNA was the only field parameter that showed strong and significant correlations across years (with SDI 2010/11; r=0.53), with outdoor NNA (r=0.51) and greenhouse NNA (r=0.62) results. It was also noted that unlike RDI, field results from assessment of SDI were better correlated with field VLDNA as well as with greenhouse and outdoor results. Regarding the

correlation of SDI and RDI, among growing seasons, there was only one case where a significant correlation was observed (i.e SDI 2011 vs SDI 2012; r=0.50). Of the two parameters used in the outdoor and greenhouse experiments, compared to NRS, NNA was more correlated with field data (Table 2.6A), somehow suggesting an effect of genotype variability in determining the extent of VL-induced stunting. This is in strong agreement with the observations of greenhouse screening experiments where some genotypes with resistant reactions in AUDPC measurements showed severe stunting (Appendix 2.3). According to the results of three years experiments in Fehmarn, compared to the susceptible control reference varieties, except SW08-190002-1 and SW 08-190002-5, the rest 12 lines were found to be promising materials. Of which genotypes SEM05-500256, SW08-190002-8, SW08-190001-25, SW08-190001-7, SW08-190001-12, SW08-190002-9, SW08-190001-11 and SW08-190002-11 have shown more or less consistent resistance responses in field experiments in different years, and under outdoor and greenhouse conditions (Fig. 2.3).

Similar to the results in Fehmarn, SDI and RDI data recorded in **2010/11** and **2011/12** experiments in **Svalöv** showed no significant differences among all genotypes (Table 2.4). In contrary to Fehmarn however, severity of SDI in 2010/11 and RDI in 2010/11 and 2011/12 were relatively lower in Svalöv. Conversely, RDI in 2010/11 was slightly higher in Svalöv than in Fehmarn. Interestingly, quantification of VL DNA from the 2011/12 season experiment in Svalöv also showed significant differences among genotypes. Unlike in Fehmarn, however, even though up to twofold VL DNA was quantified from susceptible varieties compared to resistant controls, this difference was not statistically significant. This could be because of the high variability between replicates. Regarding the different lines, seven (SEM05-500256, SW08-190001-12, SW08-190002-5, SW08-190001-11, SW08-190002-16, SW08-190001-1 and SW08-190002-9) had significantly lower amounts of VL DNA compared to the susceptible controls. Of these lines, three of them (SEM05-500256, SW08-190001-12 and SW08-190002-5) had significantly lower VL DNA than both resistant controls. Assessment of disease severity by qPCR in 2011/12 also revealed the presence of a relatively higher disease infestation (for most genotypes) in Svalöv than in Fehmarn. Since no sufficient numbers of plants remained at harvest due to loss of most plants by the hard winter in Svalöv, no data was available from **2012/13** (Table 2.4).

According to the correlation analysis results between field performance in Svalöv vs outdoor and greenhouse, the only significant correlation was found between VL DNA and greenhouse NRS (r=0.56).The other significant correlations were found between SDI and RDI in 2010/11 (r=0.73) and 2011/12 (r=0.91) (Table 2.6B). In general, it was noticed that compared to Fehmarn, results obtained from field experiments in Svalöv were poorly correlated with each other and with greenhouse and outdoor results. For instance in Fehmarn there was significant correlation (r=0.50) between the

2010/11 and 2011/12 growing seasons in SDI and RDI measurements (Table 2.6A) which was not the case in Svalöv (Table 2.6B). Furthermore, few field parameters from Fehmarn (RDI and VLDNA) at least showed a significant correlation with the outdoor NNA results (Table 2.6A), but none of the parameters from Svalöv had a significant correlation with the outdoor results (Table 2.6B). Apart from climatic and soil factors, the variation in compositions of different VL isolates in the soil and the amount of natural inoculum present in the soil might be among the possible explanations for the differences found across locations. Nevertheless, considering the results of three years of experiments in Svalöv, it was found that genotypes SEM05-500256, SW08-190001-12, SW08-190002-5, SW08-190001-7 and SW08-190001-25 were the top performing lines (Table 2.4) showing consistent resistance both under field and controlled experimental conditions.

In **Göttingen,** the trials in **2010/11** and **2011/12** were destroyed due to severe winter. As a result, data from this site was only available from **2012/13** (Table 2.5). Due to low/no natural disease infestation, the experiment at this location was conducted with additional application of inoculum (see 2.2.2.4). However, even after application of additional inoculum, level of disease severity as evidenced by phenotypic and molecular parameters was significantly lower as compared to the other two locations. Regarding SDI and RDI, with the exception of Oase and SW 08-190002-8 that had significantly lower level of disease compared to the susceptible check Laser, no significant difference was found among the rest of the DH lines and the reference varieties. The overall disease severity level in all genotypes including the susceptible controls was very low, with an average disease index less than 2.0, referring to slight infestation (Fig. 2.1).

With regard to the qPCR data however, a better differentiation of the different genotypes was achieved. More importantly, significant differences were found between the resistant and susceptible reference varieties. The highest amount of VL DNA was quantified in the two susceptible reference varieties and the DH line SW08-190001-11. On the other hand, compared to both susceptible controls, lines SW08-190001-25, SEM05-500256, SW08-190001-7, SW08-190002-18, SW08-190002-1 and the two resistant varieties had significantly lower fungal biomass (Table 2.5). As in the other two locations, compared to the phenotypic disease assessment, field evaluation of VL resistance via qPCR showed very strong and significant correlations with field SDI (r=0.85), field RDI (r= 0.68) and greenhouse AUDPC (r=0.55) recordings. Furthermore, significant correlation of RDI with SDI (r=0.83) and greenhouse NRS (r=0.56) was found (Table 2.6C). Considering a single season field result of this location, it was found that lines SW08-190001-25, SEM05-500256, SW08-190001-7, SW08-190002-9, SW08-190001-12, SW08-190001-1, SW08-190002-8, SW08-190002-16 and

SW08-190002-11 (Table 2.5) have shown a reasonably consistent and better performance in field, greenhouse and outdoor conditions.

To study the overall relationships in field performance of *B. napus* lines across the three locations, mean phenotypic and molecular disease data obtained from 18 genotypes (14 lines and 4 reference varieties) were subjected to Pearson's correlation analysis. From the 2010/11 and 2011/12 trials, since no data was available from Göttingen, correlation between results of Fehmarn and Svalöv locations was analyzed. Unfortunately, none of the disease parameters recorded in 2010/11, 2011/12 and 2012/213 showed any significant correlation between locations. Interestingly, similar to the correlations across years (Table 2.6), results from quantification of VL DNA in the 2011/12 trial from Svalöv showed a significant positive correlation (r=0.86) with the same parameter in Fehmarn. In 2012/13, no data was available from Svalöv and correlation analysis was performed for Göttingen and Fehmarn sites only. Here, none of the parameters showed any significant correlations (Table 2.7). Local differences in terms of climatic and soil factors and diversity of VL isolates that possibly determine host-pathogen interactions might be the possible reasons for the poor correlation of results across the different locations.

In conclusion, among the 14 *B. napus* lines evaluated for three years (seasons) in multiple locations, SEM05-500256, SW08-190001-12, SW08-190001-7, SW08-190001-25, SW08-190002-9, SW08-190002-8 and SW08-190002-11, that showed consistently higher resistance in greenhouse, outdoor and field trials in at least two locations, are highly recommended for use as parent materials in future breeding aiming at integration of *V. longisporum* resistance traits into commercial winter oilseed rape varieties.

Table 2.3 Response of *Brassica napus* double haploid lines to *Verticillium longisporum* infection under field conditions in **Fehmarn**. Comparisons of field performance across years and between field and greenhouse/outdoor screening results of 14 lines and 4 reference varieties screened during all season trials are shown.

Genotype code	Field trial (Fehmarn)							Outdoor		Greenhouse	
	2010/11		2011/12			2012/13					
	SDI	RDI^{ns}	SDI^{ns}	RDI^{ns}	VLDNA	SDI^{ns}	RDI^{ns}	NNA	NRS	NNA	NRS
SEM 05-500256*	2.4±0.1^{cd}	1.8±0.1	2.1±0.1	2.3±0.4	0.13±0.1^{f}	1.41±0.3	1.32±0.5	0.8±0.2^{defg}	0.8±0.4^{cde}	-0.02±0.1^{f}	0.20±0.3^{d}
SW 08-190002-8	1.8±0.1^{e}	1.4±0.1	1.7±0.3	1.7±0.2	0.17±0.0^{ef}	1.93±0.8	2.12±1.2	0.7±0.5^{efg}	0.6±0.3^{def}	1.06±0.1^{abcd}	0.54±0.4^{bcd}
SW 08-190001-25	2.5±0.3^{bcd}	1.6±0.2	1.6±0.3	1.5±0.3	0.32±0.4^{def}	2.17±0.5	1.92±0.1	0.3±0.3^{gh}	0.0±0.9^{ef}	0.95±0.4^{bcd}	0.28±0.6^{d}
SW 08-190002-5	3.4±0.1^{a}	1.9±0.1	2.4±0.2	2.1±0.2	0.48±0.3^{cdef}	2.04±0.1	2.16±0.2	1.1±0.5^{bcdef}	0.2±0.0^{ef}	1.03±0.4^{bcd}	0.63±0.6^{abcd}
SW 08-190001-7	2.3±0.4^{de}	1.6±0.1	2.0±0.6	2.0±0.4	0.54±0.3^{bcdef}	1.86±0.2	1.71±0.4	1.1±0.3^{bcde}	0.7±0.9^{cde}	0.96±0.5^{bcd}	0.31±0.3^{cd}
SW 08-190001-12	2.7±0.2^{bcd}	1.7±0.2	2.5±0.0	2.4±0.1	0.69±0.8^{cdef}	2.52±0.4	3.24±1.1	1.0±0.1^{cdef}	0.5±0.3^{def}	0.82±0.0^{cd}	0.55±0.4^{bcd}
SW 08-190002-9	2.3±0.3^{de}	1.4±0.2	2.1±0.5	2.2±0.5	0.72±0.4^{bcdef}	1.26±0.2	1.38±0.5	0.0±0.1^{hi}	2.2±1.6^{b}	0.94±0.1^{bcd}	0.60±0.4^{bcd}
Express$^{(RC)}$	2.9±0.4^{abc}	1.8±0.1	2.3±0.0	2.2±0.1	0.81±0.5^{bcde}	1.79±0.5	1.90±0.1	0.3±0.4^{gh}	0.3±0.6^{ef}	0.69±0.3^{de}	0.86±0.2^{abcd}
SW 08-190001-6	3.0±0.3^{abc}	1.6±0.1	2.1±0.1	2.3±0.1	0.98±1.0^{bcdef}	1.73±0.3	2.04±0.1	1.4±0.1^{bcd}	3.6±0.4^{a}	1.27±0.1^{ab}	0.72±0.4^{abcd}
SW 08-190002-1	2.8±0.5^{bcd}	1.9±0.2	1.8±0.5	1.9±0.6	1.14±1.2^{bcde}	2.57±1.0	2.36±0.9	1.6±0.2^{b}	1.5±0.9^{bcd}	1.18±0.5^{abc}	1.08±0.4^{ab}
SW 08-190002-18	2.7±0.1^{bcd}	1.7±0.3	2.2±0.4	2.0±0.5	1.20±0.8^{bcde}	2.06±0.1	2.30±0.4	1.4±0.3^{bc}	1.7±0.3^{bc}	1.14±0.5^{abcd}	0.78±0.7^{abcd}
SW 08-190002-16	3.0±0.3^{abc}	1.6±0.1	2.4±0.2	2.3±0.1	1.21±1.1^{bcde}	2.35±0.6	3.02±1.4	0.4±0.2^{fgh}	-1.3±0.5^{g}	1.19±0.2^{abc}	0.58±0.6^{bcd}
SW 08-190002-11	2.9±0.1^{abc}	1.6±0.0	2.2±0.3	2.3±0.3	1.33±0.5^{bc}	1.87±0.5	2.52±0.7	0.7±0.4^{efg}	-0.5±0.1^{g}	1.19±0.2^{abc}	0.77±0.4^{abcd}
Oase$^{(RC)}$	2.8±0.4^{bcd}	1.7±0.3	2.2±0.2	2.3±0.4	1.75±1.2^{bcd}	1.34±0.4	1.40±0.6	0.0±0.2^{hi}	1.0±0.4^{cde}	0.36±0.1^{ef}	0.42±0.2^{bcd}
SW 08-190001-11	2.6±0.1^{bcd}	1.5±0.1	2.4±0.1	2.4±0.1	2.09±0.2^{abc}	1.98±0.3	1.94±0.1	-0.4±0.0^{i}	0.9±0.5^{cde}	1.18±0.2^{abc}	0.99±0.1^{abc}
SW 08-190001-1	2.8±0.5^{bcd}	1.8±0.1	2.5±0.2	2.3±0.0	2.95±1.4^{ab}	2.16±0.3	2.56±0.6	0.8±0.3^{efg}	1.1±0.7^{cde}	0.97±0.2^{bcd}	0.33±0.6^{cd}
Falcon$^{(SC)}$	3.1±0.0^{ab}	1.8±0.1	2.3±1.0	2.3±1.2	9.77±5.7^{a}	2.18±0.5	2.68±0.5	1.3±1.1^{bcde}	0.3±0.5^{ef}	1.49±0.1^{a}	1.32±0.2^{a}
Laser$^{(SC)}$	3.4±0.1^{a}	1.8±0.1	2.1±0.4	2.0±0.4	11.4±6.6^{a}	1.98±0.1	1.96±0.1	2.4±0.1^{a}	2.4±0.9^{b}	1.05±0.1^{abcd}	1.06±0.1^{ab}

SDI, shoot disease index. **RDI**, root disease index. **NNA**, net normalized AUDPC. **NRS**, normalized relative stunting. **VLDNA**, *Verticillium longisporum* DNA (ng/g) in hypocotyl at BBCH80. **(RC)**, resistant control. **(SC)**, susceptible control. *****, not a double haploid line. For all parameters mean ± standard deviation data is shown. Greenhouse results are means of 60 plants derived from three independent experiments. In outdoor experiment, vernalized seedlings inoculated with VL were transplanted to pots and grown outdoor (open air condition). Genotypes are sorted according to field VLDNA quantification results that showed better correlation with other field, outdoor and GH parameters and across locations. For each parameter, means followed by the same letter are not significantly different at p=0.05. Mean values under the parameters indicated by the superscript '**ns**' are not significantly different at p=0.05.

Table 2.4 Response of *Brassica napus* double haploid lines to *Verticillium longisporum* infection under field conditions in **Svalöv**. Comparisons of field performance across years and between field and greenhouse/outdoor screening results of 14 lines and 4 reference varieties screened during all season trials are shown.

Genotype code	Field trial (Svalöv) 2010/11		2011/12			2012/13	Outdoor		Greenhouse	
	SDI^{ns}	RDI^{ns}	SDI^{ns}	RDI^{ns}	VLDNA	(ND)	NNA	NRS	NNA	NRS
SEM 05-500256*	2.0±0.2	2.1±0.1	1.2±0.1	1.3±0.1	0.26±0.1^{ef}	-	0.8±0.2^{defg}	0.8±0.4^{cde}	-0.02±0.1^{f}	0.20±0.3^{d}
SW 08-190001-12	2.6±0.7	2.8±0.8	1.4±0.2	1.5±0.2	0.34±0.2^{ef}	-	1.0±0.1^{cdef}	0.5±0.3^{def}	0.82±0.0^{cd}	0.55±0.4^{bcd}
SW 08-190002-5	1.3±0.2	2.3±0.8	1.2±0.2	1.3±0.2	0.43±0.6^{f}	-	1.1±0.5^{bcdef}	0.2±0.0^{ef}	1.03±0.4^{bcd}	0.63±0.6^{abcd}
SW 08-190001-11	2.7±0.4	2.4±0.6	2.0±0.5	2.0±0.4	0.47±0.4^{def}	-	-0.4±0.0^{i}	0.9±0.5^{cde}	1.18±0.2^{abc}	0.99±0.1^{abc}
SW 08-190002-16	2.7±1.0	2.9±0.8	1.6±0.1	1.6±0.2	0.67±0.1^{cdef}	-	0.4±0.2^{fgh}	-1.3±0.5^{g}	1.19±0.2^{abc}	0.58±0.6^{bcd}
SW 08-190001-1	2.8±0.7	2.5±0.3	1.4±0.3	1.5±0.4	0.96±0.0^{cdef}	-	0.8±0.3^{efg}	1.1±0.7^{cde}	0.97±0.2^{bcd}	0.33±0.6^{cd}
SW 08-190002-9	3.0±0.1	2.7±0.2	1.4±0.1	1.4±0.2	0.97±1.0^{cdef}	-	0.0±0.1^{hi}	2.2±1.6^{b}	0.94±0.1^{bcd}	0.60±0.4^{bcd}
SW 08-190002-1	2.5±0.2	2.0±0.4	1.1±0.1	1.2±0.1	1.05±0.5^{bcde}	-	1.6±0.2^{b}	1.5±0.9^{bcd}	1.18±0.5^{abc}	1.08±0.4^{ab}
SW 08-190001-6	2.5±1.0	2.4±0.4	1.6±0.2	1.7±0.1	1.45±0.7^{abcde}	-	1.4±0.1^{bcd}	3.6±0.4^{a}	1.27±0.1^{ab}	0.72±0.4^{abcd}
SW 08-190001-7	2.0±1.3	1.8±1.0	1.6±0.1	1.7±0.2	1.47±0.6^{abcde}	-	1.1±0.3^{bcde}	0.7±0.9^{cde}	0.96±0.5^{bcd}	0.31±0.3^{cd}
SW 08-190002-8	2.6±0.4	2.7±0.1	1.0±0.0	1.0±0.0	2.10±2.6^{bcde}	-	0.7±0.5^{efg}	0.6±0.3^{def}	1.06±0.1^{abcd}	0.54±0.4^{bcd}
SW 08-190002-18	2.9±0.3	2.8±0.0	1.8±0.3	1.9±0.3	2.14±2.5^{abcde}	-	1.4±0.3^{bc}	1.7±0.3^{bc}	1.14±0.5^{abcd}	0.78±0.7^{abcd}
SW 08-190001-25	2.4±0.2	2.3±0.2	1.4±0.2	1.4±0.2	2.95±1.4^{abcd}	-	0.3±0.3^{gh}	0.0±0.9^{ef}	0.95±0.4^{bcd}	0.28±0.6^{d}
Oase$^{(RC)}$	2.9±0.8	2.9±0.6	1.3±0.2	1.2±0.6	3.27±3.2^{abcd}	-	0.0±0.2^{hi}	1.0±0.4^{cde}	0.36±0.1^{ef}	0.42±0.2^{bcd}
Express$^{(RC)}$	1.3±0.2	1.6±0.1	1.4±0.4	1.8±0.6	3.89±1.6^{abc}	-	0.3±0.4^{gh}	0.3±0.6^{ef}	0.69±0.3^{de}	0.86±0.2^{abcd}
SW 08-190002-11	2.8±0.2	2.7±0.1	1.4±0.1	1.6±0.3	3.93±1.9^{abc}	-	0.7±0.4^{efg}	-0.5±0.1^{g}	1.19±0.2^{abc}	0.77±0.4^{abcd}
Falcon$^{(SC)}$	2.8±0.5	2.3±0.2	1.5±0.5	1.7±0.5	7.97±1.8^{ab}	-	1.3±1.1^{bcde}	0.3±0.5^{ef}	1.49±0.1^{a}	1.32±0.2^{a}
Laser$^{(SC)}$	2.5±1.0	2.4±1.0	1.7±0.2	1.7±0.3	8.82±0.9^{a}	-	2.4±0.1^{a}	2.4±0.9^{b}	1.05±0.1^{abcd}	1.06±0.1^{ab}

DI, shoot disease index. **RDI**, root disease index. **NNA**, net normalized AUDPC. **NRS**, normalized relative stunting. **VLDNA**, *Verticillium longisporum* DNA (ng/g) in hypocotyl at BBCH80. **ND**, no data due to loss of plants by hard winter. **(RC)**, resistant control. **(SC)**, susceptible control. *****, not a double haploid line. For all parameters mean ± standard deviation data is shown. Greenhouse results are mean of 60 plants derived from three independent experiments. In outdoor experiment, vernalized seedlings inoculated with VL were transplanted to pots and grown outdoor (open-air condition). Genotypes are sorted according to field VLDNA quantification results that showed better correlation with other field, outdoor and GH parameters and across locations. For each parameter, means followed by the same letter are not significantly different at p=0.05. Mean values under the parameters indicated by the superscript '**ns**' are not significantly different at p=0.05.

Table 2.5 Response of *Brassica napus* double haploid lines to *Verticillium longisporum* infection under field conditions in **Göttingen**. Comparisons of field performance across years and between field and greenhouse/outdoor screening results of 14 lines and 4 reference varieties screened during all season trials are shown.

Genotype code	Field trial (Göttingen) 2010/11 (ND)	2011/12 (ND)	2012/13 SDI	RDI	VLDNA	Outdoor NNA	NRS	Greenhouse NNA	NRS
Express$^{(RC)}$	-	-	1.25±0.2^{ab}	1.17±0.1^{ab}	0.08±0.08^{c}	0.3±0.4^{gh}	0.3±0.6^{ef}	0.69±0.3^{de}	0.86±0.2^{abcd}
SW 08-190001-25	-	-	1.07±0.1^{ab}	1.10±0.1^{ab}	0.09±0.06^{c}	0.3±0.3^{gh}	0.0±0.9^{ef}	0.95±0.4^{bcd}	0.28±0.6^{d}
SEM 05-500256*	-	-	1.20±0.2^{ab}	1.15±0.2^{ab}	0.12±0.13^{c}	0.8±0.2^{defg}	0.8±0.4^{cde}	-0.02±0.1^{f}	0.20±0.3^{d}
SW 08-190001-7	-	-	1.15±0.1^{ab}	1.12±0.1^{ab}	0.14±0.12^{c}	1.1±0.3^{bcde}	0.7±0.9^{cde}	0.96±0.5^{bcd}	0.31±0.3^{cd}
SW 08-190002-18	-	-	1.12±0.0^{ab}	1.10±0.0^{ab}	0.16±0.12^{c}	1.4±0.3^{bc}	1.7±0.3^{bc}	1.14±0.5^{abcd}	0.78±0.7^{abcd}
SW 08-190002-1	-	-	1.03±0.1^{ab}	1.07±0.1^{ab}	0.17±0.13^{c}	1.6±0.2^{b}	1.5±0.9^{bcd}	1.18±0.5^{abc}	1.08±0.4^{ab}
SW 08-190002-9	-	-	1.10±0.1^{ab}	1.21±0.2^{ab}	0.19±0.14^{c}	0.0±0.1^{hi}	2.2±1.6^{b}	0.94±0.1^{bcd}	0.60±0.4^{bcd}
SW 08-190001-12	-	-	1.24±0.1^{ab}	1.18±0.2^{ab}	0.21±0.22^{c}	1.0±0.1^{cdef}	0.5±0.3^{def}	0.82±0.0^{cd}	0.55±0.4^{bcd}
SW 08-190001-1	-	-	1.16±0.1^{ab}	1.20±0.1^{ab}	0.25±0.16^{bc}	0.8±0.3^{efg}	1.1±0.7^{cde}	0.97±0.2^{bcd}	0.33±0.6^{cd}
SW 08-190002-16	-	-	1.12±0.1^{ab}	1.04±0.0^{ab}	0.26±0.22^{bc}	0.4±0.2^{fgh}	-1.3±0.5^{g}	1.19±0.2^{abc}	0.58±0.6^{bcd}
SW 08-190002-5	-	-	1.22±0.1^{ab}	1.16±0.1^{ab}	0.28±0.24^{bc}	1.1±0.5^{bcdef}	0.2±0.0^{ef}	1.03±0.4^{bcd}	0.63±0.6^{abcd}
SW 08-190001-6	-	-	1.09±0.1^{ab}	1.22±0.2^{ab}	0.29±0.26^{bc}	1.4±0.1^{bcd}	3.6±0.4^{a}	1.27±0.1^{ab}	0.72±0.4^{abcd}
SW 08-190002-11	-	-	1.09±0.1^{ab}	1.16±0.1^{ab}	0.29±0.19^{bc}	0.7±0.4^{efg}	-0.5±0.1^{g}	1.19±0.2^{abc}	0.77±0.4^{abcd}
Oase$^{(RC)}$	-	-	1.01±0.0^{b}	1.03±0.0^{ab}	0.37±0.63^{c}	0.0±0.2^{hi}	1.0±0.4^{cde}	0.36±0.1^{ef}	0.42±0.2^{bcd}
SW 08-190002-8	-	-	1.01±0.0^{b}	1.01±0.0^{b}	0.55±0.93^{c}	0.7±0.5^{efg}	0.6±0.3^{def}	1.06±0.1^{abcd}	0.54±0.4^{bcd}
SW 08-190001-11	-	-	1.26±0.1^{ab}	1.48±0.3^{ab}	1.09±1.08^{ab}	-0.4±0.0^{i}	0.9±0.5^{cde}	1.18±0.2^{abc}	0.99±0.1^{abc}
Falcon$^{(SC)}$	-	-	1.31±0.2^{ab}	1.36±0.1^{ab}	1.47±1.78^{ab}	1.3±1.1^{bcde}	0.3±0.5^{ef}	1.49±0.1^{a}	1.32±0.2^{a}
Laser$^{(SC)}$	-	-	1.68±0.4^{a}	1.53±0.2^{a}	10.37±16.07^{a}	2.4±0.1^{a}	2.4±0.9^{b}	1.05±0.1^{abcd}	1.06±0.1^{ab}

DI, shoot disease index. **RDI**, root disease index. **NNA**, net normalized AUDPC. **NRS**, normalized relative stunting. **VLDNA**, *Verticillium longisporum* DNA (ng/g) in hypocotyl at BBCH80. **ND**, no data due to loss of plants by hard winter. **(RC)**, resistant control. **(SC)**, susceptible control. *****, not a double haploid line. For all parameters mean ± standard deviation data is shown. Greenhouse results are mean of 60 plants derived from three independent experiments. In outdoor experiment, vernalized seedlings inoculated with VL were transplanted to pots and grown outdoor (open air condition). Genotypes are sorted according to field VLDNA quantification results that showed better correlation with other field, outdoor and GH parameters and across locations. For each parameter, means followed by the same letter are not significantly different at p=0.05. Mean values under the parameters indicated by the superscript '**ns**' are not significantly different at p=0.05.

Table 2.6 Correlation between field performance and greenhouse or outdoor screening results of 14 *Brassica napus* double haploid lines and 4 reference varieties evaluated for *Verticillium longisporum* resistance in three successive years (2011, 2012 and 2013).

A. Correlation between field performance in **Fehmarn** with outdoor and greenhouse results

		Field trial (Fehmarn)						
Trial	Parameters	SDI 2011	RDI 2011	SDI 2012	RDI 2012	VLDNA 2012	SDI 2013	RDI 2013
Field (Fehmarn)	RDI 2011	**0.66****						
	SDI 2012	**0.50***	0.28^{ns}					
	RDI 2012	0.34^{ns}	0.12^{ns}	**0.85*****				
	VLDNA 2012	**0.53***	0.31^{ns}	0.16^{ns}	0.10^{ns}			
	SDI 2013	0.26^{ns}	0.32^{ns}	0.09^{ns}	-0.18^{ns}	0.14^{ns}		
	RDI 2013	0.32^{ns}	0.17^{ns}	0.41^{ns}	0.19^{ns}	0.15^{ns}	**0.84*****	
OD	NNA	0.42^{ns}	**0.51***	-0.13^{ns}	-0.21^{ns}	**0.51***	0.38^{ns}	0.27^{ns}
	NRS	0.04^{ns}	-0.03^{ns}	-0.17^{ns}	0.00^{ns}	0.19^{ns}	-0.31^{ns}	-0.38^{ns}
GH	NNA	0.27^{ns}	-0.16^{ns}	0.02^{ns}	-0.12^{ns}	0.33^{ns}	**0.54***	**0.55***
	NRS	**0.48***	0.23^{ns}	0.15^{ns}	0.14^{ns}	**0.62****	0.32^{ns}	0.29^{ns}

B. Correlation between field performance in **Svalöv** with outdoor and greenhouse results

		Field trial (Svalöv)					
Trial	Parameters	SDI 2011	RDI 2011	SDI 2012	RDI 2012	VLDNA 2012	2013 (ND)
Field (Svalöv)	RDI 2011	**0.73****					
	SDI 2012	0.26^{ns}	0.09^{ns}				
	RDI 2012	0.03^{ns}	-0.16^{n}s	**0.91*****			
	VLDNA 2012	0.11^{ns}	-0.09^{ns}	0.17^{ns}	0.23^{ns}		
OD	NNA	-0.08^{ns}	-0.17^{ns}	-0.02^{ns}	0.05^{ns}	0.43^{ns}	-
	NRS	0.18^{ns}	-0.05^{ns}	0.191^{ns}	0.11^{ns}	0.08^{ns}	-
GH	NNA	0.30^{ns}	0.13^{ns}	$0.36n^{s}$	0.37^{ns}	0.25^{ns}	-
	NRS	0.12^{ns}	-0.11^{ns}	0.30^{ns}	0.41^{ns}	**0.56***	-

C. Correlation between field performance in **Göttingen** with outdoor and greenhouse results

		Field trial (Göttingen)				
Trial	Parameters	2011 (ND)	2012 (ND)	SDI 2013	RDI 2013	VLDNA 2013
Field	RDI 2013	-	-	**0.83*****		
	VLDNA 2013	-	-	**0.85*****	**0.68****	
OD	NNA	-	-	0.46^{ns}	0.21^{ns}	**0.55***
	NRS	-	-	0.17^{ns}	0.34^{ns}	0.33^{ns}
GH	NNA	-	-	0.09^{ns}	0.28^{ns}	0.13^{ns}
	NRS	-	-	0.44^{ns}	**0.56***	0.40^{ns}

Pearson correlation analysis was performed using product-moment procedure of STATISTICA (Version 10 StatSoft, inc. 2011). Minus signs indicate negative correlations. Significant correlations are indicated in **bold** font. *****, significant at P=0.05. ******, significant at P=0.01. *******, significant at P=0.001. **ns**, not significant. **OD**, outdoor experiment. **GH**, greenhouse experiment. **SDI**, Shoot disease index. **RDI**, Root disease index. **NNA**, Net normalized AUDPC. **NRS**, Normalized net relative stunting. **VLDNA**, *Verticillium longisporum* DNA in hypocotyl tissue. **ND**, no data due to loss of plants by hard winter.

Table 2.7 Pearson correlations for field performance of *Verticillium longisporum* infected *Brassica napus* double haploid lines in three locations.

Parameters	Field trial 2010/11		
	Svalöv SDI	Svalöv RDI	Göttingen (ND)
Fehmarn SDI	-0.20^{ns}	-0.04^{ns}	-
Fehmarn RDI	-0.44^{ns}	-0.41^{ns}	-

Parameters	Field trial 2011/12		
	Svalöv SDI	Svalöv RDI	Svalöv VLDNA
Fehmarn SDI	0.37^{ns}	0.45^{ns}	-0.10^{ns}
Fehmarn RDI	0.31^{ns}	0.37^{ns}	-0.12^{ns}
Fehmarn VLDNA	0.32^{ns}	0.30^{ns}	**0.86*****

Parameters	Field trial 2012/13		
	Göttingen SDI	Göttingen RDI	Göttingen VLDNA
Fehmarn SDI	0.10^{ns}	0.00^{ns}	0.03^{ns}
Fehmarn RDI	0.07^{ns}	-0.02^{ns}	-0.06^{ns}
Svalöv (ND)	-	-	-

Pearson correlation analysis was performed using product-moment procedure of STATISTICA (Version 10 StatSoft, inc. 2011).Phenotypic and molecular disease screening results obtained from 14 *Brassica napus* double haploid lines and four reference varieties used in three consecutive field screening trials were used. Minus signs indicate negative correlations. Significant correlations are indicated in **bold** font. ***,** significant at P=0.05. ****,** significant at P=0.01. *****,** significant at P=0.001. **ns**, not significant. **OD**, outdoor experiment. **GH**, greenhouse experiment. **SDI**, shoot disease index. **RDI**, root disease index. **VLDNA**, *Verticillium longisporum* DNA in hypocotyl tissue. **ND**, no data due to loss of plants by hard winter.

2.3.2.2 Different sets of *B. napus* lines screened for *V. longisporum* resistance under field conditions for only one season (year)

Here, the field screening results of *B. napus* lines evaluated at different locations are presented in a year-by-year manner. During each season (2010/11, 2011/12 and 2012/13), different sets of 22 *B. napus* genotypes were tested.

For the DH lines evaluated in **2010/11** field trials in two locations (Fehmarn and Svalöv), disease assessment was done by SDI and RDI scoring. Of these measurements, the only parameter that showed significant differences among genotypes was Fehmarn SDI (Table 2.8) and this parameter was the only one that significantly correlated (r=0.51) with greenhouse NRS (Table 2.11A). The remaining measurements (Fehmarn RDI and Svalöv SDI and RDI) were not significantly different among all lines and reference varieties (Table 2.8). Apart from this, significant correlations were found between SDI and RDI in each location independently (Table 2.11A). Considering the only two parameters that showed significant correlations, DH lines DH196, DH69, and DH138, DH165 performed more or less similar in resistance to the reference variety Oase. In contrast, lines DH282, DH123, DH290, DH152 and DH16 showed a poor performance comparable to the susceptible reference variety Laser.

A similar trend was observed for the set of DH lines screened during **2011/12** in Fehmarn and Svalöv. The analysis of SDI and RDI data obtained from both locations showed no significant differences. In contrast, data obtained from qPCR analysis of samples collected from both locations showed significant differentiation (Table 2.9). In Fehmarn, this data significantly differed among the reference varieties. Furthermore, except for DSV-DH-Ver-9(1605-2) and DSV-DH-Ver-19(1605-2), all other DH lines had significantly lower levels of VL DNA compared to both susceptible controls. Regarding the results in Svalöv, despite the presence of a double as high level of VL DNA in the susceptible controls, the difference between resistant and susceptible controls was not statistically significant. Nevertheless, 13 DH lines with significantly lower amounts of VL DNA than in both susceptible controls were found. Of these, five of them [DSV-DH-Ver-7(1575-2), DSV-DH-Ver-14(1605-2), DSV-DH-Ver-5(1575-1), DSV-DH-Ver-22(1605-2) and DSV-DH-Ver-21(1605-1)] had significantly less VL DNA than both resistant controls. Correlation analysis of the results from DH lines screened in **2011/12** indicated that qPCR data was the highest correlated parameter across locations and between greenhouse results (Table 2.11B). Thus, qPCR results in Svalöv were significantly and positively correlated with Fehmarn VLDNA (r=0.70), greenhouse NNA (r=0.59) and greenhouse NRS (r=0.48) results. Similarly, significant correlations of Fehmarn VL DNA with Svalöv VL DNA (r=0.70), greenhouse NNA (r=0.40) and greenhouse RNS (r=0.50) results were found. Moreover, there was a significant correlation between shoot and root DI assessments in Fehmarn (0.73) and in Svalöv (r=0.99). Similar to most previously described field results, Svalöv disease index measurements showed no significant correlation with any of the other field or greenhouse parameters. Surprisingly, Fehmarn SDI and RDI measurements had significant negative correlations with Fehmarn VL DNA, Svalöv VL DNA and greenhouse NNA and NRS results (Table 2.11B). These correlations completely contradict the above mentioned qPCR (VL DNA) data of the same experiment (obtained from the same set of genotypes) that showed significant positive correlations with Svalöv VL DNA and greenhouse NNA and NRS results. Greenness of plant tissue at the time of harvesting stubble samples might potentially affect the accuracy of disease index scoring. Green stubble samples on which microsclerotia had not yet formed might possibly be scored as low disease index and this might possibly lead to the aforementioned negative correlation of Fehmarn disease index measurements with other field and greenhouse results. Nevertheless, if further work needs to be done on the lines tested in 2011/12 in Fehmarn and Svalöv, priority should be given to the following 12 lines [DSV-DH-Ver-21(1605-1), DSV-DH-Ver-22(1605-2), DSV-DH-Ver-20(1605-3), DSV-DH-Ver-16(1605-2), DSV-DH-Ver-8(1605-2), DSV-DH-Ver-10(1605-2), DSV-DH-Ver-11(1605-2), DSV-DH-Ver-14(1605-2), DSV-DH-Ver-12(1605-2), DSV-DH-Ver-4(1575-3), DSV-DH-Ver-6(1575-1) and DSV-DH-Ver-18(1605-2)] that have shown better performance than the resistant reference varieties both in the field and under greenhouse conditions (Table 2.9).

Results in **2012/13** with 22 *B. napus* lines were only obtained from Göttingen and Fehmarn since the plants in Svalöv were lost due to the strong winter (Table 2.10). Regarding the results from Fehmarn, only data from disease index assessments (SDI and RDI) were available and both parameters showed no significant differences among the genotypes. In Göttingen, disease assessment was carried out by disease index scoring and quantification of VL DNA. With both parameters, genotypes displayed significant differences. Regarding SDI, except for the susceptible control cultivar Laser and the DH line DM12-02 that showed a significantly higher SDI than KWS195, the differences among the rest of the reference controls and *B. napus* lines was not significant. Similarly, the only observed significant difference with regard to RDI was between the susceptible cultivar Laser and KWS195.

Interestingly, a better differentiation of the lines was achieved by quantification of VL DNA. In particular, the resistant and susceptible reference varieties showed significant differences. Accordingly, the highest VL DNA was quantified from Laser (1.53ng/g) and this amount was significantly higher than in Oase and Express. VL DNA in cultivar Falcon was also significantly higher than in Express, from which the least amount of VL DNA (0.08ng/g) was quantified. Among the remaining lines, SW12-4, NICK220, NICK221, KWS200, SW08-190002-17 and SYN10091232 had significantly lower VL DNA than both susceptible controls. In general, the observed disease levels in Göttingen were very low resulting in a disease index less than 2.0 (very slight infestation) and VL DNA below 0.5ng/g for most genotypes. Nevertheless, unlike all previously described field results and despite a very low level of infection, data obtained from the 22 *B. napus* lines and 4 reference varieties screened in 2012/13 in Göttingen showed significant correlations between field disease index (SDI and RDI), field VL DNA and greenhouse (NNA and NRS) results (Table 2.11C). One of the possible explanations for the rare significant correlation between field disease index and greenhouse results may possibly be the uniform exposure of all genotypes to the same type and amount of inoculum and the use of artificial inoculation. Considering these correlations, promising lines to be suggested for further research are NICK 221, NICK 219, SYN10091232, SW08-190002-2, DSV, SW12-2, KWS 195, SW08-190002-25 and KWS 187. The ones that showed weak performance comparable to the susceptible controls were DM12-01 and DM12-01 (Table 2.10).

In summary, the field experimental results described in the previous two sections of this chapter (2.4.4.1 and 2.4.4.2) provide evidence that field evaluation of *V. longisporum* resistance in OSR based on assessment of stubble disease index seems to be inconsistent. With this parameter, a high variability among results of the same season trials conducted in different locations was found. Moreover, the same genotype frequently showed considerably different levels of disease in different season's trials of the same location. Furthermore, despite the variation in several agro-ecological

Table 2.8 Response of 22 *Brassica napus* double haploid lines and four reference varieties evaluated for *Verticillium longisporum* resistance under field conditions in the **2010/11** growing season. Comparisons of field performance across locations and field vs greenhouse screening results are shown.

Genotype code	Fehmarn		Göttingen	Svalöv		Greenhouse	
	SDI	RDI^{ns}	(ND)	SDI^{ns}	RDI^{ns}	NNA	NRS
DH 88	2.57+0.0^{ef}	1.46+0.0	-	2.70+0.6	2.48+0.4	1.52+0.7^{bc}	0.90+0.5^{bc}
DH 69	2.68±0.2^{def}	1.50±0.1	-	2.57±0.1	2.56±0.2	0.92±0.4^{fgh}	0.64+0.3^{ab}
DH 179	2.80±0.3^{cdef}	1.60±0.3	-	2.45±0.4	2.50±0.5	1.19±0.8^{cdefg}	0.96+0.5^{fg}
DH 165	2.68±0.5^{def}	1.52±0.2	-	2.82±0.6	2.76±1.1	0.80±0.3^{fghij}	0.62+0.2^{ab}
DH 272	2.50±0.7^{ef}	1.63±0.2	-	2.96±0.1	3.00±0.1	0.94±0.5^{fgh}	0.73+0.4^{fg}
Oase$^{(RC)}$	2.80±0.4^{bcdef}	1.68±0.3	-	2.86±0.8	2.92±0.6	0.24±0.1^{k}	0.27+0.2^{f}
DH 138	2.68±0.1^{def}	1.68±0.0	-	2.04±1.4	1.95±1.3	0.70±0.3^{hij}	0.48+0.2^{cd}
DH 152	3.25±0.2^{abcd}	1.68±0.1	-	3.12±0.5	2.74±0.4	1.85±0.6^{ab}	1.43+0.4^{cd}
DH 126	2.93±0.4^{abcdef}	1.70±0.1	-	2.18±1.1	2.04±1.1	1.42±0.5^{bcd}	1.22+0.4^{ef}
Express$^{(RC)}$	2.85±0.4^{cdef}	1.76±0.1	-	1.28±0.2	1.56±0.1	0.56±0.5^{ijk}	0.68+0.4^{def}
Laser$^{(SC)}$	3.40±0.1^{abcde}	1.78±0.1	-	2.48±1.0	2.44±1.0	0.98±0.5^{efgh}	0.96+0.5^{cd}
DH 123	3.23±0.2^{abcd}	1.78±0.1	-	2.28±0.9	2.25±0.8	1.58±0.6^{bc}	1.36+0.4^{ab}
DH 290	3.23±0.2^{abcd}	1.78±0.4	-	2.99±0.2	3.04±0.1	1.84±0.4^{ab}	1.43+0.4^{ab}
DH 196	2.47±0.3^{f}	1.80±0.2	-	2.93±0.2	2.68±0.2	0.74±0.3^{ghij}	0.45+0.2^{def}
DH 294	3.06±0.1^{abcde}	1.80±0.2	-	2.12±0.4	2.00±0.2	0.49±0.3^{jk}	0.47+0.2^{def}
DH 359	2.52±0.3^{ef}	1.80±0.1	-	1.91±0.7	1.86±0.5	1.05±0.5^{defgh}	0.69+0.3^{de}
Falcon$^{(SC)}$	3.07±0.0^{ab}	1.82±0.1	-	2.78±0.5	2.28±0.2	1.44±0.7^{bcde}	1.32+0.6^{ab}
DH 101	2.88±0.3^{abcdef}	1.82±0.1	-	2.10±1.0	2.09±0.8	1.47±0.3^{bc}	0.67+0.3^{fg}
DH 108	2.83±0.2^{bcdef}	1.82±0.1	-	3.24±0.2	3.12±0.1	1.02±0.3^{defgh}	0.52+0.1^{def}
DH 282	3.45±0.3^{a}	1.82±0.1	-	2.64±0.3	2.40±0.2	1.14±0.3^{cdef}	0.98+0.3^{a}
DH 16	3.27±0.4^{abc}	1.86±0.0	-	3.00±0.6	3.00±0.4	2.42±0.6^{a}	1.63+0. 3^{fg}
DH 24	3.19±0.1^{abcd}	1.87±0.1	-	2.60±0.3	2.51±0.2	2.20±0.7^{a}	1.47+0.5^{def}
DH 7	3.21±0.0^{abcd}	1.88±0.1	-	3.09±0.2	2.88±0.1	0.96±0.7^{fgh}	0.46+0.7^{def}
DH 140	3.29±0.4^{abc}	1.88±0.3	-	2.12±1.4	2.08±1.1	0.84±0.4^{fghi}	0.73+0.4^{fg}
DH 118	3.19±0.3^{abcd}	1.94±0.1	-	3.36±0.3	3.06±0.1	0.88±0.4^{fgh}	0.73+0.3^{fg}
DH 141	3.03±0.0^{abcdef}	2.04±0.2	-	3.22±0.1	3.16±0.1	0.81±0.2^{fghi}	0.55+0.1^{def}

SDI, shoot disease index. **RDI**, root disease index. **NNA**, net normalized AUDPC. **NRS**, normalized relative stunting. **ND**, no data due to loss of plants by hard winter. **(RC)**, resistant control. **(SC)**, susceptible control. For all parameters mean ± standard deviation data is shown. Greenhouse results are mean of 60 plants derived from three independent experiments. Genotypes are sorted according to Fehmarn SDI results that showed significant correlation with GH NRS results. For each parameter, means followed by the same letter are not significantly different at p=0.05. Mean values under parameters indicated by the superscript '**ns**' are not significantly different at p=0.05.

Table 2.9 Response of 22 *Brassica napus* double haploid lines and four reference varieties evaluated for *Verticillium longisporum* resistance under field conditions in the **2011/12** growing season. Comparisons of field performance across locations and field vs greenhouse screening results are shown.

Genotype code	Fehmarn			Göttingen	Svalöv			Greenhouse	
	SDI^{ns}	RDI^{ns}	VLDNA	(ND)	SDI^{ns}	RDI^{ns}	VLDNA	NNA	NRS
DSV-DH-Ver-8(1605-2)	1.9±0.2	2.0±0.2	0.18±0.1^{d}	-	1.37±0.5	1.36±0.6	2.17±2.5^{abcdefg}	0.64±0.3^{j}	0.03±0.5^{bcde}
DSV-DH-Ver-7(1575-2)	1.9±0.5	1.7±0.3	0.23±0.1^{cd}	-	1.59±0.4	1.32±0.2	2.05±2.8^{fghi}	0.72±0.4^{defgh}	0.46±0.5^{bc}
DSV-DH-Ver-10(1605-2)	1.7±0.1	1.7±0.0	0.39±0.1^{cd}	-	1.16±0.2	1.16±0.2	1.19±1.4^{cdefgh}	0.13±0.1^{fghij}	0.18±0.2^{g}
DSV-DH-Ver-1(1575-1)	2.4±0.2	2.3±0.4	0.40±0.4^{cd}	-	1.05±0.0	1.05±0.0	0.39±0.3^{defghi}	0.41±0.2^{bcde}	0.68±0.4^{ef}
DSV-DH-Ver-3(1575-2)	2.5±0.6	2.1±0.4	0.41±0.5^{cd}	-	1.84±0.4	1.67±0.3	0.61±0.5^{cdefghi}	0.48±0.3^{abc}	1.00±0.4^{cdef}
DSV-DH-Ver-11(1605-2)	1.9±0.5	1.8±0.4	0.52±0.0^{cd}	-	-	-	2.29±2.3^{abcdef}	0.08±0.3^{ij}	0.05±0.2^{gh}
DSV-DH-Ver-14(1605-2)	2.8±0.1	2.6±0.2	0.52±0.0^{cd}	-	1.17±0.1	1.11±0.1	0.08±0.1^{i}	-0.63±0.3^{hij}	0.09±0.4^{lm}
DSV-DH-Ver-12(1605-2)	2.1±0.1	2.0±0.1	0.54±0.4^{cd}	-	1.26±0.2	1.43±0.2	0.33±0.3^{efghi}	0.30±0.1^{fghij}	0.23±0.3^{fg}
DSV-DH-Ver-4(1575-3)	2.0±0.2	1.9±0.2	0.56±0.0^{cd}	-	1.26±0.1	1.32±0.6	1.20±1.1^{bcdefgh}	0.26±0.2^{bcde}	0.70±0.5^{fg}
DSV-DH-Ver-6(1575-1)	2.4±0.3	2.2±0.2	0.56±0.5^{cd}	-	1.20±0.0	1.00±0.0	2.47±2.1^{abcde}	0.45±0.4^{defg}	0.48±0.6^{def}
DSV-DH-Ver-18(1605-2)	2.6±1.0	2.5±0.8	0.72±0.0^{cd}	-	1.29±0.5	1.38±0.6	0.49±0.5^{cdefghi}	-0.45±0.2^{efghi}	0.30±0.4^{jkl}
Express$^{(RC)}$	2.3±0.0	2.2±0.1	0.81±0.5^{cd}	-	1.44±0.4	1.77±0.6	3.89±1.6^{abc}	0.84±0.1^{defg}	0.44±0.3^{b}
DSV-DH-Ver-5(1575-1)	2.2±0.1	2.2±0.2	0.95±0.9^{cd}	-	1.30±0.1	1.20±0.1	0.13±0.0^{hi}	0.59±0.3^{bcd}	0.84±0.5^{bcde}
DSV-DH-Ver-15(1605-2)	2.2±0.5	2.2±0.4	0.97±1.0^{cd}	-	1.58±0.4	1.39±0.3	1.12±1.2^{cdefgh}	-0.53±0.3^{defg}	0.47±0.3^{kl}
DSV-DH-Ver-13(1605-1)	2.2±0.3	2.0±0.2	1.05±0.9^{cd}	-	2.04±0.3	2.96±0.9	7.87±2.8^{ab}	0.42±0.2^{efgh}	0.39±0.5^{def}
DSV-DH-Ver-20(1605-3)	1.8±0.0	2.1±0.0	1.15±0.8^{cd}	-	1.42±0.5	1.30±0.6	0.43±0.2^{cdefghi}	-0.83±0.4^{ghij}	0.16±0.3^{m}
DSV-DH-Ver-2(1575-2)	2.0±0.2	2.2±0.2	1.21±0.6^{cd}	-	1.21±0.1	1.21±0.1	3.29±3.7^{abcde}	0.42±0.1^{bcd}	0.78±0.2^{def}
DSV-DH-Ver-22(1605-2)	1.9±0.1	1.8±0.2	1.26±0.0^{cd}	-	1.29±0.1	1.27±0.1	0.14±0.0^{ghi}	-0.16±0.3^{efgh}	0.37±0.4^{hi}
DSV-DH-Ver-17(1605-2)	2.3±0.0	2.1±0.1	1.29±1.0^{cd}	-	1.34±0.1	1.64±0.2	4.40±4.9^{abcd}	-0.21±0.3^{a}	1.31±0.4^{ij}
DSV-DH-Ver-19(1605-2)	2.2±0.0	2.0±0.1	1.42±0.8^{bcd}	-	1.39±0.1	1.39±0.2	0.27±0.1^{efghi}	-0.35±0.2^{ab}	1.03±0.5^{ijk}
Oase$^{(RC)}$	2.2±0.2	2.3±0.4	1.75±1.2^{cd}	-	1.34±0.2	1.20±0.6	3.27±3.2^{abcde}	0.65±0.2^{defg}	0.48±0.3^{bcde}
DSV-DH-Ver-9(1605-2)	2.4±0.3	2.4±0.2	1.78±0.3^{abc}	-	1.57±0.3	1.70±0.3	0.81±0.8^{cdefgh}	0.68±0.3^{cdef}	0.60±0.5^{bcd}
DSV-DH-Ver-16(1605-2)	2.0±0.1	2.2±0.5	1.99±2.8^{cd}	-	1.34±0.2	1.38±0.2	0.43±0.5^{efghi}	-0.60±0.3^{fghij}	0.20±0.4^{klm}
DSV-DH-Ver-21(1605-1)	2.4±0.3	2.3±0.3	2.09±2.6^{cd}	-	1.37±0.2	1.36±0.2	0.06±0.0^{i}	-0.85±0.3^{K}	-0.42±0.3^{m}
Falcon$^{(SC)}$	2.3±1.1	2.3±1.2	9.77±5.7^{ab}	-	1.49±0.5	1.65±0.5	7.97±1.8^{ab}	1.16±0.6^{a}	1.56±1.1^{a}
Laser$^{(SC)}$	2.1±0.4	2.0±0.4	11.4±6.6^{a}	-	1.68±0.2	1.74±0.3	8.82±0.9^{a}	1.10±0.3^{abc}	1.04±0.8^{a}

SDI, Shoot disease index. **RDI**, Root disease index. **NNA**, Net normalized AUDPC. **NRS**, Normalized relative stunting. **VLDNA**, *Verticillium longisporum* DNA (ng/g) in hypocotyl at BBCH80. **ND**, no data due to loss of plants by hard winter. **(RC)**, resistant control. **(SC)**, susceptible control. For all parameters mean ± standard deviation data is shown. Greenhouse results are mean of 60 plants derived from three independent experiments. Genotypes are sorted according to Fehmarn VLDNA quantification results that showed significant correlation with Svalöv field, GH and OD screening results. For each parameter, means followed by the same letter are not significantly different at p=0.05. Mean values under parameters indicated by the superscript '**ns**' are not significantly different at p=0.05.

Table 2.10 Response of 22 *Brassica napus* accessions and four reference varieties evaluated for *Verticillium longisporum* resistance under field conditions in the **2012/13** growing season. Comparisons of field performance across locations and field vs greenhouse screening results are shown.

Genotype code	Fehmarn		Göttingen			Svalöv	Greenhouse	
	SDI^{ns}	RDI^{ns}	SDI	RDI	VLDNA	(ND)	NNA	NRS
Express$^{(RC)}$	1.79±0.5	1.90±0.1	1.25±0.2^{ab}	1.17±0.1^{ab}	0.08±0.08^{e}	-	1.1±0.8^{bcd}	1.1±0.5^{abcd}
SW 12-4	2.24±0.4	1.88±0.2	1.24±0.2^{ab}	1.14±0.1^{ab}	0.15±0.12^{de}	-	0.7±0.2^{defg}	0.9±0.1^{cdef}
NICK 220	2.65±0.4	2.6±20.5	1.21±0.1^{ab}	1.13±0.1^{ab}	0.17±0.11^{de}	-	0.7±0.2^{defg}	0.4±0.1^{k}
NICK 221	1.90±0.3	1.90±0.1	1.20±0.2^{ab}	1.13±0.1^{ab}	0.17±0.13^{de}	-	0.9±0.4^{cde}	0.6±0.3^{hijk}
KWS 200	2.31±0.3	2.54±0.7	1.16±0.1^{ab}	1.15±0.1^{ab}	0.18±0.14^{e}	-	0.7±0.2^{defg}	0.6±0.2^{hijk}
NICK 219	2.03±0.3	1.74±0.4	1.20±0.2^{ab}	1.17±0.2^{ab}	0.18±0.09^{bcde}	-	0.8±0.3^{cde}	0.9±0.2^{defg}
SYN 10091232	2.16±0.8	2.70±1.8	1.06±0.1^{ab}	1.07±0.1^{ab}	0.20±0.23^{de}	-	0.3±0.2^{i}	0.5±0.2^{jk}
DM 12-02	2.33±0.1	2.56±0.6	1.51±0.2^{a}	1.46±0.2^{ab}	0.21±0.04^{bcde}	-	1.4±0.4^{a}	1.3±0.4^{ab}
DSV 1	2.27±0.2	2.60±0.6	1.40±0.2^{ab}	1.26±0.1^{ab}	0.21±0.21^{bcde}	-	0.5±0.5^{ghi}	0.6±0.4^{jk}
SW 08-190002-2	1.78±0.5	1.82±0.3	1.10±0.0^{ab}	1.19±0.1^{ab}	0.21±0.21^{bcde}	-	0.8±0.3^{cde}	0.5±0.3^{efghi}
SYN 09033483	2.39±0.2	2.34±0.9	1.16±0.1^{ab}	1.18±0.1^{ab}	0.23±0.29^{cde}	-	0.9±0.3^{bcd}	1.0±0.2^{bcde}
DSV 3	2.41±0.9	2.88±1.6	1.20±0.1^{ab}	1.18±0.1^{ab}	0.29±0.23^{bcde}	-	0.6±0.5^{efgh}	0.7±0.5^{ghij}
KWS 187	2.10±0.4	2.44±0.8	1.04±0.1^{ab}	1.12±0.2^{ab}	0.30±0.32^{bcde}	-	0.4±0.3^{hi}	0.6±0.2^{ijk}
SW 08-190002-17	2.06±0.1	2.30±0.4	1.13±0.1^{ab}	1.13±0.0^{ab}	0.31±0.34^{de}	-	0.8±0.3^{cde}	0.8±0.3^{jk}
SW 12-1	1.94±0.2	2.08±0.1	1.28±0.1^{ab}	1.22±0.2^{ab}	0.33±0.48^{bcde}	-	0.6±0.3^{efgh}	0.8±0.2^{defgh}
SYN 11091465	2.23±0.4	1.92±0.1	1.29±0.1^{ab}	1.20±0.1^{ab}	0.33±0.22^{bcde}	-	1.0±0.5^{bcd}	1.0±0.4^{bcde}
DSV 2	2.23±0.6	3.00±1.4	1.10±0.1^{ab}	1.12±0.1^{ab}	0.34±0.36^{bcde}	-	0.8±0.6^{def}	0.8±0.2^{defgh}
SW 12-2	2.26±0.4	2.00±0.0	1.08±0.1^{ab}	1.09±0.2^{ab}	0.34±0.26^{bcde}	-	0.5±0.3^{fghi}	1.0±0.3^{bcde}
Oase$^{(RC)}$	1.34±0.4	1.40±0.6	1.01±0.0^{ab}	1.03±0.0^{ab}	0.37±0.63^{cde}	-	0.4±0.1^{hi}	0.7±0.2^{fghij}
SW 12-3	1.89±0.3	1.88±0.2	1.13±0.1^{ab}	1.11±0.1^{ab}	0.38±0.44^{bcde}	-	0.7±0.3^{cdef}	0.9±0.2^{cdef}
SW 08-190001-10	1.59±0.2	1.94±0.1	1.18±0.1^{ab}	1.21±0.1^{ab}	0.41±0.42^{bcde}	-	0.9±0.2^{bcd}	0.7±0.1^{ghij}
KWS 195	2.07±0.4	2.38±0.9	1.00±0.0^{b}	1.01±0.0^{b}	1.31±0.87^{ab}	-	0.5±0.3^{efghi}	0.8±0.2^{efghi}
Falcon$^{(SC)}$	2.18±0.5	2.68±0.5	1.31±0.2^{ab}	1.36±0.1^{ab}	1.47±1.78^{abc}	-	1.5±0.5^{a}	1.3±0.3^{a}
SW 08-190002-25	2.04±0.1	2.16±0.2	1.11±0.1^{ab}	1.11±0.1^{ab}	1.50±1.50^{abcd}	-	0.4±0.4^{hi}	0.8±0.3^{efghi}
DM 12-01	2.21±0.2	2.48±0.7	1.62±0.4^{ab}	1.46±0.3^{ab}	1.76±3.15^{abcd}	-	1.3±0.4^{ab}	1.2±0.3^{abc}
Laser$^{(SC)}$	1.98±0.1	1.96±0.1	1.68±0.4^{a}	1.53±0.2^{a}	10.37±16.07^{a}	-	1.1±0.5^{abc}	1.0±0.4^{bcde}

SDI, shoot disease index. **RDI**, root disease index. **NNA**, net normalized AUDPC. **NRS**, normalized relative stunting. **VLDNA**, *Verticillium longisporum* DNA (ng/g) in hypocotyl at BBCH80. **ND**, no data due to loss of plants by hard winter. **(RC)**, resistant control. **(SC)**, susceptible control. For all parameters mean ± standard deviation data is shown. Greenhouse results are mean of 60 plants derived from three independent experiments. Genotypes are sorted according to Göttingen VLDNA quantification results that showed significant correlation with field screening results. For each parameter, means followed by the same letter are not significantly different at p=0.05. Mean values under parameters indicated by the superscript '**ns**' are not significantly different at p=0.05.

Table 2.11 Correlation analyses between field and greenhouse screening results of *Brassica napus* lines evaluated under field conditions for individual seasons (years).

A. Correlation between field and greenhouse results of 22 *Brassica napus* double haploid lines and four reference varieties screened during **2010/11** field trial.

	Fehmarn SDI	Fehmarn RDI	Svalöv SDI	Svalöv RDI	GH NNA
Fehmarn RDI	**0.55****				
Svalöv SDI	0.15^{ns}	0.15^{ns}			
Svalöv RDI	0.10^{ns}	0.12^{ns}	**0.95*****		
Göttingen (ND)	-	-	-	-	-
GH NNA	0.38^{ns}	0.07^{ns}	0.20^{ns}	0.15^{ns}	
GH NRS	**0.51****	0.06^{ns}	0.09^{ns}	0.03^{ns}	**0.90*****

B. Correlation between field and greenhouse results of 22 *Brassica napus* double haploid lines and four reference varieties screened during **2011/12** field trial.

	Fehmarn SDI	Fehmarn RDI	Fehmarn VLDNA	Svalöv SDI	Svalöv RDI	Svalöv VLDNA	GH NNA
Fehmarn RDI	**0.73*****						
Fehmarn VLDNA	-0.35^{ns}	-0.31^{ns}					
Svalöv SDI	-0.01^{ns}	-0.06^{ns}	-0.09^{n}s				
Svalöv RDI	-0.01^{ns}	0.06^{ns}	-0.09^{ns}	**0.99*****			
Svalöv VLDNA	**-0.52****	**-0.50****	**0.70*****	0.02^{ns}	0.02^{ns}		
Göttingen (ND)	-	-	-	-	-	-	-
GH NNA	**-0.68*****	**-0.88*****	**0.40***	-0.03^{ns}	-0.05^{ns}	**0.59****	
GH NRS	**-0.48***	**-0.48***	**0.50***	-0.22^{ns}	-0.22^{ns}	**0.48***	**0.50***

C. Correlation between field and greenhouse results of 22 *Brassica napus* accessions and four reference varieties screened during **2012/13** field trial.

	Fehmarn SDI	Fehmarn RDI	Göttingen SDI	Göttingen RDI	Göttingen VLDNA	GH NNA
Fehmarn RDI	**0.71*****					
Göttingen SDI	0.22^{ns}	0.10^{ns}				
Göttingen RDI	0.16^{ns}	0.15^{ns}	**0.94*****			
Göttingen VLDNA	-0.08^{ns}	-0.10^{ns}	**0.58****	**0.58****		
Svalöv (ND)	-	-	-	-	-	-
GH NNA	0.10^{ns}	0.07^{ns}	**0.68*****	**0.77*****	0.26^{ns}	
GH NRS	0.07^{ns}	-0.04^{ns}	**0.53****	**0.58****	0.24^{ns}	**0.73*****

Pearson correlation analysis was performed using product-moment procedure of STATISTICA (Version 10 StatSoft, inc. 2011). For each season analysis, data obtained from 22 lines and 4 reference varieties were used. Minus signs indicate negative correlations. Significant correlations are indicated in **bold** font. ***,** significant at P=0.05. ****,** significant at P=0.01. *****,** significant at P=0.001. **ns**, not significant. **GH**, greenhouse experiment. **OD**, outdoor experiment. **SDI**, shoot disease index. **RDI**, root disease index. **VLDNA**, *Verticillium longisporum* DNA (ng/g) in hypocotyl. **NNA**, net normalized AUDPC. **NRS**, normalized net relative stunting. **ND**, no data due to loss of plants by hard winter.

conditions across locations or seasons, in most cases (experiments) disease index results were hardly or only occasionally correlated with field qPCR as well as with NNA and NRS results from the greenhouse. Besides this, again in most cases the disease index showed no significant differences among genotypes, even between the well-known resistant and susceptible varieties. Understanding disease threshold levels by stubble rating at or after crop maturity stage (after grain harvest) is not useful anymore, particularly with regard to taking immediate control action. Because, as plants have already attained the maximum stage of development, application of any possible control measure (such as fungicide spray) at this time point may not be economical. Nevertheless, this does not mean that the information obtained from stubble disease index assessment is completely irrelevant. It rather provides valuable information on the amount of pathogen resting propagules (microsclerotia) in a certain field. Estimating the amount of inoculum in the soil is one of the basic and most important inputs required for forecasting disease pressure and this information will help to plan future production schemes such as decision making in crop rotation programs. In addition, the method can still be considered as one alternative of disease assessment in resistance screening studies.

The other alternative for measurement of VL disease severity in field-grown OSR plants is quantification of fungal biomass by qPCR. According to the results of the present multi-site study conducted in multiple seasons, it was found that this method provides more reliable information at a relatively earlier time point (plant growth stage). Furthermore, the results obtained from this parameter were mostly correlated with the results in controlled environments. More importantly, with this method, it was possible to detect even very slight levels of infection (up to few picograms of fungal DNA per gram of plant tissue). In conclusion, quantification of *V. longisporum* biomass by qPCR seems to be the most consistent, fast and possibly cheaper alternative for field disease survey or evaluation of resistance in OSR. Nevertheless, identifying the right time point (crop growth stage) at which VL can specifically and precisely be detected or quantified from field grown plants is crucial. From this knowledge, further extrapolation of future disease epidemics (ahead of crop maturity) is possible and this helps timely decision making on selection and application of possible disease control measures. This topic is partly addressed in the following chapter.

2.3.2.3 Determination of the critical crop growth stage for molecular detection and differential quantification of *Verticillium longisporum* disease severity in field grown winter oilseed rape plants

In order to perform timely application of appropriate control measures, early detection and accurate quantification of VL infection in OSR production is very important. With this rationale, the present experiment was conducted with the objective of identifying the critical crop developmental growth stage for detection of *V. longisporum* infection from stem tissues of field grown winter OSR plants using qPCR. Furthermore, verifying the feasibility of this method as an alternative for assessment of VL disease severity or VL resistance in OSR was the focus of this experiment. For this purpose, stem samples of resistant and susceptible OSR cultivars from multi-locational field trials were collected at three growth stages (BBCH65, BBCH70 and BBCH80). The growth stages represent full flowering, fruit development and beginning of ripening stages, respectively. The amount of VL biomass in the DNA samples extracted from stem samples were quantified based on a standard curve (Fig. 2.5A) produced from known concentrations of VL genomic DNA. PCR amplification was performed using gene specific primer pairs targeting a 261bP ribosomal ITS (internal transcribed spacer) region in the *V. longisporum* genome (Knüfer, 2013). Furthermore, since the analysis was done on DNA samples extracted from field grown plants samples potentially harboring multiple pathogen species, specificity of PCR reaction was verified by melting curve analysis.

PCR results show high amplification efficiency (Fig. 2.5A) and a single melting peak (Fig. 2.5B) indicating the amplification of a single and specific DNA sequence. qPCR results revealed that irrespective of experimental locations, VL infection can be detected at growth stage BBCH65 (50% flowering). However, fungal biomass in stem tissue at this growth stage was extremely low and consequently, no significant difference was found between susceptible and resistant varieties. At BBCH70, relatively higher (than BBCH65) fungal biomass was quantified from both susceptible and resistant materials. Nevertheless, irrespective of experimental locations, no significant difference between resistant and susceptible materials was observed until this time point which marks the stage of pod development. At BBCH80, unlike earlier time points, relatively higher fungal biomass which significantly differed between resistant and susceptible materials was found (Fig. 2.6).

At this time point, VL DNA in the resistant cultivars Oase and Express in Göttingen (Fig. 2.6A) was 0.083 and 0.370ng/g, respectively. In this location, the highest amount of VL DNA (10.4ng/g) which is significantly higher than the amounts detected in both resistant varieties was quantified from the

susceptible Laser. In cultivar Falcon, 1.47ng/g which was significantly more than in Oase was found (Fig. 2.6A).

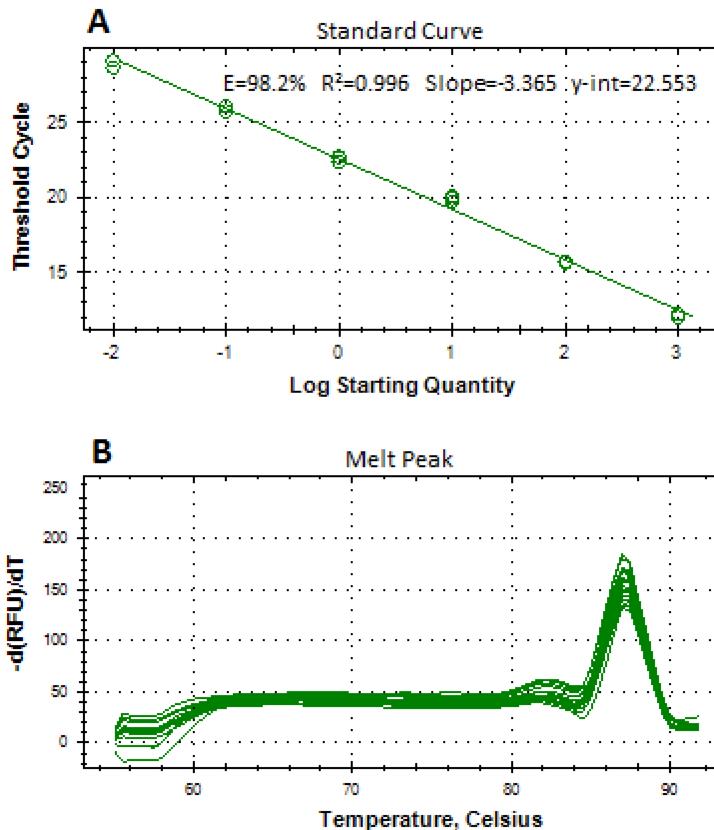


Figure 2.5 Standard curve (A) and melting peak (B) from real-time PCR amplification of tenfold dilution series of *Verticillium longisporum* genomic DNA. PCR was performed using ABsolute Blue SYBR Green Fluorescein Mix in a CFX-384 real-time PCR system (Bio-Rad laboratories, Inc).

In Fehmarn (Fig. 2.6B), the highest level of infection (79.9ng) at BBCH80 which was significantly higher than all other varieties was found in Falcon. Regarding the other susceptible cultivar Laser, even though relatively high VL biomass (22.0ng/g) was quantified, this amount was not significantly different from the quantity detected in the resistant material Oase (5.9ng/g) or Express (13.3ng/g).

In Svalöv (Fig. 2.6C) compared to the resistant cultivars, more than 300fold VL DNA was quantified from the susceptible varieties. Accordingly, the highest infestation was found in Laser (3252.6ng/g) and this amount was significantly higher than the amount quantified from Oase (21.3ng/g) and Express (27.0ng/g). In cultivar Falcon, even though statistically insignificant, a still quite higher value (554.3ng/g) than in both resistant varieties was found.

The other important observation in this particular experiment was the variation of VL disease severity across locations. In general, the relatively highest infestation occurred in Svalöv followed by Fehmarn. In Göttingen, where the experiment was conducted with addition of inoculum (section 2.2.2.4), infestation levels were extremely low (Fig. 2.6). For instance, infection levels as measured by qPCR at BBCH80 were 7 (in Oase), 36 (in Express), 221 (in Laser), and 54 (in Falcon) fold higher VL DNA in Fehmarn compared

to Göttingen. Similarly, compared to Göttingen, the respective increase in Svalöv were 26, 73, 313 and 377 fold in cultivars Oase, Express, Laser and Falcon, respectively. When the two locations with relatively higher disease infestation were compared with each other, a 4 (in Oase), 2 (in Express), 142 (in Laser) and 7 (in Falcon) fold amount of VL DNA was quantified in Svalöv compared to Fehmarn. Furthermore, despite very low levels of infestations across all locations at earlier growth stages (BBCH65 and BBCH70), qPCR results still showed a relatively higher fungal biomass in Fehmarn and Svalöv than in Göttingen (Fig. 2.6). The above described local variations in VL disease infestations was consistent with disease index assessment results of previous studies conducted with other sets of oilseed rape genotypes (Eynck, 2008; Knüfer, 2013).

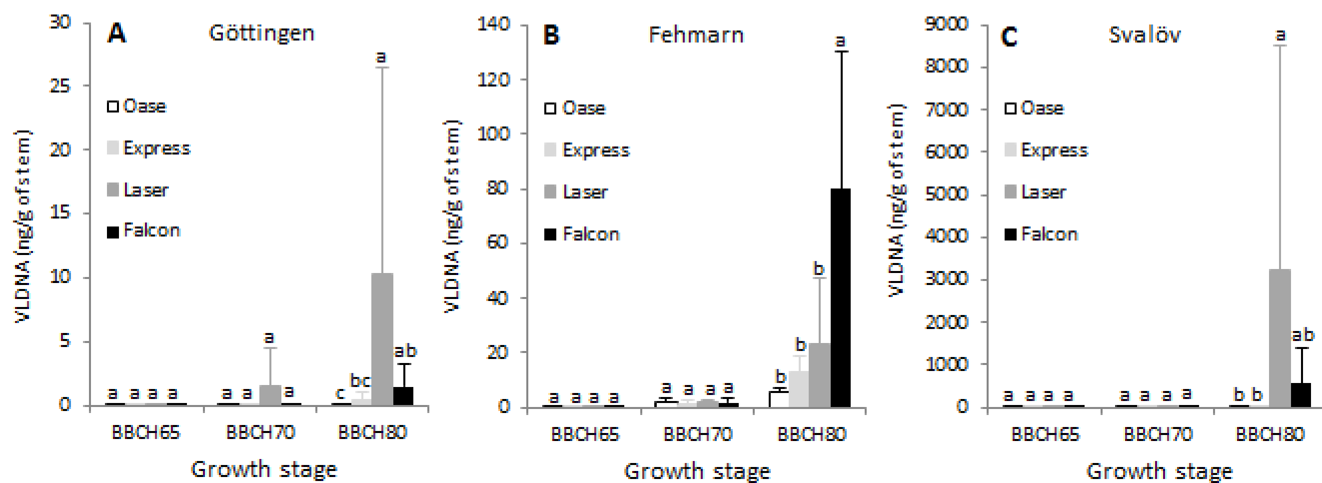


Figure 2.6 qPCR analysis of *Verticillium longisporum* infection at different developmental stages of resistant and susceptible winter oilseed rape (*Brassica napus*) varieties grown under field conditions in 2012/13. Mean data obtained from four biological replicates of a DNA sample extracted from 10 pooled stems per replicate is shown. Bars represent standard deviations of biological replicates. For each developmental stage, means followed by the same letter are not significantly different at p=0.05. **VLDNA**, *Verticillium longisporum* DNA.

One of the possible explanations for the locational differences in VL disease levels could be the presence of variable climatic and soil conditions (Table 2.2). The amount of inoculum in the soil which is positively associated with long tradition of intensive oilseed rape cultivation in Svalöv (Johansson et al., 2006) and Fehmarn (Eynck, 2008) might be also among the most determining factors that contributed for the high levels of infestation in these locations. Also, the contribution of possible variations in composition, diversity or abundance of aggressive *V. longisporum* isolates among the different locations should not be ignored or underestimated. In general, the information gathered from stubble disease index assessment seems insufficient particularly with regard to immediate decision making on the use of possible control measures. This is mainly because plants at this stage have already attained the maximum stage of development and taking any action at this time point may not be economical.

Nevertheless, determining disease levels on stubble samples provide reliable information about the amount of VL microsclerotia that join the soil bank and this is the major input required for forecasting disease epidemics. This intern helps early decision-making regarding choice of control options such as selection of resistant varieties, crop rotation schemes and possible use of pre emergence fungicides.

2.3.3 Outdoor experiment

As described previously, the major objective of this experiment was to find out the causes of variations between field and greenhouse *V. longisporium* symptoms in oilseed rape (particularly stunting and excessive branching). The study was conducted based on the assumption that differences in climatic factors, type of inoculum or inoculation methods are among the major factors contributing for the above mentioned variations in symptoms. To investigate possible effects of these factors, the outdoor experiment was conducted using the same type of inoculum and an identical inoculation method as in the greenhouse experiments. To mimic field conditions, inoculated plants were transplanted to pots and grown in outdoor conditions where they experienced similar climatic conditions as field-grown plants. Details of the methodologies are found in section 2.2.3.2 and 2.2.3.3.

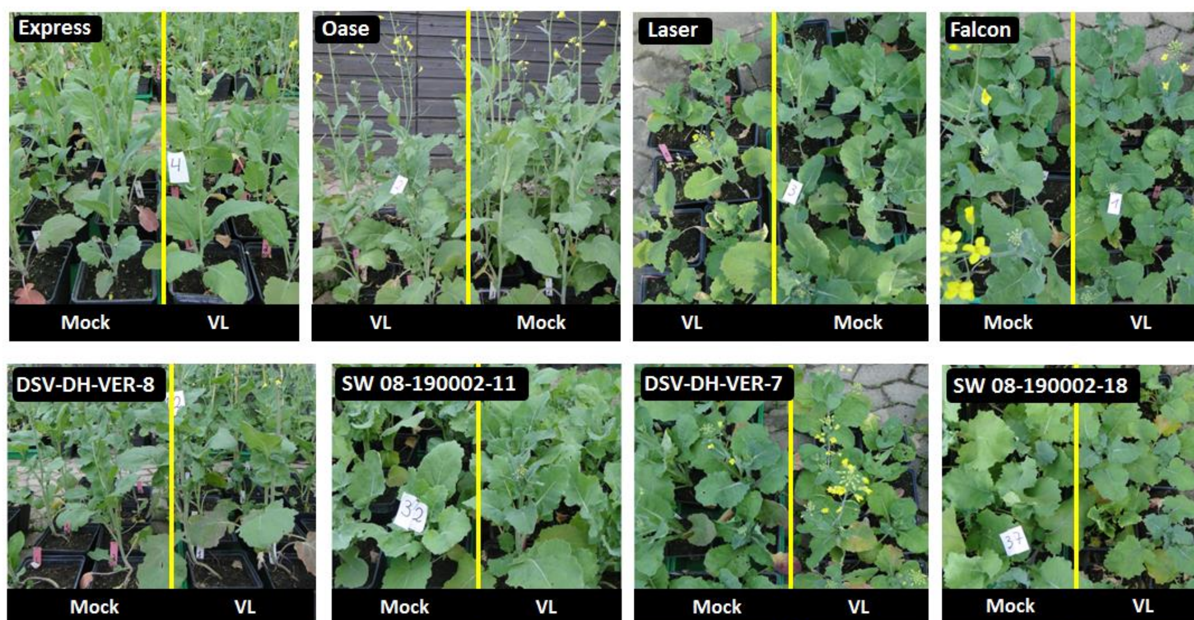


Figure 2.7 Phenotypes of mock and VL infected *Brassica napus* reference varieties (Top) and representative double haploid lines (bottom) showing resistance and susceptible responses towards *Verticillium longisporum* infection in the outdoor experiment at 49DPI. Inoculation of vernalized seedlings (BBCH14) was performed following a standard cut-root inoculation method. **Mock**, control plants infected with sterile water. **VL**, infected with *Verticillium longisporum*. Express and Oase are resistant reference varieties. Laser and Falcon were used as susceptible references. DH lines DSV-DH-VER-8 and SW08-190002-11 indicate resistance response while lines DSV-DH-VER-7 and SW-08-190002-18 show susceptible reactions. Labels shown in white tags in each genotype are plot numbers.

Disease assessment (net AUDPC) results showed considerable differences between susceptible and resistant reference varieties. The remaining 36 DH lines tested in this experiment also showed a wide range of performances (Fig. 2.8A). Phenotypes of reference varieties and four lines representing susceptible and resistant individuals of a DH population are shown in Fig. 2.7. Except for few lines, severe stunting was observed in most genotypes that had high net AUDPC values (Fig. 2.8B).

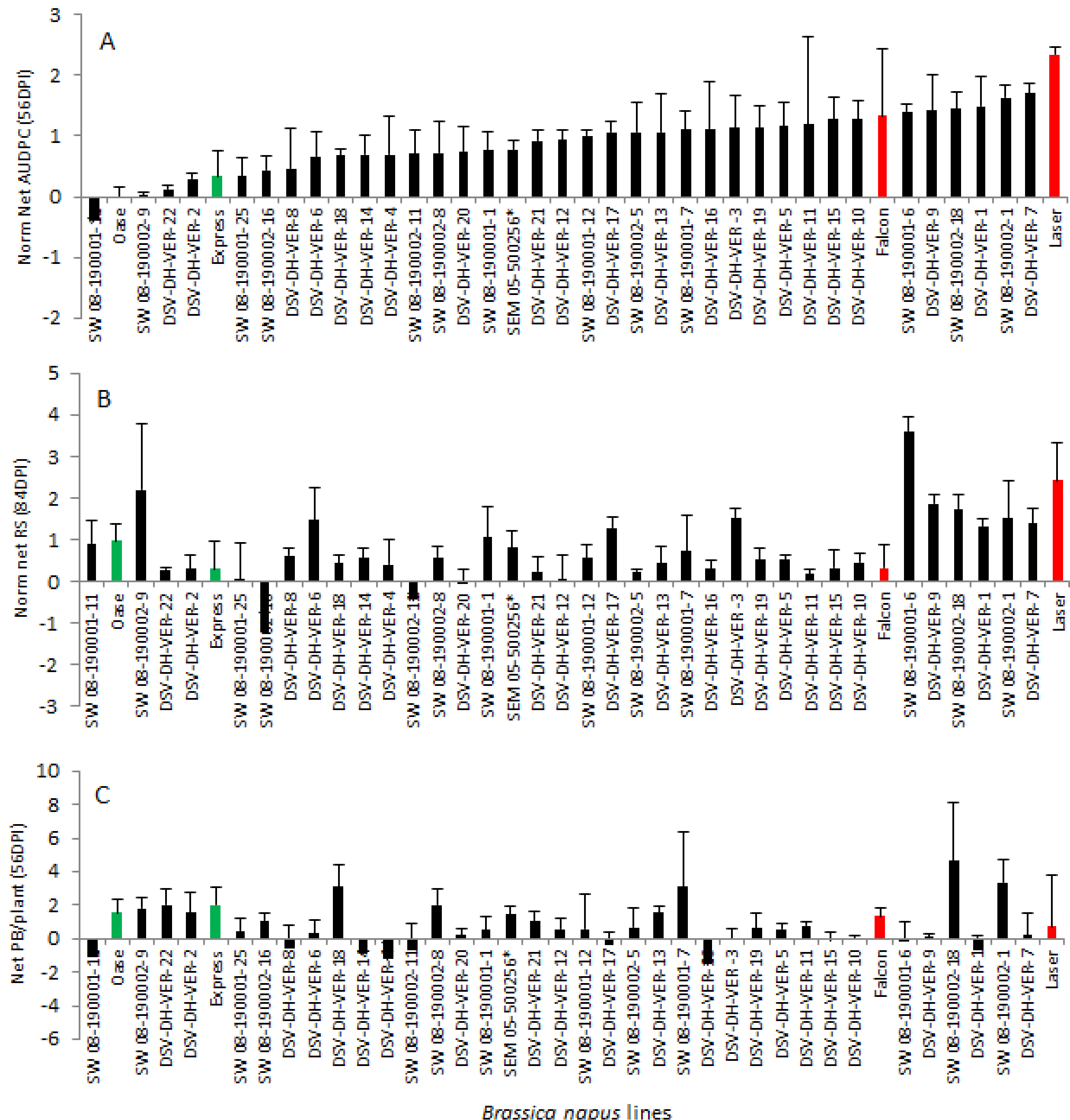


Figure 2.8 Normalized net AUDPC (A), Normalized net relative stunting (B) and net primary branches per plant (C) measured from *Brassica napus* double haploid lines screened for *Verticillium longisporum* resistance in the outdoor experiment at 56DPI. **AUDPC**, Area under disease progress values. **RS**, relative stunting. **PB**, primary branches. Green and red bars represent values of resistant and susceptible controls, respectively. *****, not a double haploid line. For all parameters mean +standard deviation data obtained from three biological replicates is shown. Genotypes are sorted according to increasing net AUDPC values.

With regard to the branching phenotype, it was found that upon infection with VL, some lines (37.5% of the total) produced at least one additional primary branch per plant compared to uninfected plants. On the other hand, in a few other DH lines (7.5% of the total), infection reduced branching. For a considerable proportion (55% of the total), VL infection had no effect at all (Fig. 2.8C). This result suggests that the characteristic excessive branching usually observed in infected plants is not exclusively related to either susceptible or resistant reactions of OSR to VL.

Table 2.12 Correlation between outdoor performance *vs* field and greenhouse screening results of *Brassica napus* double haploid lines evaluated for resistance against *Verticillium longisporum*.

	Fehmarn (2012)			Svalov (2012)			Greenhouse		Outdoor		
	SDI	RDI	VLDNA	SDI	RDI	VLDNA	NNA	NRS	NGS	NPBPP	NRS
OD NNA	-0.15^{ns}	-0.21^{ns}	**0.37***	-0.016^{ns}	-0.01^{ns}	0.24^{ns}	-0.08^{ns}	0.18^{ns}	-0.03^{ns}	0.01^{ns}	**0.38***
OD NRS	-0.07^{ns}	0.047^{ns}	0.18^{ns}	0.09^{ns}	-0.01^{ns}	0.04^{ns}	0.09^{ns}	0.32^{ns}	**-0.37***	0.06^{ns}	
OD PBPP	-0.09^{ns}	-0.13^{ns}	0.01^{ns}	-0.12^{ns}	-0.13^{ns}	-0.03^{ns}	0.11^{ns}	0.05^{ns}	-0.18^{ns}		
OD NGS	-0.08^{ns}	-0.26^{ns}	-0.14^{ns}	-0.19^{ns}	-0.19^{ns}	-0.08^{ns}	-0.24^{ns}	-0.18^{ns}			

Pearson correlation analysis was performed using product-moment procedure of STATISTICA (Version 10 StatSoft, inc. 2011). Data obtained from 36 lines and 4 reference varieties were used. Minus signs indicate negative correlations. Significant correlations are indicated in **bold** font. ***,** significant at P=0.05. ****,** significant at P=0.01. *****,** significant at P=0.001. **ns**, not significant. **GH**, greenhouse experiment. **OD**, outdoor experiment. **SDI**, field shoot disease index. **RDI**, field root disease index. **VLDNA**, *Verticillium longisporum* DNA in hypocotyl. **NNA**, net AUDPC. **NRS**, normalized relative stunting. **NPBPP**, net primary branches per plant. **NGS**, net growth stage.

The above observations were strongly supported by results of correlation analyses (Table 2.12). Stunting was significantly and positively correlated with net AUDPC (r=0.38). Despite the potential impact of climatic and soil factors, inoculation of plants by soaking cut-roots in spore suspensions might induce strong and rapid infections which may result in early changes in internal plant metabolism (such as hormonal imbalance) leading to stunted growth. Under field conditions however, since the major sources of inoculum are dormant resting structures (microsclerotia), it might take longer until the fungus receives a host signal, initiates germination, starts infection and finally spreads into the shoot. This time gap may provide the host plant a sufficient time to attain the maximum possible plant height and that is probably why VL is not causing stunting symptoms in the field. The negative significant correlation (r=-0.37) found between stunting and plant developmental growth stage also strongly supports the above hypothesis. Thus, compared to fast growing plants, genotypes with slow growth rates might be more vulnerable to stunting caused by VL infection. Branching did not show any correlation with any of the field, greenhouse or outdoor disease evaluation parameters (Table 2.12). This indicates that increased branching in OSR due VL infection is genotype dependent and not related

to susceptibility to *V. longisporum*. Another interesting relationship was the strong and significant correlation found between field qPCR and outdoor net AUDPC (r=37), suggesting the reliability and consistency of the qPCR method for assessment of VL resistance in oilseed rape under various experimental or environmental conditions.

Further effects of VL infection on plant growth rate were assessed by evaluating developmental growth stages of mock and VL-infected plants. The mean growth stage of all genotypes at the last time point of disease index assessment (56DPI) is shown in Fig. 2.9. For most genotypes, differences in growth stages of infected and control plants were not exaggerated. Furthermore, except for the only correlation with outdoor NRS, this parameter showed no significant relationship with all the other disease evaluation results from greenhouse, field and outdoor experiments (Table 2.12). Nevertheless, it was interesting that correlation of growth stage with all other disease parameters (be it significant or not) had negative coefficients, which might suggest a growth retarding effect of VL.

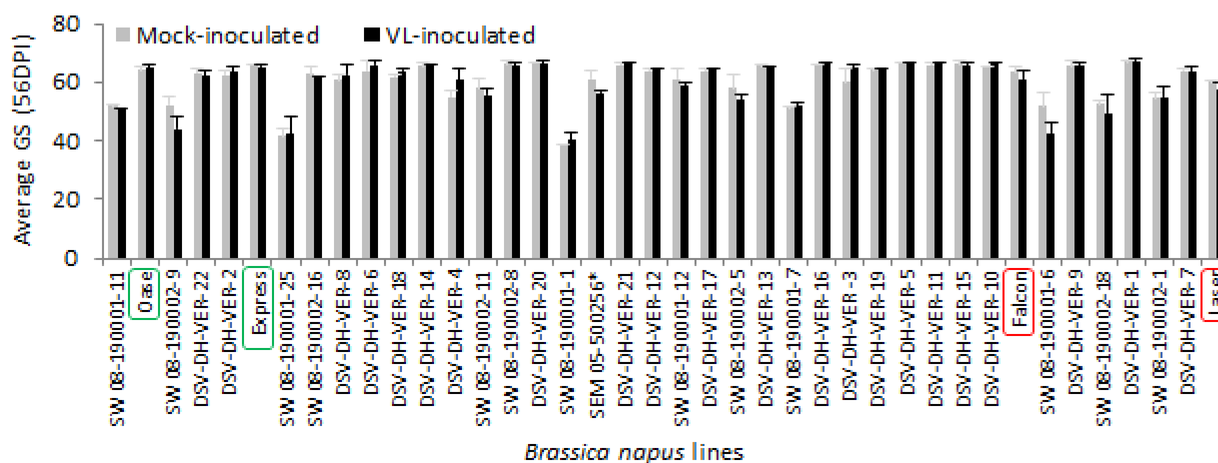


Figure 2.9 Average growth stage of *Brassica napus* double haploid lines and reference varieties screened for *Verticillium longisporum* resistance in the outdoor experiment. Inoculation of vernalized seedlings (BBCH14) was performed following a standard cut-root inoculation method. Resistant and susceptible controls are indicated in green and red marks, respectively. *****, not a double haploid line. **GS**, growth stage according to BBCH scale. Mean ±standard deviation data obtained from three biological replicates is shown. Genotypes are sorted according to increasing net AUDPC values.

2.4 References

Berg G., Frankowski J. and Bahl H. 1999. Biocontrol of verticillium wilt in oilseed rape by chitinolytic *Serratia Plymuthica*. Proceedings of 10th International Rapeseed Congress. Canberra, Australia.

Carré P. and Pouzet A. 2014. Rapeseed market, worldwide and in Europe. *Oilseeds and fats crops lipids*, 21 (1) D102.

Dunker S., Keunecke H., Steinbach P., von Tiedemann A. 2008. Impact of *Verticillium longisporum* on yield and morphology of winter oilseed rape (*Brassica napus*) in relation to systemic spread in the plant. *Journal of Phytopathology* 156: 698-707.

Eynck C. 2008. Identification of resistance sources and characterization of resistance factors in *Brassica* species to *Verticillium longisporum*. PhD thesis. Georg-August-Universität Göttingen, Göttingen, Germany.

Eynck C., Koopmann B. and von Tiedemann A. 2009a. Identification of *Brassica* accessions with enhanced resistance to *Verticillium longisporum* under controlled and field conditions. *Journal of Plant Diseases and Protection*, 116(2), 63-72.

Farkas Á. and Zajácz E. 2007. Nectar production for the hungarian honey industry. *The European Journal of Plant Science and Biotechnology*, 1(2):125-151.

França S. C., Spiessens K., Pollet S., Debode J., De Rooster L., Callens D. and Höfte M. 2013. Population dynamics of *Verticillium* species in cauliflower fields: Influence of crop rotation, debris removal and ryegrass incorporation. *Crop Protection*, 54:134-141.

Gladders P. 2009. Relevance of Verticillium wilt (*Verticillium longisporum*) in winter oilseed rape in the UK. *HCGA Research Review*, 72:1-39.

Goicoechea N. 2009. To what extent are soil amendments useful to control Verticillium wilt? *Pest Management Science*, 65:831-839.

Happstadius I., Ljungberg A., Kristiansson B. and Dixelius C. 2003. Identification of *Brassica oleracea* germplasm with improved resistance to Verticillium wilt. *Plant Breeding*, 122:30-34

Inderbitzin P., Davis R. M., Bostock R. M. and Subbarao K. V. 2011a. The Ascomycete *Verticillium longisporum* is a hybrid and a plant pathogen with an expanded host range. *PLoS ONE*, 6(3): e18260.

Karapapa V. K., Bainbridge B. W. and Heale J. B. 1997. Morphological and molecular characterization of *Verticillium longisporum* comb. nov., pathogenic to oilseed rape. *Mycological Research*, 101:1281-1294.

Klosterman S. J., Atallah Z. K., Vallad G. E. and Subbarao K. V. 2009. Diversity, pathogenicity, and management of *Verticillium* species. *Annual Review of Phytopathology*. 47:39-62.

Knüfer J. 2013. Improvement of winter oilseed rape resistance to *Verticillium longisporum*– Assessment of field resistance and characterization of ultrastructural plant responses. PhD thesis. Georg-August-Universität Göttingen, Göttingen, Germany.

Leino M. 2006. Fungal diseases on oilseed rape and turnip rape. Norrköping, Sweden.

Orlovius K. 2003. Oilseed rape. In: Kirbky, E. A. (Ed.).Fertilizing for high yield and quality, Bulletin 16. International Potash Institute. Basel, Switzerland.

Rygulla W., Friedt W., Seyis F., Lühs W., Eynck C., von Tiedemann A. and R. J. Snowdon. 2007a. Combination of resistance to *Verticillium longisporum* from zero erucic acid *Brassica oleracea* and oilseed *Brassica rapa* genotypes in resynthesized rapeseed (*Brassica napus*) lines. *Plant Breeding*, 126:596-602.

Rygulla W., Snowdon R. J., Eynck C., Koopmann B., von Tiedemann A., Lühs W. and Friedt W. 2007b. Broadening the genetic basis of *Verticillium longisporum* resistance in *Brassica napus* by interspecific hybridization. *Phytopathology*, 97:1391-1396.

Sattell R., Dick R., Ingham R., Karow R., Kaufman D. and D. McGrath. 1998. Rapeseed (*Brassica campestris*/*Brassica napus*). Oregon State University, USA.

Zeise K. and von Tiedemann A. 2002. Host specialization among vegetative compatibility groups of *Verticillium dahliae* in relation to *Verticillium longisporum*. *Journal of Phytopathology,* 150, 112-119.

3. Searching for cultivar-related resistance factors to *Verticillium longisporum* in oilseed rape (*Brassica napus)* xylem sap*

*This chapter have been submitted for publication to Phytopathology. Manuscript ID PHYTO-07-16-0281-R: Lopisso T. D., Knüfer J., Koopmann B. and von Tiedemann A. **Growth of *Verticillium longisporum* in *Brassica napus* xylem sap is independent from cultivar resistance but promoted by plant ageing.**

Department of Crop Sciences, Division of Plant Pathology and Crop Protection, Georg August University, Grisebachstr. 6, 37077 Göttingen, Germany; [2]Current address: Strube Research GmbH & Co. KG, 38387 Söllingen, Germany

3.1 Introduction

Verticillium species are among the most important fungal pathogens and cause vascular diseases in several economically important crop species. Among these, the recently evolved species *Verticillium longisporum* (Inderbitzin *et al.,* 2011a) is becoming a potential threat in oilseed rape (OSR) production particularly in the Northern European regions (Heale and Karapapa*,* 1999) where production of this crop is particularly widespread (Carré and Pouzet, 2014). One of the most peculiar characteristics of *V. longisporum* (VL) which makes it different from other Verticillium species is its host specificity towards members of the *Brassicaceae* including OSR (Karapapa *et al.,* 1997; Zeise and Tiedemann, 2002). In addition, despite effective colonisation and successful spread throughout the plant vascular system, VL does not induce wilting. Typical symptoms are rather leaf chlorosis, stunted growth and premature senescence and ripening (Karapapa *et al.,* 1997; Leino, 2006) potentially resulting in substantial yield losses particularly under conditions of high disease incidence (Dunker et al., 2008). Systemic infection and long term soil survival of this pathogen significantly limit the options available to manage this disease in OSR with fungicides. Hence, besides preventive measures such as crop rotation, improvement of cultivar resistance is the sole potential control strategy. Accordingly, a range of studies from identification of resistance sources (Happstadius *et al.,* 2003; Rygulla *et al.,* 2007a; Rygulla *et al.,* 2007b, Rygulla *et al.,* 2008) to field performance evaluation experiments (Eynck *et al.,* 2009a) have been undertaken in the past. These studies have been successful in identifying promising lines from which resistance traits can be derived. Nevertheless, there is lack of knowledge regarding the mechanisms of resistance and this can potentially hamper the effort of introgression of VL resistance traits into commercial OSR varieties.

Plant defence to vascular plant pathogens can generally consist of physical or chemical defence responses. The most common physical defence mechanisms that prevent or contain vertical and lateral xylem spread of vascular pathogens include formation of tyloses accompanied with accumulation of gels and gums, vascular coating, callose deposition, xylem wall swelling and vascular HR (Hypersensitive response). In addition, chemical defence compounds accumulating in the xylem and killing or inhibiting fungal growth are common strategies. Among these, phenols, phytoalexins and several proteins and enzymes play a major role (Yadeta and Thomma *et al.,* 2013).

Several potential mechanisms of VL resistance in OSR have been proposed. Eynck *et al.* (2009b) was the first to reveal the involvement of vascular occlusions, phenolics and lignin in OSR resistance against VL. Later on, other studies have shown the crucial role of phenylpropanoids (Obermeier *et al.,* 2013; Kamble *et al.,* 2013). Singh *et al.*, (2012) demonstrated that OSR xylem sap is capable of inhibiting the *in vitro* growth of VL and suggested a possible role of pre-existing xylem sap proteins in defence. Despite the fact that VL infection caused no change in total xylem sap protein content, xylem sap extracted from susceptible infected *B. napus* plants significantly reduced *in vitro* growth of VL (Floerl *et al.,* 2008). Another study aiming at the general analysis of xylem sap proteins in *B. napus* identified more than 60 different proteins, some of which were associated with plant defence against pathogens (Kehr *et al.,* 2005). In a study on the interaction of *B. napus* with VL, an enhanced accumulation of defence related antifungal proteins (including endochitinase, peroxidase PR-proteins and glucanase) were identified in the leaf apoplastic washing fluids of VL-susceptible *B. napus* (Floerl *et al.* (2008). In contrast, an expression analysis of the Salicylic acid (SA) signalling pathway marker gene PR1 by Kamble *et al.* (2013) showed no role of this gene in *B. napus* resistance against VL. In another study focusing on plant hormones involved in resistance signalling pathways, VL infection caused an increased accumulation of SA in the xylem sap or in hypocotyl tissue extract of OSR but had no effect on jasmonic acid (JA) and abscisic acid (ABA) concentrations (Ratzinger *et al.,* 2009; Kamble *et al.,* 2013). Johansson *et al.* (2006) reported that VL-resistance in Arabidopsis is regulated by JA and ethylene associated signals, but not by SA. According to Veronese *et al.* (2003), impairment of JA and SA signalling caused no sensitivity of Arabidopsis plants to *Verticillium dahliae*.

The complex changes occurring in xylem tissue of *B. napus* as a consequence of VL infection is a clear indication for the hypocotyl tissue to be the key site of crucial processes regulating the host-pathogen interaction. According to previous field and greenhouse resistance evaluation studies, it is clear that there is significant variation in the level of VL-resistance among *B. napus* lines. Moreover, distinct differences between resistant and susceptible genotypes with regard to the rate and intensity of

physical resistance factors as well as tissue extract chemical responses are known. However, except for a limited number of reports on the existence of xylem sap compounds involved in general resistance responses, nothing is known about the presence of cultivar-related VL- resistance factors in the xylem sap of *B. napus*. In the past, most reports focusing on VL-resistance factors in *B. napus* or Arabidopsis xylem sap were based on only a single mainly susceptible genotype. Moreover, as mentioned above, there are conflicting reports on the role or involvement of plant hormones and metabolites in VL resistance of *B. napus* and Arabidopsis. As a result, concrete and comprehensive information regarding the existence and role of cultivar-related VL-resistance factors in the xylem sap of OSR is not available. The present study therefore aims to compare the composition and growth inhibitory effect of xylem sap extract from susceptible and resistant OSR genotypes and to explore whether xylem sap play a major role in cultivar-related resistance of OSR against *V. longisporum*.

3.2 Materials and methods

3.2.1 Treatments and experimental design

The overall study was carried out in two independent experiments each consisting of greenhouse trials and *in vitro* bioassays. In the first experiment in the greenhouse, a completely randomized design with four replications was chosen. Two OSR genotypes (VL-resistant SEM 05-500256 and VL-susceptible Falcon) and two inoculation factors (mock and VL-inoculated) resulted in four treatments (Table 3.1). For the phenotypic disease evaluation, 20 plants per treatment (5 plants per replication) were used. For xylem sap extraction at two time points (21 and 28 days post inoculation, DPI), at least 45 extra plants per treatment were included, since this was a destructive sampling. The *in vitro* bioassay consisted of 10 treatments derived from the combination of genotype, disease and xylem sap filtration (Table 3.2), and was conducted in 96-well microtitre plates. In the greenhouse experiment, mock-inoculated plants were used as control whereas in the *in vitro* bioassay, two controls, sterile distilled water with conidial suspension and sterile distilled water alone, represented the control treatments (Table 3.1 and 3.2). The second experiment was conducted in a climate chamber with the additional objective to investigate VL-resistance factors in xylem sap of plants at different stages of ageing. In this study, a different VL-resistant *B. napus* genotype (Aviso) was used (Table 3.1) and xylem sap samples were collected in 2-3 day intervals (From 13 to 28 DPI). All other conditions and settings of the climate chamber and bioassay experiments were similar as described above.

3.2.2 Plant material and cultivation

Three *B. napus* genotypes were used. Seeds for the VL-susceptible cultivar Falcon were purchased from NPZ (Norddeutsche Pflanzenzucht, Hans-Georg Lembke KG, Hohenlieth, Germany). Seeds for the VL-

tolerant genotypes SEM and Aviso were obtained from Lantmännen SW Seed (Svalöv, Sweden) and Danisco Seed (Sweden), respectively. Before sowing, seeds were double surface sterilized with 70% ethanol for 2 min under constant shaking and rinsed with autoclaved tap water. Seeds were grown on autoclaved silica sand in a climate-controlled chamber (day/night conditions of 14h/10h at 23°C) for two weeks and then inoculated with VL as described below.

Table 3.1 Treatment descriptions and designations for greenhouse/climate chamber experiments.

No.	Designation	Experimental factors and descriptions	
		Genotype	Disease
1	SM	SEM 05-500256	Mock-inoculated
2	SVL	SEM 05-500256	VL-inoculated
3	FM	Falcon	Mock-inoculated
4	FVL	Falcon	VL-inoculated
5	AM	Aviso	Mock-inoculated
6	AVL	Aviso	VL-inoculated

S, SEM 05-500256. **F**, Falcon. **A**, Aviso. **M**, mock-inoculated. **VL**, *Verticillium longisporum* inoculated.

Table 3.2 Treatment descriptions and designations for bioassay studies.

No.	**Designation**	**Experimental factors and descriptions**		
		Genotype	**Disease**	**Xylem sap filtration**
1	SM	SEM 05-500256	Mock-inoculated	unfiltered
2	SMf	SEM 05-500256	Mock-inoculated	filtered
3	SVL	SEM 05-500256	VL-inoculated	unfiltered
4	SVLf	SEM 05-500256	VL-inoculated	filtered
5	FM	Falcon	Mock-inoculated	unfiltered
6	FMf	Falcon	Mock-inoculated	filtered
7	FVL	Falcon	VL-inoculated	unfiltered
8	FVLf	Falcon	VL-inoculated	filtered
9	AMf	Aviso	Mock-inoculated	filtered
10	AVLf	Aviso	VL-inoculated	filtered
11	BDWVL	VL growth on sterile bi-distilled water		
12	BDW	Sterile bi-distilled water alone		

S, SEM 05-500256. **F**, Falcon. **A**, Aviso. **M**,mock-inoculated. **VL**, *Verticillium longisporum* inoculated. **f**, filtered xylem sap, **BDW**, sterile bi-distilled water.

3.2.3 Fungal growth and inoculation technique

Verticillium longisporum isolate VL43 (Fig. 1.2), a highly virulent isolate on OSR (Zeise and Tiedemann, 2002) was grown on potato extract glucose broth (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for 10

days at 23°C in the dark with constant shaking. A standard non-cut root dip inoculation technique was used. Briefly, roots of two weeks old seedlings (BBCH 12) were washed under a running tap water and submerged for 1 h in a conidial suspension with a density of 10^6 conidia/ml. Mock-inoculated plants were treated in the same way with autoclaved tap water. Treated seedlings were transferred to plastic pots (200ml) filled with a 3:1 (v/v) mixture of commercial soil (Fruhstorfer Erde, Type ; pH 6.0; Hawita-Gruppe, Vechta, Germany) and sand substrate. Transplants were grown in the greenhouse/climate chamber under similar conditions as described above.

3.2.4 Xylem sap extraction

Xylem sap was extracted from the lower hypocotyl part of different aged plants using the pressure bomb technique (Scholander *et al.,* 1965). Briefly, plants were uprooted and soil materials adhering to roots were washed off under running tap water. The shoot part was removed by cutting the stem above the hypocotyl. The remaining hypocotyl part with the roots was immediately inserted into the chamber of the Scholander bomb (Model 600; PMS Instrument Company; Albany, USA). Xylem sap was collected by gradually increasing the chamber pressure until xylem sap appears at the cut surface. In a repeated experiment, in order to collect a sufficient amount of xylem sap, extraction was done by cutting plants at the tip of hypocotyl and collecting the exuding xylem sap from the cutting end using hand held pipette. Each treatment had three biological replications. Aliquots of approximately 1ml of xylem sap were collected from 15 plants pooled together and considered as one replication. Xylem sap samples collected from control and infected plants were immediately stored at -20°C until further use.

3.2.5 Disease evaluation

Disease severity evaluation was done in a weekly interval from 7 to 28 DPI using a nine class assessment key modified from Zeise, 1992 (Table 4.2). Disease severity recordings were used to calculate AUDPC values (Campbell & Madden, 1990) from which Net AUDPC ($AUDPC_{net}$) values were calculated by subtracting the AUDPC value of control treatments from that of the values of inoculated treatments. That is $AUDPC_{net}= AUDPC_{inoc} - AUDPC_{mock}$ where $AUDPC_{net}$, $AUDPC_{inoc}$ and $AUDPC_{mock}$ refer to net AUDPC, AUDPC of VL-inoculated treatments and AUDPC of mock-inoculated treatments, respectively (Eynck *et al,* 2009b). Stunting was measured by recording plant height (from base of hypocotyl to the tip of the central main stem) at 28 DPI. At the same time, stem diameter (at the tip of hypocotyl) was precisely measured using a digital calliper. Quantification of fungal DNA by quantitative real time PCR was performed following the method described in 4.2.7.3. Cultivar's response to VL-infection was further determined by comparing the above hypocotyl biomass determined after oven drying (70°C for 24 h).

3.2.6 *In vitro* bioassay

Verticillium longisporum isolate VL43 was grown on potato extract glucose broth potato extract glucose broth (PEGB) (*Carl Roth GmbH + Co. KG*, Karlsruhe, Germany) as described in section 2.2.4. To remove mycelial masses, the culture was filtered with sterile cheesecloth. From the filtered culture, conidia were harvested by centrifugation for 10 min at 1046 g Conidia were washed three times by resuspending in sterile distilled water and the final conidial density was adjusted to $5x10^3$ conidia/ml using a haemocytometer. To eliminate any bacterial growth during *in vitro* assay, filter sterilized streptomycin (20ppm) was added to the final spore suspension. To prepare filtered xylem sap treatments, xylem sap was filtered through a 0.2µm sterile Anotop 10 plus disposable syringe filter with aluminum oxide membrane material (Whatman GmbH; Dassel, Germany). Measurement of fungal growth in xylem sap was performed using the method of Broekaert *et al.,* 1990. The assay was set up as follows. First, 50µL of VL spore suspension ($5x10^3$ conidia/ml) and 200µl of xylem sap were loaded in 96-well microtitre plates in three technical replicates. Prior to incubation, spores were allowed to settle for 30 min and the initial absorbance was taken at 580nm in a microplate spectrophotometer (µQuant, Bio-Tek Instruments, Bad Friedrichshall, Germany). Plates were placed in a moist chamber and incubated at 23°C for seven days in the dark. Fungal growth was monitored daily by taking the mean absorbance value of 25 readings taken from different spots in a single well. Fungal growth at different DAI (days after incubation) was obtained by subtracting initial absorbance values (0 DAI) from the daily absorbance recordings.

3.2.7 Total protein assay

The total protein content in xylem sap was measured with the Bradford method (Bradford, 1976). A standard curve was developed using a series of bovine serum albumin (BSA) standards in the range of 600 to 100µg/ml. Then, 276µl of 1X-Roti-Quant staining solution (Carl Roth GmbH + Co. KG; Karlsruhe, Germany) was mixed with 5µl of BSA standard solutions, xylem sap or double distilled water (used as blank) and loaded into the microtitre plate wells in triplicates. After incubation for 5 min at room temperature, absorbance at 595nm was measured using a microplate spectrophotometer (µQuant, Bio-Tek Instruments, Bad Friedrichshall, Germany). Total protein content in xylem sap was estimated by referring to the standard curve produced from known concentrations of BSA.

3.2.8 Quantification of xylem sap total carbohydrate content

Xylem sap total carbohydrate (CHO) content was determined following the microplate format phenol-sulphuric acid protocol (Masuko *et al.,* 2005). Briefly, aliquots of standard glucose (Glc) solutions were prepared in double distilled water. Then, 100µl of Glu standard solution or xylem sap samples were

mixed with 300µl of concentrated sulphuric acid, the solution was immediately amended with 60µl of 5% phenol and incubated for 5 min at 90°C in a static water bath. Subsequently, the solution was cooled for 5 min in a water bath at room temperature and 230µl of the reaction was loaded into 96-well plates in triplicates. Absorbance was measured at 490nm, the absorption maximum of most sugars including mannose, xylose, frucose, galactose and glucose (Masuko et al., 2005). To calculate xylem sap total CHO content, mean absorbance values of xylem sap samples were referred to the standard curve produced from known concentrations of Glc.

3.2.9 Data analysis

Each experiment was repeated at least twice. All statistical analyses were performed using STATISTICA version 10 data analysis software (StatSoft, inc. 2011). One way ANOVA with Fisher Least Significant Difference (LSD) test was used to analyse means obtained from four or three biological replicates of greenhouse and laboratory experiments, respectively. Differences were considered statistically significant when P-values were less than 0.05. Before analysis of variance, all data sets were tested for normal distribution using the Shapiro-Wilk test and whenever required, ANOVA was performed on transformed data (Gomez and Gomez, 1984). Relationships between fungal growth and xylem sap total protein or carbohydrate concentration were determined by Pearson (product-moment) correlation analysis.

3.3 Results

The overall study was carried out in two different experimental series each consisting of a greenhouse trial, an *in vitro* bioassay and biochemical analyses. Greenhouse and *in vitro* bioassay experiments were repeated at least twice and provided consistent results. Results obtained from a single experiment are presented as follows.

3.3.1 Greenhouse experiments

Response of the two *B. napus* genotypes to VL infection was examined under greenhouse conditions. In this part of the study, phenotypic and molecular disease parameters that evidently demonstrate resistant or susceptible reactions of OSR to VL infection were measured. Evaluation of disease severity, from which net AUDPC was generated, was performed on a weekly basis using a standard disease index key. In addition, quantification of VL-DNA in hypocotyl tissue, measurement of stem thickness and determination of stunting were carried out four weeks after inoculation. Results obtained from all disease evaluations clearly showed strong resistance and susceptible reactions of genotype SEM and Falcon, respectively. Four weeks after inoculation, infected SEM plants remained green, vigorous and

healthy while diseased Falcon plants showed extremely stunted growth with shrivelled, yellow and black veined leaves, which are typical symptoms of VL infection (Fig. 3.1). Disease levels as measured by net AUDPC showed five times higher disease severity in cultivar Falcon compared to the resistant genotype SEM. Accordingly, average net AUDPC values at 28 DPI recorded from infected SEM and Falcon plants were 10.2 and 49.7, respectively (Fig. 3.2A). Similarly, quantification of VL DNA from hypocotyl tissue of infected plants by quantitative PCR showed a 74fold accumulation of fungal biomass in Falcon (1,419.2ng/g) compared to SEM (19.3ng/g) (Fig. 3.2B).

Figure 3.1 Phenotypes of resistant (left) and susceptible (right) *Brassica napus* plants 28 days after inoculation with *Verticillium longisporum*. Plants were inoculated by submerging non-cut roots of 10 day old seedlings in spore suspension (10^6conidia/ml) for one hour. Mock-inoculated plants were similarly dipped in autoclaved tap water. **SM**, genotype SEM mock-inoculated. **SVL**, genotype SEM VL-inoculated. **FM**, cultivar Falcon mock-inoculated. **FVL**, cultivar Falcon VL-inoculated.

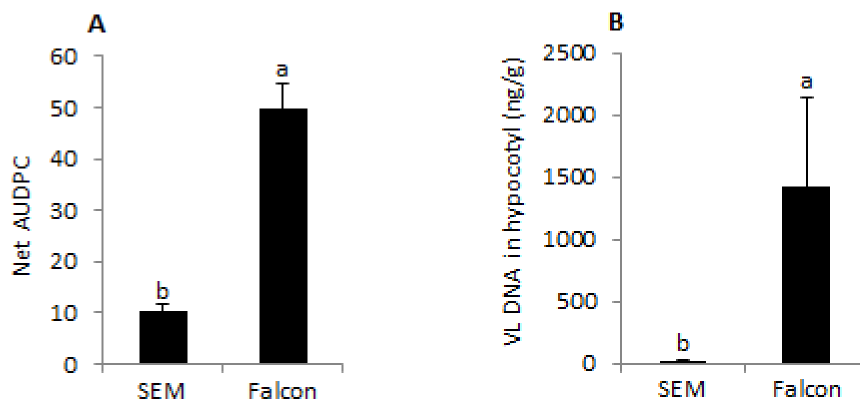


Figure 3.2 Evaluation of disease levels in resistant (SEM) and susceptible (Falcon) *Brassica napus* genotypes 28 days after inoculation with *Verticillium longisporum*. **A**, net area under disease progress curve values. **B**, quantification of *Verticillium longisporum* DNA in hypocotyl tissue by quantitative PCR. Bars indicate standard deviations of four biological replicates. Different letters on the bars indicate significant differences at P≤0.05.

With regard to stem diameter, the two genotypes showed significant differences under both mock- and VL-inoculation conditions. In the absence of infection, the stem diameter of the VL-resistant genotype was significantly larger than the susceptible genotype. That means SEM had genetically wider stem thickness than Falcon. Four weeks after inoculation, infected SEM plants had a similar hypocotyl

thickness (3.8mm) as mock-inoculated plants. In cultivar Falcon however, infection significantly reduced hypocotyl thickness by 24% (Fig. 3.3A).

Regarding plant height, unlike the previously described disease parameters, VL infection caused significant effects both in resistant and susceptible plants. However, compared to SEM, the stunting effect of VL on the susceptible cultivar Falcon was significantly more severe. In this cultivar, reduction of plant height due to VL infection 28 days after inoculation was 69% while in genotype SEM, a relatively lower but still significant stunting (20%) was recorded. As for stem diameter, the two genotypes had genetically different with regard to plant height where the VL-susceptible cultivar possessed significantly longer phenotype than the resistant genotype SEM (Fig. 3.3B). The responses of the resistant and susceptible genotypes to VL-infection were further shown in dry matter yield assessment. As expected dry matter yield in the susceptible genotype Falcon was significantly reduced by 68.6%. In contrast, the reduction in SEM was not significant (Fig 3.3C). The resistance and susceptible responses of the two genotypes used in climate chamber experiments was also verified by disease index assessment (data not shown).

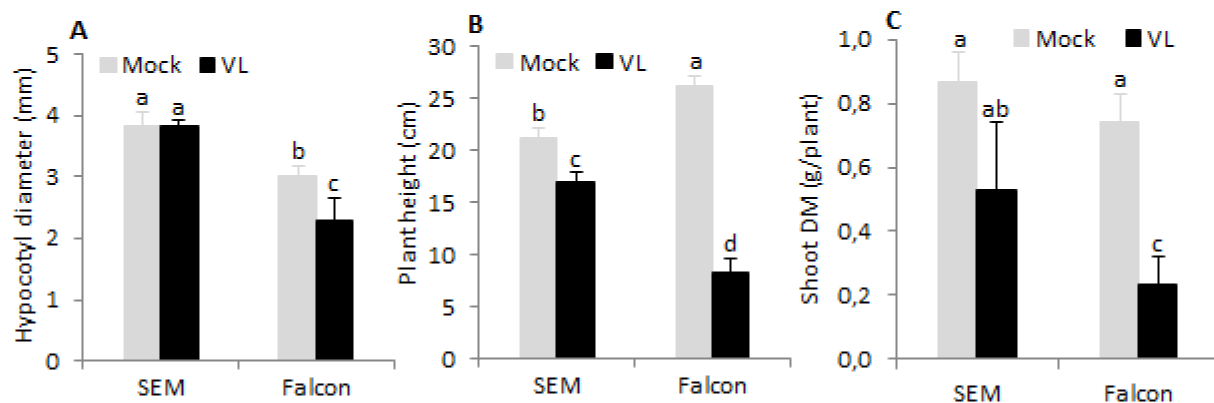


Figure 3.3 Assessment of stem thickness, plant height and shoot dry biomss in resistant (SEM) and susceptible (Falcon) *Brassica napus* genotypes infected with Verticillium *longisporum.* Measurement was done 28 days after inoculation. Bars indicate standard deviations of four biological replicates. Different letters on the bars indicate significant difference at P≤0.05. **Mock**, control plants inoculated with sterile water. **VL**, *Verticillium longisporum* inoculated treatments. **DM**, dry matter.

3.3.2 Analysis of *Verticillium longisporum* growth on *Brassica napus* xylem sap

3.3.2.1 Xylem sap bioassay

To investigate the presence or absence of cultivar-specific xylem sap-residing VL resistance factors, growth of *V. longisporum* on xylem sap extracted from resistant and susceptible plants was analysed *in vitro*. With the intention of excluding the effect of xylem sap endophytes that might interfere with the bioassay and to further investigate the effect of xylem sap filtration on fungal growth, filtered xylem sap

treatments were also included in this assay. The growth of VL on *B. napus* xylem sap was monitored on a daily basis using a spectrophotometer. Results showed that spore germination and further growth of VL begun after 2 to 3 days of incubation. Unlike the water control where no or only slight growth was detected, an enhanced and significantly higher level of fungal growth was observed in xylem sap medium from all treatment combinations. Analysis of variance showed that irrespective of VL infection or xylem sap filtration, the rate and level of VL growth in xylem sap extracted from resistant or susceptible plants was not significantly different (Fig. 3.4). Even after one week ofincubation (7 DAI), VL growth on filtered or unfiltered xylem sap extracts derived from either mock or VL-inoculated plants of both genotypes was similar (Fig. 3.5). Compared to filtered xylem sap however, a slightly higher growth of VL on unfiltered xylem sap of both genotypes was observed. Furthermore, again irrespective of the genotype, a slightly higher fungal growth on xylem sap extracted from VL-infected plants was noticed (Fig. 3.4; Fig. 3.5). Similar analyses of fungal growth measurements from 2 to 6 DAI showed no significant difference among all treatment combinations (Appendix 3.3).

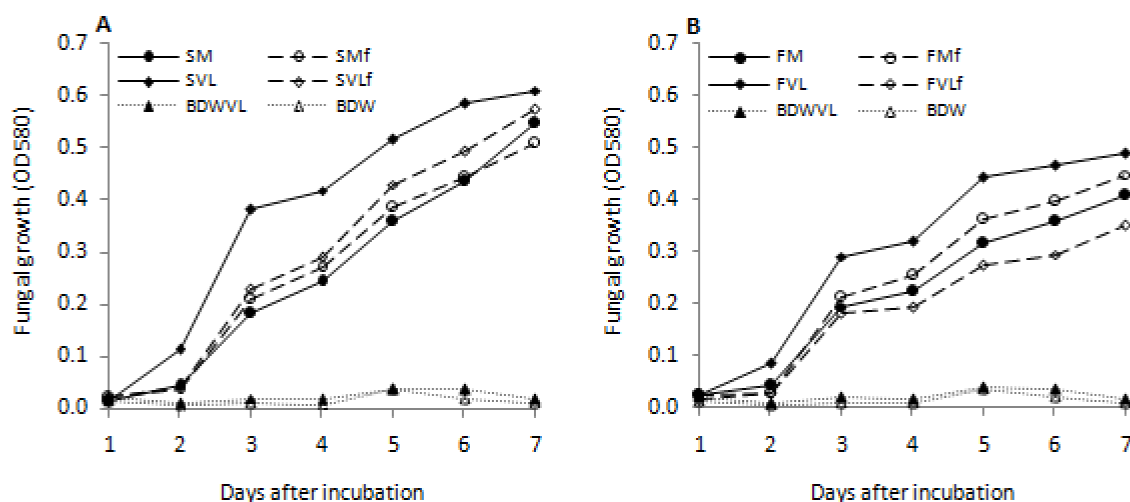


Figure 3.4 Spectrophotometric measurement of *Verticillium longisporum* growth on xylem sap extracted from resistant (SEM) and susceptible (Falcon) *Brassica napus* plants. Xylem sap was extracted 28 days after inoculation *with Verticillium longisporum*. **A.** Fungal growth in xylem sap extracted from genotype SEM. **SM** and **SMf**, respective fungal growth in unfiltered and filtered xylem sap extracted from mock-inoculated plants. **SVL** and **SVLf**, respective fungal growth in unfiltered and filtered xylem sap extracted from VL-inoculated plants. **B**. Fungal growth in xylem sap extracted from genotype Falcon. **FM** and **FMf**, respective fungal growth on unfiltered and filtered xylem sap extracted from mock-inoculated plants. **FVL** and **FVLf**, respective fungal growth in unfiltered and filtered xylem sap extracted from VL-inoculated plants. **BDWVL**, Fungal growth in sterile bi-distilled water. **BDW**, sterile bi-distilled water without VL inoculum. At no DAI, there was any significant difference in fungal growth in the different xylem sap samples (Appendix 3.3).

3.3.2.2 Xylem sap total protein content

Protein analysis was performed in order to investigate the effect of VL infection on xylem sap protein concentration and to further find out whether changes in protein concentration are related to resistance

or susceptible responses of *B. napus* genotypes to *V. longisporum*. Accordingly, the total soluble protein content of xylem sap samples used in fungal growth bioassays was analysed with the Bradford method. Results revealed the presence of considerable amounts of proteins in *B. napus* xylem sap. In mock-inoculated plants, the quantities of total proteins in xylem sap of SEM and Falcon plants were 104.9 and 101.8µg/ml, respectively. When infected with VL, a slightly higher (109.4 and 106.0µg/ml, respectively) but insignificant increase was found. Analysis of variance showed no significant difference in total soluble protein concentration of xylem sap samples extracted from resistant and susceptible genotypes. It was also found that VL infection and xylem sap filtration did not significantly alter protein concentrations. Nevertheless, a slight increase of total protein concentration due to VL infection and a reduction due to filtration was noticed in both genotypes (Table 3.3).

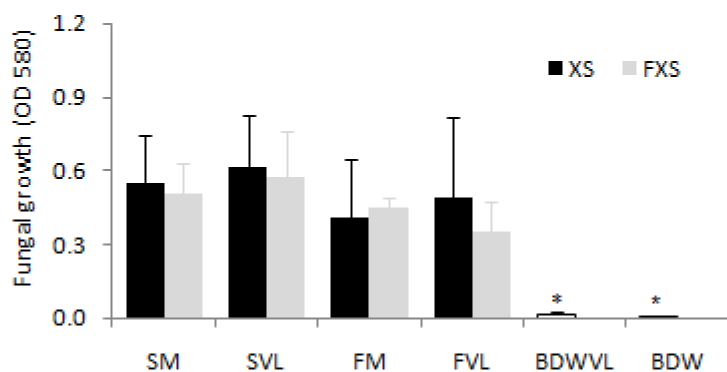


Figure 3.5 Spectrophotometric measurement of *Verticillium longisporum* growth seven days after incubation in xylem sap extracted from resistant (SEM) and susceptible (Falcon) *Brassica napus* plants. Xylem sap was extracted 28 days after inoculation. **FXS**, filtered xylem sap, **XS**, unfiltered xylem sap. **SM,** Growth of *V. longisporum* in xylem sap extracted from mock-inoculated SEM plants. **SVL**, fungal growth in xylem sap extracted from VL-inoculated SEM plants. **FM**, fungal growth in xylem sap extracted from mock-inoculated Falcon plants. **FVL**, fungal growth in xylem sap extracted from VL-inoculated Falcon plants. **BDWVL**, fungal growth on sterile bi-distilled water. **BDW**, sterile bi-distilled water without VL inoculum. *****, significantly different at P=0.05.

Table 3.3 Concentrations of total soluble protein in xylem sap of resistant (SEM) and susceptible (Falcon) *Brassica napus* genotypes 28 days after inoculation with *Verticillium longisporum*.

	Treatments	Total protein (µg/ml)	
		unfiltered xylem	Filtered xylem sap
SEM	Mock-inoculated	104.9±17^{a}	99.1±11^{a}
	VL-inoculated	109.4±14^{a}	98.5±12^{a}
Falcon	Mock-inoculated	101.8±14^{a}	99.0±12^{a}
	VL-inoculated	106.0±12^{a}	100.5±14^{a}

Protein content was determined by with the Bradford method using bovine serum albumin (BSA) as a standard. Means ± standard deviation were obtained from three biological replicates. Mean values assigned with the same superscript are not significantly different at P=0.05.

3.3.3 Analysis of *V. longisporum* growth in xylem sap extracted from plants at different age

In this independent study, in addition to the cultivar-related VL resistance, the growth of VL in xylem sap extracted from different aged plants was investigated. Similar to the previous experiment (section 3.4.2), this study was accomplished in two settings, greenhouse experiments and *in vitro* bioassays. Further to analyse the relationship between xylem sap constituents and *in vitro* fungal growth, the sugar concentration in xylem sap samples collected from different aged plants was quantified. Two *B. napus* genotypes expressing contrasting levels of resistance to VL, namely Aviso (resistant) and Falcon (susceptible) were used. Xylem sap was extracted from mock and VL-infected plants in 2-3 day intervals during the period between 13 to 28 DPI. For the bioassay, xylem saps extracted from different aged plants were inoculated with spores of VL and fungal growth was daily measured with a spectrophotometer as above.

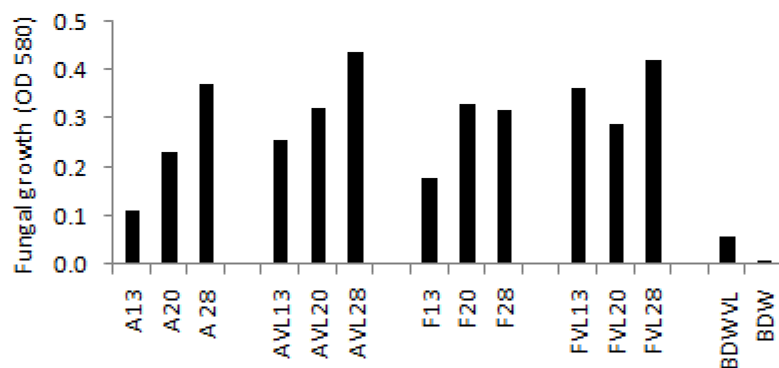


Figure 3.6 Spectrophotometric measurement of *Verticillium longisporum* growth five days after incubation on xylem sap extracted from resistant (Aviso) and susceptible (Falcon) *Brassica napus* plants. Treatments: **A**, Aviso mock-inoculated. **AVL**, Aviso VL-inoculated. **F**, Falcon mock-inoculated. **FVL**, Falcon VL-inoculated. Numbers after treatments refer to DPI at which xylem sap was extracted. **BDWVL**, Fungal growth on sterile bi-distilled water. **BDW**, sterile bi-distilled water without VL inoculum.

Table 3.4 Average growth rate of *V. longisporum* in xylem sap extracted from different aged mock and *Verticillium longisporum* inoculated resistant and susceptible *Brassica napus* genotypes.

	Treatments	Mean daily fungal growth rate (OD 580)		
		13 DPI	20 DPI	28 DPI
Aviso	Mock-inoculated	0.023	0.039	0.072
	VL-inoculated	0.052	0.059	0.090
Falcon	Mock-inoculated	0.033	0.072	0.066
	VL-inoculated	0.069	0.053	0.085
Control	VL growth on bi-distilled water		0.013	
	Bi-distilled water without VL inoculum		0.000	

Daily fungal growth rate was calculated by subtracting absorbance value of previous day measurements of each treatment. Data shown is average of four time points (2, 3, 4 and 5 DPI). **DPI**, days post incubation.

3.3.3.1 Xylem sap bioassay

Results obtained from the *in vitro* bioassay were consistent and strongly comparable with the independently conducted first experimental series with two different genotypes (section 3.4.2). Xylem sap derived from mock and VL-infected plants of both resistant and susceptible genotypes was equally suitable for the growth of VL (Fig. 3.7). Unlike the control medium (sterile bi-distilled water), a significantly higher fungal growth was measured after two days of incubation. However, the growth of VL in xylem sap from resistant and susceptible plants was not significantly different. Likewise, xylem sap from VL-infected resistant and susceptible same age plants did not cause significant change on the *in vitro* growth of VL (Fig. 3.6). Analysis of variance of fungal growth in xylem sap extracted from earlier time points (13, 18, 20, 22, and 26 DPI) also showed comparable growth of VL in all xylem sap samples (data not shown).

Interestingly, comparison of fungal growth in xylem sap from plants at different age showed enhanced fungal growth of VL in xylem sap from older plants. Analysis of the daily fungal growth rate in xylem sap samples extracted in a weekly interval also showed a significantly positive correlation between *in vitro* fungal growth rate and plant age (Table 3.6). A slightly increased fungal growth rate was observed in xylem sap samples of VL-inoculated Aviso plants. Regarding the pattern of fungal growth in xylem sap derived from the susceptible genotype Falcon, a similar increase in growth rate was recorded from older plant xylem sap samples. It is noteworthy to mention, that the average daily fungal growth rate recorded in xylem sap at 13 DPI of mock and VL-inoculated plants was OD 0.033 and 0.069, respectively. The respective growth rate values at 28DPI were OD 0.066 and 0.085. Growth rates at 20 DPI seemed to be inconsistent. As with genotype Aviso, an increased rate of fungal growth at 13 and 28 DPI was also noticed in xylem sap samples collected from VL infected Falcon plants.

3.3.3.2 Xylem sap total carbohydrate content

In order to investigate the relationship between fungal growth and xylem sap sugar content, the total carbohydrate (CHO) concentration in xylem sap samples used in the bioassays was quantified using a glucose standard (Appendix 3.2). Results obtained from different aged plants showed comparable CHO concentrations in xylem sap of susceptible and resistant genotypes. Even though statistically insignificant, plant age dependent effect of VL infection was noticed. At 14, 18 and 21 DPI, CHO levels in xylem sap of VL-infected plants were slightly increased. In contrast, CHO levels in xylem sap at 28 DPI of VL-infected plants were relatively lower. Interestingly, it was found that xylem sap sugar concentration was significantly dependent on plant age. Compared to earlier time points, total xylem sap CHO

concentration in 28 DPI old plants of both mock or VL-infected resistance and susceptible genotypes was significantly higher than in younger plants (Table 3.5).

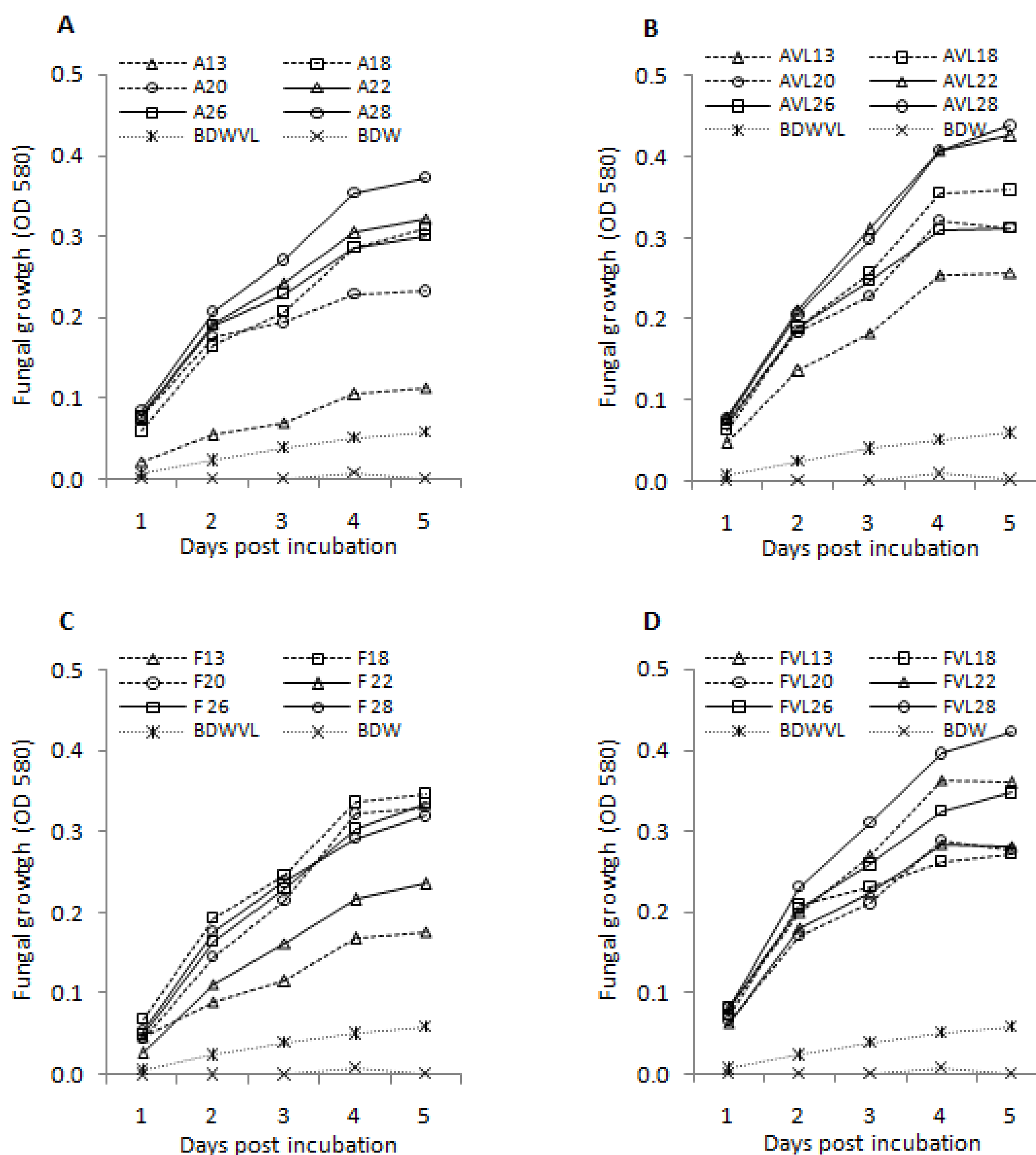


Figure 3.7 Spectrophotometric measurement of *Verticillium longisporum* growth in xylem sap extracted from resistant (Aviso) and susceptible (Falcon) *Brassica napus* genotypes. **A**, fungal growth in xylem sap extracted from Aviso, mock-inoculated. **B**, fungal growth in xylem sap extracted from Aviso, VL-inoculated. **C**, fungal growth in xylem sap extracted from Falcon, mock-inoculated. **D**, fungal growth in xylem sap extracted from Falcon, VL-inoculated. Treatments: **A**, Aviso mock-inoculated. **AVL**, Aviso VL-inoculated. **F**; Falcon mock-inoculated. **FVL**, Falcon VL-inoculated. Numbers after treatments refer to DPI at which xylem sap was extracted. **BDWVL**, Fungal growth on sterile bi-distilled water. **BDW**, sterile bi-distilled water without VL inoculum.

3.3.4 Correlation analysis

With correlation analysis, the relationship between *in vitro* fungal growth and xylem sap sugar and protein concentrations was studied. Furthermore, the correlation between plant age and fungal growth rate in xylem sap and the correlation among disease parameters were analysed. Results showed a signif-

Table 3.5 Concentrations of xylem sap total carbohydrate in *Brassica napus* genotypes resistant (Aviso) and susceptible (Falcon) to *Verticillium longisporum*.

	Treatments	Xylem sap carbohydrate content (µg/ml)			
		14 DPI	18 DPI	21 DPI	28 DPI
Aviso	Mock-inoculated	22.9 ± 2^{cde}	29.5 ± 5^{cde}	16.2 ± 7^{ef}	80.0 ± 4^{a}
	VL-inoculated	24.5 ± 10^{cde}	22.5 ± 8^{cde}	40.3 ± 4^{bc}	59.9 ± 8^{ab}
Falcon	Mock-inoculated	20.7 ± 5^{def}	21.3 ± 2^{cdef}	11.7 ± 5^{f}	94.4 ± 10^{a}
	VL-inoculated	33.6 ± 15^{bcd}	27.6 ± 15^{cde}	33.6 ± 13^{bcd}	57.9 ± 4^{ab}

Carbohydrate content was determined using the phenol-sulphuric acid method. Known concentrations of glucose were used as standards. Means ± standard deviation were obtained from three biological replicates. Mean values assigned with the same superscript are not significantly different at P=0.05.

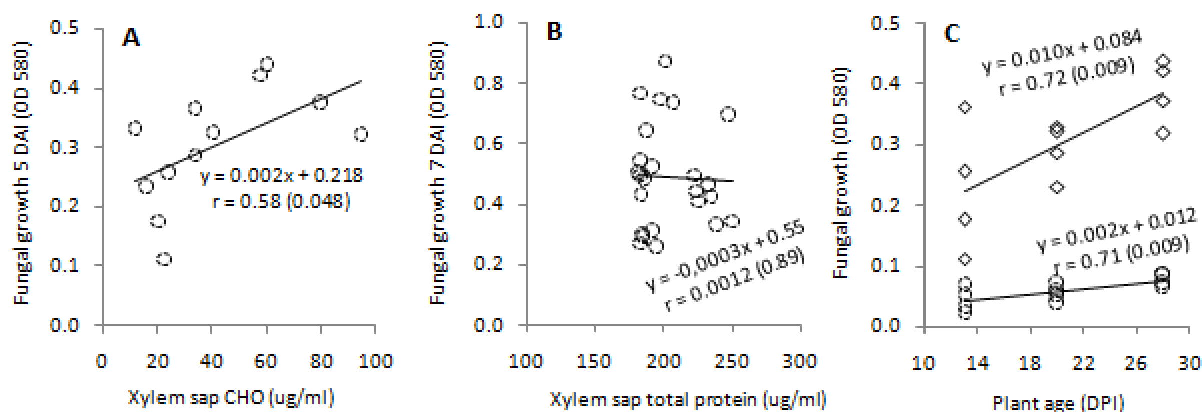


Figure 3.8 Relationships between *Verticillium longisporum* growth and constituents and age of xylem sap extracted from mock and VL-inoculated resistant and susceptible *Brassica napus* plants. P-values are indicated in brackets. P-values less than 0.05 indicate significant correlations.

A. Pearson's correlation between total xylem sap protein and fungal growth five days after incubation. Spores of VL were grown in xylem sap extracted 13, 20 and 28 DPI. Total carbohydrate concentration in xylem sap extracted at 14, 21 and 28 DPI was quantified using known concentrations of glucose standard solutions.

B. Pearson's correlation between total xylem sap proteins and fungal growth seven days after incubation. Spores of VL were grown on xylem sap extracted at 28 DPI. Total protein concentration in xylem sap extracted at 28 DPI was quantified using known concentrations of BSA standard solutions. **DPI**, Days post inoculation with *Verticillium longisporum*. **DAI**, days after incubation in xylem sap. **CHO**, carbohydrate.

C. Pearson's correlation between plant age at the time of xylem extraction and fungal growth five days after incubation (diamond) or daily fungal growth rate (circles). Data from weekly interval samples (13, 20 and 28 DPI) were used.

-cant and strong positive correlation between xylem sap sugar concentration and growth of VL in xylem sap (Fig. 3.8A). A similarly strong correlation was identified between fungal growth and plant age at the time of xylem sap extraction (Fig. 3.8C). In contrast, no correlation was found between rate of fungal growth and concentrations of total proteins in xylem sap (Fig. 3.8B). Results of Pearson's correlation

analysis of phenotypic and molecular disease parameters from greenhouse experiments also showed strong relationships. The highest correlation was found between AUDPC and plant height (r=-0.92) followed by AUDPC and VL DNA (r=0.89). Very strong negative correlations (ranging between -0.73 and -0.79) existed among the rest of disease parameters except for plant height and stem diameter (r=0.49) where the correlation was not significant. The relationship between disease and SDM yield was significantly strong, verifying the consistent responses of the genotypes to VL infection (Table 3.6).

Table 3.6 Correlation coefficients describing relationships among disease and agronomic parameters measured in the greenhouse experiments at 28 DPI.

	Net AUDPC	Plant height	Hypocotyl diameter	Shoot DM
VL DNA	0.89^{***}	-0.79^{***}	-0.73^{**}	-0.69^{*}
Net AUDPC		-0.92^{***}	-0.77^{***}	-0.81^{**}
Plant height			0.49^{ns}	0.78^{**}
Hypocotyl diameter				0.61^{*}

Analysis was performed using Pearson (product-moment) correlation (STATISTICA version 10 StatSoft, inc. 2011). Minus signs indicate negative correlations. **ns**, not significant. ***,** significant at P=0.05. ****,** significant at P=0.01. *****,** significant at P=0.001. **DPI**, Days post inoculation with *Verticillium longisporum*. **Net AUDPC**, net area under disease progress curve, **VL DNA**, *Verticillium longisporum* DNA in hypocotyl. **DM**, dry matter.

3.4 Discussion

The focus of this study was identification of xylem sap residing cultivar-related *V. longisporum* resistance factors in oilseed rape. The study utilized three *B. napus* genotypes with differential degree of resistance against VL. The overall study was accomplished by different experimental settings involving greenhouse experiments, *in vitro* bioassays and biochemical analyses. Initially, the greenhouse experiments were conducted with the aim of collecting xylem sap and verifying the responses of the three *B. napus* genotypes to VL infection. Analysis of net AUDPC, stunting effects and stem thickness together with quantification of VL DNA showed significantly lower levels of disease in genotypes SEM and Aviso, confirming resistance of these genotypes to VL. In contrast, infected Falcon plants showed a fast development of disease symptoms and a significantly higher level of disease severity (see section 3.4.1). The strong positive correlation between AUDPC values and qPCR data as well as the negative correlation of AUDPC and fungal biomass with plant height, stem thickness and shoot biomass measurements (Table 3.6) also consistently verified resistance and susceptible responses of the genotypes used in this study. In general, molecular and phenotypic greenhouse disease evaluation results confirmed the observations of previous field and greenhouse resistance evaluations that showed VL-resistance of

genotype Aviso (Keunecke, 2009) and SEM (Keunecke, 2009; Eynck *et al.,* 2009b) and susceptibility of cultivar Falcon (Knüfer, 2013; Eynck *et al.,* 2009b; Rygulla *et al,* 2007b).

In the first experiment, the antifungal activity of xylem sap samples extracted from 28 DPI old plants was investigated. This time point was chosen based on previous studies that identified this stage as critical period for significant accumulation of VL-resistance factors in resistant *B. napus* plants (Eynck *et al.,* 2009b; Obermeier *et al.,* 2013). Results showed that *B. napus* xylem sap provides a suitable medium for the growth of VL. Spore germination and further fungal growth were detected 2-3 days after incubation. From 5 DPI onwards, proliferated fungal growth that covered the whole area of the microtitre plate wells were observed. Comparison of fungal growth in xylem sap of resistant and susceptible plants however showed no significant difference until 7 DPI (Fig. 3.4 and 3.7; Appendix 3.3). Further comparisons of fungal growth in xylem sap of both genotypes revealed the absence of infection induced VL-resistance factor contributing to a significant reduction of *in vitro* fungal growth. Singh *et al.*, (2012) however suggested a possible role of pre-existing xylem sap proteins in defence of OSR against VL. In contrast, we observed slightly better fungal growth in xylem sap derived from infected plants, compared to control plants. Enhanced growth of the *V. dahlia*e in vascular fluid of nematode infected cotton cultivars have been reported previously (Katsantonis *et al.,* 2005). Despite the slightly reduced fungal growth on filtered xylem sap treatments, no significant effect of xylem sap filtration on the rate and level of VL growth was noticed. This observation is partly explained by the similar concentrations of total soluble protein measured in xylem fluid of the resistant and susceptible genotypes (Table 2) which is comparable concentration to previous reports in *B. napus* (Buhtz *et al.,* 2004), cotton (Singh *et al.,* 2013) and olive seedlings (Neumann *et al.,* 2010). On the other hand, the relatively higher protein concentrations found in infected plants indicate a slight, cultivar independent and infection-induced increase of protein levels in *B. napus* xylem sap. Since fungi use proteins as a source of nitrogen and sulphur (Walker and White, 2011), it is not surprising that enhanced fungal growth was observed in xylem sap samples with high protein concentrations. It was also observed that total soluble protein concentrations were slightly reduced in filtered xylem samples. This might be due to a slight protein binding property of the syringe filter (Whatman Anotop 10 plus sterile) used for the purification of xylem sap samples. Still, reduction of protein content in filtered xylem sap samples was not significantly different from the non-infected samples. This again is in agreement with the fungal growth bioassay results where insignificant difference in fungal growth between filtered and unfiltered xylem sap samples was observed.

The bioassay results from the present study are in agreement with some of the observations reported by Floerl *el al.* (2008) who compared leaf apoplast and xylem sap compositions of non-infected and VL-infected susceptible *B. napus* plants. According to this study, *B. napus* xylem sap provides a suitable medium for growth of VL and VL infection did not affect concentrations of xylem sap total proteins. In contrast, the study also showed a significant reduction of VL growth on xylem sap derived from infected VL-susceptible *B. napus* plants. As possible factors of VL growth inhibition, enhanced accumulation of two xylem sap proteins (PR-4 and β-1,3-glucanase) were suggested (Floerl *el al.,* 2008). More than 69 pre-existing secreted enzymes and proteins with various potential functions related to plant defence, cell wall modification and strengthening are known to exist in xylem sap of *B. napus* (Kehr *et al.,* 2005). However, whether the concentrations or the occurrence of these proteins is variable among *B. napus* varieties is not known. In addition, it is not known whether pathogenic or symbiotic interactions cause changes in *B. napus* xylem sap composition. In tomato for example, infection of resistant and susceptible plants with virulent and avirulent strains *V. albo-atrum* caused a general increase in the xylem sap amino acid concentration (Dixon and Pegg, 1972). In contrast, infection with the symbiotic bacterium *Bradyrhizobium japonica,* unlike the elicitor of the pathogenic fungi *Phytophthora sojae,* caused no significant increase in concentrations of xylem sap proteins in soybean (Subramanian *et al,* 2009).

With regard to defence related plant hormones, Ratzinger et al. (2009) reported the pre-existence of salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) in xylem sap of VL-susceptible rapid cycling rape (*B. napus*). This study clearly showed that following VL infection, concentration of SA is significantly increased while levels of JA and ABA are unaffected. Other studies also showed a lack of correlation between SA levels and VL-resistance in *B. napus*. Kamble et al. (2013) reported VL induced increased levels of SA in hypocotyl and leaf extracts of a susceptible winter OSR cultivar. This study also showed no role of the SA pathway marker gene (PR-1) in VL-resistance. Another study that compared susceptible and resistant genotypes revealed that VL infection induced significantly higher levels of SA in stem extracts of VL-susceptible cultivar compared to a resistant genotype (Siebold, 2012).

The most striking observation of the present study was the differential growth of VL in xylem saps from different aged plants. Unlike the sap of younger (13 to 21 DPI old) plants, xylem sap from 28 DPI old resistant and susceptible plants provided the highest level of fungal growth (Fig. 3.8; Table 3.4). This finding may explain disease development under field conditions. During early growth stages of *B. napus* in the field, VL has a characteristic long latency period. Once the crop reaches maturity stages, however, first symptoms as one-sided brownish discoloration of the stem are developed (Knüfer, 2013). Subsequently, the fungus grows rapidly and produces microsclerotia, which later burst out of the stem

tissue (Leino, 2006). VL might sense signals of crop maturity stages that might be present in xylem tissue. With these signals, the fungus might detect that the plant is on the way to complete its life cycle and this situation might induce an increase in growth of VL resulting in bursting out of the vascular tissues in the shoot and produce resting structures.

Given the fact that large amounts of sugar is present and transported via xylem (Loescher *et al.,* 1990; Iwai *et al.,* 2003) and increased sugar concentrations were found in xylem sap of older plants (Table 3.5), it is not surprising that we observed enhanced growth of VL in xylem sap of older plants. Increased concentration of sugar is suggested as an indigenous signal for transition from juvenile to adult stages of different plant species (Yu *et al.,* 2013). In tomato, xylem sap sugar concentration in healthy and nematode infected plants sharply increased with increase in plant age (Wang and Bergeson, 1974). In Arabidopsis, compared to 15 day old plants, significantly higher levels of sugars (Glc, Suc and Fruc) were detected in shoot extracts of 60 days old plants (Yu *et al.,* 2013). In the present study, the presence of about equal amounts of CHO irrespective of plant genotype also explains the equal growth of VL in xylem sap of resistant and susceptible plants. Similar results showing no correlation between xylem sap sugar content and resistance to the vascular dutch elm disease in different Ulmaceae species have been reported long time ago (Singh and Smalley, 1969).

Overall, our results support previous studies that showed that physical or cell wall bound resistance factors play a key role in cultivar-related resistance of *B. napus* against VL. Eynck et al. (2009b) first identified the hypocotyl tissue as a major battlefield of the *B. napus*-VL interaction and demonstrated the significance of physical defence structures. These mechanisms include *de novo* formation and a significant build-up of lignin as well as reinforcement of tracheary elements with cell wall bound phenolics and lignin. In another recent study focusing on the identification QTLs involved in VL resistance in different *B. napus* genetic backgrounds, a strong correlation between phenylpropanoids and VL resistance was found. More importantly, this study showed lower concentrations of the major lignin precursor (caffeic acid) in resistant lines compared to susceptible plants, suggesting substantial incorporation of this compound into lignin of cell walls (Obermeier *et al.,* 2013), thus again indicating the substantial role of physical barriers in cultivar-related VL resistance in *B. napus*. In general, the findings of the present study suggest that soluble xylem sap constituents are not playing a role as major resistance factors for cultivar-related winter OSR resistance against *V. longisporum*.

3.5 References

Atwell B. J., Kriedemann P. E., Turnbull C.G.N. 1999. Plants in action: adaptation in nature, performance in cultivation. Macmillan Education. South Yarra, Melbourne, Australia.

Bradford M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72: 248-254.

Broekaert W. F., Terras F. R. G., Cammue B. P. A., Vanderleyden J. 1990. An automated quantitative assay for fungal growth inhibition. *FEMS Microbiology Letters*, 69: 55-60.

Buhtz A., Kolasa A., Arlt K., Walz C. and Kehr J. 2004. Xylem sap protein composition is conserved among different plant species. *Planta*, 219: 610618.

Carré P. and Pouzet A. 2014. Rapeseed market, worldwide and in Europe. Oilseeds and fats crops lipids, 21 (1) D102.

Dixon G. R. and Pegg G. F. 1972.Changes in amino-acid content of tomato xylem sap following infection with strains of *Verticillium albo-atrum*. *Annals of Botany*, 36:147-54.

Dunker S., Keunecke H., Steinbach P., von Tiedemann A. 2008. Impact of *Verticillium longisporum* on yield and morphology of winter oilseed rape (*Brassica napus*) in relation to systemic spread in the plant. *Journal of Phytopathology,* 156: 698-707.

Eynck C., Koopmann B. and von Tiedemann A. 2009a. Identification of *Brassica* accessions with enhanced resistance to *Verticillium longisporum* under controlled and field conditions. *Journal of Plant Diseases and Protection*, 116(2), 63-72.

Eynck C., Koopmann B., Karlovsky P., and von Tiedemann A. 2009b. Internal resistance in winter oilseed rape inhibits systemic spread of the vascular pathogen *Verticillium longisporum*. *Phytopathology*, 99:802-811.

Floerl S**.,** Druebert C.**,** Majcherczyk A.**,** Karlovsky P.**,** Kües U. and Polle A. 2008. Defence reactions in the apoplastic proteome of oilseed rape (*Brassica napus* var. *napus*) attenuate *Verticillium longisporum* growth but not disease symptoms. *BMC Plant Biology,* 8:129.

Gomez K. A. and Gomez A. A. 1984. Statistical procedures for agricultural research, 2nd ed., John Wiley and Sons, New York.

Happstadius I., Ljungberg A., Kristiansson B. and Dixelius C. 2003. Identification of *Brassica oleracea* germplasm with improved resistance to *Verticillium wilt*. *Plant Breeding*, 122:30-34.

Heale J. B., and Karapapa K. V. (1999) The *Verticillium* threat to Canada's major oilseed crop: Canola. *Canadian Journal of Plant Pathology*, 21(1):1-7.

Iwai H., Usui M., Hoshino H., Kamada H., Matsunaga T., Kakegawa K., Ishii T. and Satoh S. 2003. Analysis of sugars in squash xylem xap. *Plant and Cell Physiology*, 44(6): 582-587.

Inderbitzin P., Davis R. M., Bostock R. M. and Subbarao K. V. 2011a. The Ascomycete *Verticillium longisporum* is a hybrid and a plant pathogen with an expanded host range. *PLoS ONE*, 6(3): e18260.

Johansson A., Goud J. C. and Dixelius C. 2006 Plant host range of *Verticillium longisporum* and microsclerotia density in Swedish soils. *European Journal of Plant Pathology*, 114:139-149.

Kamble A., Koopmann B. and von Tiedemann A. 2013. Induced resistance to *Verticillium longisporum* in *Brassica napus* by b-aminobutyric acid. *Plant Pathology*,62:552-561.

Karapapa V. K., Bainbridge B. W. and Heale J. B. 1997. Morphological and molecular characterization of *Verticillium longisporum* comb. nov., pathogenic to oilseed rape. *Mycological Research*, 101:1281-1294.

Kehr J., Buhtz A. and Giavalisco P. 2005. Analysis of xylem sap proteins from *Brassica napus*. *BMC Plant Biology*, 5(11):1-13.

Keunecke H.. 2009. Einfluss von Kohlfliegenbefall auf die Infektion und Schadwirkung von *Verticillium longisporum* und *Phoma lingam* an Raps. PhD thesis. Georg-August-Universität Göttingen, Göttingen, Germany.

Knüfer J. 2013. Improvement of winter oilseed rape resistance to *Verticillium longisporum* – Assessment of field resistance and characterization of ultrastructural plant responses. PhD thesis. Georg-August-Universität Göttingen, Göttingen, Germany.

Leino M. 2006. Fungal diseases on oilseed rape and turnip rape. Norrköping, Sweden.

Loescher W. McCamant H., T., and Keller J. D. 1990. Carbohydrate reserves, translocation, and storage in woody plant roots. *HortScience*, 25(3):274-281.

Masuko T., Minami A., Iwasaki N., Majima T., Nishimura S. and Lee Y. C. 2005. Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. *Analytical Biochemistry*, 339:69-72.

Neumann P. M., Weissman R., Stefano G. and Mancuso S. 2010. Accumulation of xylem transported protein at pit membranes and associated reductions in hydraulic conductance. *Journal of Experimental Botany*, 61(6):1711-1717.

Obermeier C., Hossain M. A., Snowdon R., Knüfer J., von Tiedemann A. and Friedt W. 2013. Genetic analysis of phenylpropanoid metabolites associated with resistance against *Verticillium longisporum* in *Brassica napus*. *Molecular Breeding*, 31:347-361.

Ratzinger A., Riediger N., von Tiedemann A. and P. Karlovsky. 2009. Salicylic acid and salicylic acid glucoside in xylem sap of *Brassica napus* infected with *Verticillium longisporum*. *Journal* of *Plant Research*, 122:571-579.

Rygulla W., Friedt W., Seyis F., Lühs W., Eynck C., von Tiedemann A. and R. J. Snowdon. 2007a. Combination of resistance to *Verticillium longisporum* from zero erucic acid *Brassica oleracea* and oilseed *Brassica rapa* genotypes in resynthesized rapeseed (*Brassica napus*) lines. *Plant Breeding*, 126:596-602.

Rygulla W., Snowdon R. J., Eynck C., Koopmann B., von Tiedemann A., Lühs W. and Friedt W. 2007b. Broadening the genetic basis of *Verticillium longisporum* resistance in *Brassica napus* by interspecific hybridization. *Phytopathology*, 97:1391-1396.

Rygulla W., Snowdon R. J., Friedt W., Happstadius I., Cheung W. Y. and Chen D. 2008.Identification of quantitative trait loci for resistance against *Verticillium longisporum* in oilseed rape (*Brassica napus*). *Phytopathology,* 98(2):215-221.

Scholander P. F., Hammel H. T., Bradstreet E. D., and Hemmingsen E. A. 1965. Sap pressure in vascular plants: Negative hydrostatic pressure can be measured in plants. *Science*, 16,148(3668):339-46.

Siebold M. 2012. Effects of rising air and soil temperatures on the life cycle of important pathogens in oilseed rape (*Brassica napus* L.) in Lower Saxony. PhD thesis. Georg-August-Universität Göttingen, Göttingen, Germany.

Singh D. and Smalley E. B. 1969. Nitrogenous and carbohydrate compounds in the xylem sap of *Ulmaceae* species varying in resistance to Dutch elm disease. *Canadian Journal of Botany*, 47(2):335-339.

Singh H., Dixit S., Verma P. C., and Singh P. K. 2013. Differential peroxidase activities in three different crops upon insect feeding. *Plant Signaling & Behavior*, 8(10):e25615.

Singh S., Braus-Stromeyer S. A., Timpner C., Valerius O., von Tiedemann A., Karlovsky P., Druebert C., Polle A., and Braus G. H. 2012. The plant host *Brassica napus* induces in the pathogen *Verticillium longisporum* the expression of functional catalase peroxidase which is required for the late phase of disease. *Molecular Plant-Microbe Interactions*, 25(4):569-581.

Subramanian S., Cho U. H., Keyes C. and Yu O. 2009. Distinct changes in soybean xylem sap proteome in response to pathogenic and symbiotic microbe interactions. *BMC Plant Biology*, 9(119):1-11.

Veronese P., Narasimhan M. L., Stevenson R. A., Zhu J. K., Weller S. C., Subbarao K. V. and Bressan R. A. 2003. Identification of a locus controlling Verticillium disease symptom response in *Arabidopsis thaliana*. The *Plant Journal,* 35(5):574-587.

Wang E. L. H. and Bergeson G. B. Biochemical changes in root exudate and xylem sap of tomato plants infected with *Meloidogyne incognita*. *Journal of Nematology,* 6(4): 194-202.

Yadeta K. A. and Thomma B. P. H. J. 2013. The xylem as battleground for plant hosts and vascular wilt pathogens. *Frontiers in Plant* Science, 4(97):1-12.

Yu S., Cao L., Zhou C. M., Zhang T. Q., Lian H., Sun Y., Wu J., Huang J., Wang G. and Wang J. W. 2013. Sugar is an endogenous cue for juvenile-to-adult phase transition in plants. *eLife*, 2:e00269.

Zeise K. and von Tiedemann A. 2002. Host specialization among vegetative compatibility groups of *Verticillium dahliae* in relation to *Verticillium longisporum*. *Journal of Phytopathology,* 150, 112-119.

4. Physiology, gene expression and agronomic performance of winter oilseed rape under drought stress and infection with *Verticillium longisporum**

*Parts of this chapter have been submitted for publication to Phytopathology. Manuscript ID PHYTO-07-16-028/0-R: Lopisso T. D., Knüfer J., Koopmann B. and von Tiedemann A. **The vascular pathogen *Verticillium longisporum* does not affect water relations and plant responses to drought stress of its host, *Brassica napus*.**

Department of Crop Sciences, Division of Plant Pathology and Crop Protection, Georg August University, Grisebachstr. 6, 37077 Göttingen, Germany; 2Current address: Strube Research GmbH & Co. KG, 38387 Söllingen, Germany

4.1 Introduction

Oilseed rape (OSR) ranks among the top four largely produced oil crops in the world. The economic importance of OSR is increasing overtime and at present it is intensively cultivated in more than 60 countries worldwide (FAOSTAT, 2014). However, the increasing intensive production particularly due to the growing demands for biodiesel (Carré and Pouzet, 2014; Zentková and Cvengrošová, 2013) favored the emergence of new economically important diseases such as *Verticillium longisporum*. *V. longisporum* recently evolved as a host specific vascular pathogen with host specificity to cruciferous species (Karapapa *et al.,* 1997; Inderbitzin *et al.,* 2011a) and a potential to cause substantial yield losses (Dunker *et al.*, 2008). Since the 1990s, it is becoming a major threat to oilseed rape production particularly in northern European countries (Dunker *et al,* 2008). Simultaneously, the geographical distribution of *V. longisporum* is widening and more recently its presence is reported from Japan, Illinois and the UK (Babadoost *et al.,* 2004; Ikeda *et al.,* 2012; Gladders *et al.,* 2011; CABI/EPPO, 2011).

As a soil-borne pathogen, VL infects OSR by direct penetration of lateral roots and root hairs. Once it is inside the root cortex cells, it starts to systemically colonize root and shoot xylem vessels where it spends most of its life cycle (Zhou *et al.,* 2006; Eynck *et al.,* 2007). The typical symptoms caused by VL begin with the development of one-sided yellowing of leaves followed by vein clearing, complete foliar chlorosis and premature senescence and ripening. Artificially inoculated susceptible plants under controlled conditions also show severe stunting of shoots, reduction of root length and excessive production of side branches. Towards crop maturity, infected plants show brownish stripes along the stems and subsequently, the pathogen bursts out of the xylem vessels and produces blackish layers of elongate microsclerotia underneath the stem epidermis, in the stem pith and roots. When diseased plants start senescence and during crop harvest, enormous amounts of highly durable microsclerotia are

released in the soil where they may survive in dormancy for more than a decade (Heale and Karapapa, 1999; Berg *et al.,* 1999; Leino, 2006; Eynck, 2008).

The nature of systemic colonization and the extremely durable survival of VL greatly have hampered its control and limited the options available for combating this disease. To date, there are no fungicides that effectively work against VL and cultural methods such as crop rotation are not reliable. Consequently, current control of VL solely relies on the use of OSR genotypes with enhanced resistance (Dunker *et al.,* 2008). Previous breeding efforts aimed at developing resistant varieties appeared to be successful. At present, OSR genotypes that show different levels of resistance towards VL are available (Eynck *et al.,* 2009a; Rygulla *et al.,* 2007b; Rygulla *et al.,* 2007a). It is also known that pathogen-induced histological and biochemical changes in the vascular tissue of infected OSR, including accumulation of soluble and cell wall-bound phenolics, vascular occlusions and cell wall-bound lignin, are major resistance mechanisms responsible for restricting VL below the hypocotyl and preventing its further spread into the shoot (Eynck *et al.,* 2009b). Obermeier *et al.* (2013) who recently conducted a QTL analysis of phenylpropanoid metabolites associated with VL resistance provided additional evidence for the involvement of phenolic compounds and lignin in *B. napus* resistance against VL. Nevertheless, whether these defence mechanisms and structural changes in the xylem vessels are at the expense of yield or have further negative secondary consequences on water and nutrient relations, particularly during drought seasons, is not known.

In an agronomic context, drought refers to a situation where the amount of moisture in the soil no longer meets the needs of a particular crop (Stanke *et al.,* 2013). This is one of the most adverse abiotic factors threatening crop growth and productivity (Anjum *et al.,* 2011). Drought was long considered a phenomenon affecting mainly developing countries in the arid regions. But, this may change since these days some areas in the temperature climate regions are also experiencing drought. For instance, due to an increase in frequency, duration or intensity of low flows, the drought situation in some European regions has become more severe in the last decades. Besides, a further increase of drought driven by climate change particularly in southern European areas is expected (Lehner and Döll, 2001). According to long-term climate change projections, there could be more frequent and severe droughts in certain areas of the world and such impact could also become more marked in climate zones where oilseed rape is grown.

Drought generally affects plant growth and development by interfering with water and nutrient relations, photosynthesis, assimilate partitioning, and other related metabolic processes. Disturbance of

the aforementioned processes in turn leads to reduced growth, wilting, leaf senescence and finally severe yield losses and complete death of plants (Farooq *et al.,* 2009). In Brassica crops, drought stress severely affects leaf chlorophyll content, photosynthesis, transpiration and stomatal conductance (Din *et al.,* 2011; Singh *et al.,* 2009). It causes a delayed and prolonged period of flowering and reduces leaf area index, branching, silique production, plant biomass formation, and grain yield (Sadaqat *et al.,* 2003; Müller *et al.,* 2010; Zakirullah *et al.,* 2000). Drought also reduces seed oil content (Sinaki *et al.,* 2007) and increases the glucosinolate content which in turn influences oil quality (Moghadam *et al.,* 2011; Jensen *et al.,* 1996) and susceptibility to diseases and insect herbivory (Mithen, 1992).

As a response to drought stress, plants display a range of physiological, morphological, biochemical and molecular changes. Mechanisms of drought resistance in plants vary among species, varieties and even depend on plant age. Nevertheless, all mechanisms involve either avoidance or tolerance of dehydration and this can be achieved either by enhancement of water use efficiency or reduction of transpiration losses (Blum, 2005). The mechanisms of drought tolerance in plants include possession of prolific and deep root systems, smaller and succulent leaves, reduced numbers of stomata, and production of trichomes or waxes on leaves. Accumulation of higher amounts of compatible solutes, amino acids, organic acids, and sugar alcohols are reported as crucial factors to sustain cellular functions during drought periods. Plant growth hormones like abscisic acid also regulate the response of plants towards drought stress (Farooq *et al.,* 2009; Valliyodan and Nguyen, 2006). Several genes, transcription factors or proteins are known to be induced by drought stress and to regulate the abovementioned morpho-physiological and biochemical changes in various crop species (Xiang *et al.,* 2007; Cong *et al.,* 2008; Jun-Wei *et al.,* 2006; Tang *et al.,* 2005).

Like in other crop species, a remarkable variation regarding drought tolerance exists among *Brassica* species. Ashraf and Mehmood (1990) compared drought resistance of different *Brassica species* and found *B. napus* and *B. carinata* to be the most drought-tolerant and drought-sensitive species, respectively. The crop growth stage also significantly determines the sensitivity of *B. napus* to drought stress (Naderikharaji *et al.,* 2008). Different morphological, biochemical and genetic traits are known as key indicators of drought stress resistance in *B. napus*. These include possession of deep root system and osmotic adjustment (Mahmood *et al.,* 2004; Kumar and Singh, 1998), accumulation of enzymes (cystein proteinases and acid peroxidases) involved in xylem differentiation and lignification (Fernandez-Garcia *et al.,* 2011) and increased accumulation of free proline in leaf and stem tissues (Din *et al,* 2011; Xue *et al.,* 2009). Furthermore, although only very few are well studied, a number of putative genes

involved in drought and dehydration stress resistance have been identified in *B. napus.* Examples are the Drought-responsive element binding factor (*DREB*) transcription factors which control the expression of several drought inducible genes (Yang *et al.,* 2010) known to be induced by drought (Kagale *et al.,* 2007) and cold stress (Gao *et al.,* 2002; Lindemose *et al.,* 2013). DREBs also regulate chloroplast development and photosynthetic capacity during cold stress (Savitch *et al.,* 2005). The recently identified Calcineurin B-like (CBL) protein interacting calcium-dependent protein kinases (CPKs), called CIPK genes, are also known to be induced by high salinity and osmotic stresses (Chen *et al,* 2012). Another group of drought induced genes called HD-zip (Homeodomain leucine-zipper) genes are known to be up-regulated by osmotic treatment (Yu *et al.,* 2005). Late embryogenesis abundant (LEA) genes are responsible for enhanced growth and yield under drought stress conditions (Park *et al.,* 2005; Dalal *et al.,* 2009). The abiotic stress related plant hormone ABA, which mediates stomatal closure and developmental changes during water stress (Hartung *et al.,* 1999), is increased by drought stress (Qaderi *et al.,* 2006) but not affected by VL-infection in *B. napus* (Ratzinger et al., 2009).

When plants are exposed to drought stress in the presence of pathogen stress, they may either benefit or suffer from the complex interactions between the pathogen and the abiotic stress (drought). In general, three possible interactions may be expected. First, drought stress can have a direct effect on pathogen survival. Second, drought by changing host and climatic conditions, may indirectly affect the pathogen potential. And third, drought alone and its interaction with the pathogen can potentially affect the host plant resistance (Desprez-loustau *et al.,* 2006). The effect of drought stress on disease development and host susceptibility seems to be dependent on the pathosystem. On one side, drought may be helpful for the plant if it leads to the so called cross-resistance. Conversely, it may aggravate the disease development or increase host susceptibility to pathogens. For instance, drought stress may enhance resistance of barley to powdery mildew caused by *Blumeria graminis* f. sp. *hordei* (Wiese *et al.,* 2004); it also resulted in a significant decrease in *Botrytis cinerea* infection of tomato *(Lycopersicon esculentum)(*Achuo *et al.,* 2006), and reduced *Verticillium albo-atrum* disease symptoms in a highly susceptible alfalfa clone (Pennypacker *et al.,* 1991). Reduced root growth caused by drought stress can reduce the chance of root contact with pathogen propagules in the soil leading to lower incidence of infection. Drought can also activate plant defense pathways (Pertot and Elad, 2012). Foliar pathogens in general tend to have lower infection success under dry conditions (Garrett *et al.,* 2006). However, contradicting results are reported from other pathosystems. Newton and Young (1996) showed that drought causes loss of barley resistance to powdery mildew (*Erysiphe graminis* f.sp. *hordei*). It also increased the severity of Phytophthora root rot in tomato (Ristaino and Duniway, 1989). Drought may

alter host plant physiology favoring pathogen colonization. For example increased concentration of sugars (carbohydrates) and amino acids (proline, asparagine, alanine) in drought stressed forest trees may stimulate growth of different fungal pathogens (Desprez-loustau *et al.,* 2006).

The diverse effects of drought in different pathosystems suggests that studying the significance of drought stress on plant resistance as well as on the extent of disease development particularly for vascular pathogens whose effect mimic drought stress, is very critical. Nevertheless, the effect of drought stress in *B. napus* - *V. longisporum* interaction is not yet understood. More importantly, it is not known whether VL-resistance related defence mechanisms or structural changes in the host plant are associated with physiological effects or yield penalties. Understanding the *B. napus* - *V. longisporum* pathosystem under drought conditions provides a hint on how to deal with the effects of this host specific pathogen during periods of water deficit. With this background, the present comprehensive study therefore aimed to address the following objectives:

Objectives:

- to evaluate the level and rate of *V. longisporum* disease development during drought stress periods
- to investigate the effects of *V. longisporum* infection on plant physiology and host response to drought stress
- to study the possible consequences of *B. napus* internal *V. longisporum* resistance mechanisms on plant performance, yield attributes and resistance to drought stress
- to assess the main and interactive effects of VL infection and drought stress factors on plant physiology, agronomic features and expression of drought induced genes
- to verify whether *B. napus* resistance towards *V. longisporum* is stable under drought stress conditions

4.2 Materials and methods

4.2.1 Treatments and experimental conditions

A factorial pot experiment was installed in a Completely Randomized Design (CRD) under greenhouse conditions and repeated twice. The study consisted of a combination of three experimental factors resulting in 12 treatments. The experimental factors were genotype (two winter oilseed rape genotypes 'Falcon' and 'SEM 05-500256'), disease (mock-inoculated and VL-inoculated) and three watering levels (100, 60 and 30% field capacity) (Table 4.1). Treatments were arranged in a randomized fashion (Fig. 4.1)

with three biological replicates each composed of 5 plants grown independently in separate pots. Mock- and/or VL-inoculated plants supplied with water at 100% field capacity were used as control.

Table 4.1 Treatment designation and descriptions

No.	Designation	Experimental factors and descriptions		
		Genotype	Disease	Drought
1	SM1	SEM 05-500256	Mock-inoculated	100% FC
2	SM2	SEM 05-500256	Mock-inoculated	60% FC
3	SM3	SEM 05-500256	Mock-inoculated	30% FC
4	SV1	SEM 05-500256	VL-inoculated	100% FC
5	SV2	SEM 05-500256	VL-inoculated	60% FC
6	SV3	SEM 05-500256	VL-inoculated	30% FC
7	FM1	Falcon	Mock-inoculated	100% FC
8	FM2	Falcon	Mock-inoculated	60% FC
9	FM3	Falcon	Mock-inoculated	30% FC
10	FV1	Falcon	VL-inoculated	100% FC
11	FV2	Falcon	VL-inoculated	60% FC
12	FV3	Falcon	VL-inoculated	30% FC

FC, Field capacity. **100% FC**, maximum watering. **60% FC**, moderate drought stress. **30% FC**, severe drought stress.

Figure 4.1 Partial view of experimental arrangement in a completely randomized design in the greenhouse experiment (14 DPI).

4.2.2 Determination of field capacity in the soil substrate

A substrate composed of commercial soil (Fruhstorfer Erde, Type; pH 6.0; Hawita-Gruppe, Vechta, Germany) and river sand in a 3:1 ratio was used. For determination of substrate gravimetric moisture content at field capacity the method described in Grzesiak *et al.* (2013) and Somasegaran and Hoben (1985) was adopted. Briefly, plastic cylinders (2.5 cm radius and 20 cm height) with a drainage plastic net at the bottom were filled with gravel (20-50 mm diameter) up to 3 cm in six replications and placed for 15 min in a container filled with tap water. Cylinders were then covered with aluminum foil and placed on a wire grid for 15 min to drain the excess water. Subsequently their weight was measured using an analytical balance (Wt_1). Then, 150 g of oven dried (105°C for 24 hours) substrate was added to each cylinder and cylinders were placed in the water container for two hours. Additional water was gently poured on the surface of the substrate until it was fully saturated. After saturation, cylinders were removed from the water container and allowed to stand on a wire grid for two hours to drain the excess water through holes in the bottom of the cylinders (until drainage equilibrium) with the top covered to prevent evaporation. Cylinders were then placed on absorbent paper for 15 min to drain the excess water held in the spaces between gravel particles and then a second weight was recorded (Wt_2). Moisture content of the substrate at this stage was assumed to be equal to field capacity and calculated as FC_{100}= [(Wt_2 - substrate oven dry weight) - Wt_1)]. This water status was considered as moisture content at field capacity (FC_{100}) and was maintained throughout the experiment period for control treatments. Accordingly, 60 and 30% of the water content at FC_{100} were used to make 60% (FC_{60}) and 30% (FC_{30}) field capacity treatments, respectively (Abedi and Pakniyat , 2010).

4.2.3 Plant material and growing conditions

Two winter oilseed rape genotypes expressing contrasting levels of resistance against *V. longisporum* were used. Seed of the VL- susceptible commercial winter oilseed rape cultivar Falcon was purchased from Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (NPZ, Hohenlieth, Germany). The VL-tolerant *B. napus* accession SEM 05-500256 seed was obtained from Lantmännen SW Seed (Svalöv, Sweden). Seeds of both genotypes were double surface sterilized with 70% ethanol for two minutes under constant shaking and rinsed with autoclaved tap water. Seeds were then sown in multi-pot trays containing autoclaved silica sand and soil (1:1) and grown in a climate-controlled chamber (20°C temperature, 16 hours light and 60% relative humidity). Two weeks after sowing (BBCH 12), seedlings were subjected to vernalization for 13 weeks by gradually lowering the temperature and day length to 4°C and 10 hours, respectively. Before seedlings were inoculated with VL, acclimatization was performed for three days by keeping vernalized seedlings under 10°C and 16 hours light conditions. After

inoculation, plants were transplanted to plastic pots (1.5 liter) filled with a substrate composed of commercial soil (section 4.2.2) and river sand in a 3:1 ratio and grown under greenhouse conditions. Average climatic conditions during the whole experimental period in the greenhouse were 16 hours light, 24°C temperature and 70% relative humidity. All other agronomic practices such as fertilization (*Hakaphos* COMPO, Germany) were equally applied to all treatments according to the manufacturer's recommendations.

4.2.4 Fungal isolate and inoculation procedures

Verticillium longisporum isolate VL43 (Fig. 1.2), originally isolated from *B. napus* in Northern Germany and highly virulent on OSR (Zeise and Tiedemann, 2001) was used. For inoculum preparation, 500ul of VL43 spore stock solution (VL conidial suspensions in PEGB supplemented with 25% glycerol and stored at -80°C) was added to 250 ml of PEGB and propagated for 10 days at 23°C in the dark with constant shaking. The culture was subsequently filtered through sterile gauze and spore concentration was adjusted to 10^6 conidia/ml using a haemocytometer (Thoma chamber, 0.100 mm depth, Germany). Inoculation was performed following a standard cut-root dip inoculation technique where roots of vernalized seedlings (BBCH 14) were washed, injured by cutting the tips with a sterile scissor, and submerged in the spore suspension for 30 min. Control plants were dipped in the same way using autoclaved tap water. After inoculation, seedlings were transplanted into plastic pots and grown in the greenhouse under standardised conditions as described in section 4.2.3.

4.2.5 Determination of soil water content and maintenance of water regime

The amount of water to be supplied to plants in each treatment was calculated considering the initial pot weight at different field capacity levels and plant biomass increment as well (Earl, 2003; Champolivier and Merrien, 1996; Grzesiak *et al.,* 2013). Initial pot weight in each treatment was determined from the weight of substrate (1 kg), amount of water required to make up the different field capacities (considering initial substrate moisture content) and plant weight. To determine the weekly plant biomass increment, 15 randomly selected plants from each treatment were carefully uprooted, roots were washed under running tap water to remove adhering soil particles and the total fresh weight was determined. During the first three weeks after inoculation (until 21 DPI), all plants were uniformly supplied with water at FC_{100}. For drought stress treatments, watering at FC_{60} and FC_{30} was started at 21 DPI when a reasonable acclimatization of transplanted seedlings, successful colonization of roots by the pathogen and sufficient extent of resistance responses were attained (Eynck *et al.,* 2009b). The water

consumption in each pot was daily monitored by weighing the pots. Re-watering was done to restore the soil water content according to the individual field capacity treatment.

4.2.6 Plant sample collection and preservation

For non-destructive measurements (disease severity, physiological and agronomic data), a total of 30 plants per treatment, 15 VL-inoculated and 15 mock-inoculated, each representing three biological replicates consisting of 5 plants, were used. Similar numbers of plants from each treatment were weekly harvested for biochemical and molecular analyses involving destructive sampling. Leaf and hypocotyl samples were frozen in liquid nitrogen immediately after harvest and stored at -80°C until further analysis.

4.2.7 Disease assessment

4.2.7.1 Area Under Disease Progress Curve

Disease evaluation was performed using an assessment key with nine classes (Table 4.2). AUDPC values were calculated from weekly disease severity recordings using the formula:

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

Where y_i = disease severity at the ith observation, t_i = the time (days after inoculation) at the ith observation and n= total number of observations (Campbell and Madden, 1990). To obtain the Net AUDPC values ($AUDPC_{net}$), mock-inoculated plants were assessed in the same way for natural (age-mediated) yellowing and senescence ($AUDPC_{mock}$) and this value was subtracted from corresponding VL-inoculated treatments ($AUDPC_{inoc}$). That is $AUDPC_{net} = AUDPC_{inoc} - AUDPC_{mock}$ (Eynck *et al.,* 2009b).

4.2.7.2 Relative stunting

Stunted growth is one of the typical greenhouse symptoms observed in susceptible OSR plants infected with *V. longisporum*. To evaluate this effect, plant height (from the base of hypocotyl to the tip of the central main shoot) was weekly measured with a ruler. Relative stunting (RS) referring to the reduction of plant height in inoculated plants (h_{inoc}) relative to plant height of mock-inoculated (h_{mock}) plants was calculated as RS (%) = 100-[(h_{inoc} x 100)/(h_{mock})].

Table 4.2 Disease scoring index for assessment of *Verticillium longisporum* disease severity in *Brassica napus* (modified from Zeise, 1992).

Index	Disease symptom
1	Healthy plants with no symptoms
2	Up to 10% of leaves on the main stem show either yellowing and/or black veins or are dead
3	11-20% of leaves on the main stem show either yellowing and/or black veins or are dead
4	21-40% of leaves on the main stem show either yellowing and/or black veins or are dead
5	41-60% of leaves on the main stem show either yellowing and/or black veins or are dead
6	61-80% of leaves on the main stem show either yellowing and/or black veins or are dead
7	81-100% of leaves on the main stem show either yellowing and/or black veins or are dead
8	Only the terminal bud (apex) is still alive
9	The plant is dead

4.2.7.3 RT PCR based quantification of *V. longisporum* infection

DNA extraction

A modified CTAB DNA extraction protocol (Brandfass and Karlovsky, 2008) was used for extraction of genomic DNA from hypocotyl tissue. Briefly, hypocotyl samples from VL- and mock-inoculated plants were collected at 28 and 49 DPI as described in section 4.2.4, immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Hypocotyl samples were lyophilized for 72 h (freeze dryer ALPHA 1-4, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and grinded with a mixer mill (MM 200, Retsch GmbH, Haan, Germany). Subsequently, 100mg of hypocotyl powder was transferred to 2ml tubes and 1ml of CTAB buffer, 1µL of proteinase K (20 mg/mL), and 2µL mercaptoethanol were added and the sample was mixed by vortexing. Then 3µL of RNase A (10mg/ml) solution was added and the sample was incubated at 37°C for 15 min. After chloroform-isoamyl alcohol extraction, the sample was centrifuged (16,060 x g) and the upper aqueous phase (600µL) was transferred to 1.5mL tubes containing 194µL of 30% PEG solution and 100µL of 5M NaCl. The DNA was pelleted by centrifugation (16,060 x g), washed twice with 70% ethanol and let to dissolve overnight in 200µl of TE buffer at 4°C. The quality and concentration of DNA was examined by loading 5µL of DNA sample on 1% (w/v) agarose gel (Agarose low EEO*,* AppliChem GmbH, Germany) prepared in TBE buffer. Electrophoresis was carried out at 3 V/cm for 60 min and gel was visualized by ethidium bromide staining and documented using a digital imaging system (Bio-Rad Gel Doc 1000). Using DNA of bacteriophage Lambda as a standard, concentration of DNA was quantified by densitometry using

Quantity One® software (Version 4.5.0 Bio-Rad Laboratories). DNA samples were stored at -20°C until qPCR analysis.

Preparation of VL DNA standard for qPCR

Total genomic DNA of *V. longisporum* was extracted from isolate VL43 grown on potato extract glucose broth following the protocol described in section 2.2.4. Briefly, fungal mycelium was filtered through filter paper, freeze dried and then crushed in liquid nitrogen. Subsequently, DNA was extracted using a modified CTAB DNA extraction protocol (Brandfass and Karlovsky, 2008). Concentration of DNA was quantified as described in section 4.2.7.4 and a dilution series of 1ng to 0.001pg of *V. longisporum* DNA with a dilution factor of 10 was produced in 1X TE buffer.

Quantitative real-time PCR (qPCR)

In this procedure, a highly species specific primer pair developed for quantification of *V. longisporum* infection in OSR (Knüfer, 2013) was used. Using these primers (sense primer OLG70 5´CAGCGAAACGCGATATGTAG 3´ and antisense primer OLG71 5´GGCTTGTA GGGGTTTAGA 3´) which amplify a 261 bp fragment of the ITS region, a real time PCR was performed using ABsolute Blue SYBR Green Fluorescein Mix (ABgene, Hamburg, Germany) in a CFX384 real-time PCR detection system (Bio-Rad laboratories, Inc). Each RT-qPCR reaction had 5-10ng of template DNA, 5µl of ABsolute Blue QPCR SYBR Green Fluorescein Mix, 0.3 µM of forward and reverse primers and ddH_2O to give a final volume of 10µl. The amplification protocol was as follows: initial enzyme activation step 15 min at 95°C and 40 cycles of 95°C for 10 s (denaturation), 60°C for 15 s (primer annealing) and 72°C for 15 s (extension). Reaction was completed with a final elongation step of 72°C for 2 min. For evaluation of the amplification specificity, a melting curve analysis (55°C to 95°C) with a heating rate of 0.5°C/5 s was used. PCR for all treatment samples were performed with three biological and technical replicates and data was analyzed using CFX Manager Software (Bio-Rad laboratories, Inc).

4.2.8 Physiological data

4.2.8.1 Gas exchange

The leaf gas exchange parameters, photosynthesis rate (A), transpiration rate (E) and stomatal conductance (gs), were measured using a portable infrared gas analyzer LCpro+ (ADC BioScientific Ltd., Hertfordshire, UK) equipped with a broad leaf chamber and laser-trimmed humidity sensor. Measurement was performed *in situ* from the upper 3rd fully developed leaves at 28 and 49 DPI (one and four weeks after the beginning of the drought stress treatment, respectively) representing the beginning of flowering and fruit development stages, respectively. Conditions during measurement were as

follows: projected leaf surface area was 6.25 cm^2; PAR (Photosynthetically Active Radiation) at the leaf surface was set to 700 µmol m^{-2} s^{-1}; and relative humidity and CO_2 concentration were set to ambient greenhouse conditions, 70% and 500µmol mol^{-1}, respectively. Each parameter was measured from 5 independently grown individual plants from each treatment. Water use efficiency (WUE) was computed as the ratio of photosynthesis to transpiration rate.

4.2.8.2 Relative water content

Leaf relative water content (RWC) was determined gravimetrically from the 3rd upper fully expanded leaves (Mationn *et al.,* 1989). Briefly, leaf discs (≈10cm^2) were excised (excluding the midrib) and fresh weight (FW) was immediately determined on an analytical balance. Subsequently, leaves were immersed in double distilled water in petri dishes for three hours in the dark and the turgid weight (TW) was taken after blotting. Thereafter, dry weight (DW) was obtained after oven drying of the leaves overnight at 70°C. Percent leaf relative water content was calculated using the formula: RWC = [(FW-DW) / (TW-DW)] x 100. For each replication, measurement was done from five leaf samples taken from five independently grown plants.

4.2.8.3 Proline content

Accumulation of free proline content in leaf and hypocotyl samples was determined according to the method of Bates *et al.,* 1973. In this procedure, 100mg of leaf or hypocotyl milled samples prepared as described in section 2.2.7.4 were homogenized in 2ml of 3% sulfosalicylic acid. After centrifugation (9,503 x g) for 10 min at room temperature (RT), 500µl of the supernatant was reacted with equal volumes of acid-ninhydrin solution and glacial acetic acid in a water bath for 1 h at 100°C. Known concentrations of L-proline (Fluka Biochemika) ranging between 150 - 0.5 µg/ml were prepared in 3% aqueous sulfosalicylic acid and treated the same way as plant extract samples. The reaction was terminated by placing tubes on ice and proline was extracted by adding 800µl of toluene into 1,200µl of the reaction mixture. Subsequently, 200µl of the chromospheres containing toluene was loaded on 96-well microtitre plate and absorbance was read in triplicates at 520 nm using a Microplate Spectrophotometer (µQuant, Bio-Tek Instruments, Bad Friedrichshall, Germany). Toluene was used as a blank and concentration of proline in plant samples was estimated by referring to the standard curve produced from known concentrations of L-proline. Finally, µmoles of proline per gram of leaf and hypocotyl dry weight samples were calculated using the formula: [(µg proline/ml × ml toluene) / (115.5µg/µmole)] / [(g sample) / (5)].

4.2.9 Measurement of yield and agronomic variables

In order to assess the magnitude of yield reduction and changes in morphological traits due to VL-infection under drought stress conditions, several agronomic parameters were measured. Production of side shoots was assessed by counting the number of primary branches per plant. Growth rate was determined by evaluating phenological growth stages according to the BBCH scale (Appendix 4.13). Days to 50% flowering were calculated as the number of days (DPI) required for 50% or more plants in a treatment to reach growth stage BBCH 65. Assessments of biomass yield and hypocotyl thickness were performed at the end of the experiment (49 DPI). Stem diameter at the tip of hypocotyl was measured using a digital calliper. Root fresh weight was estimated after removing soil and water adhering to the root surface by washing and blot drying. For determination of shoot fresh biomass yield, the whole portion of the above-ground plant part (above the hypocotyl) was weighed immediately after harvest. To determine dry biomass yield, root and shoot samples were oven dried (70°C for 24 h) and weighed separately. Total fresh and dry matter was expressed as the sum of root and shoot biomass weights.

4.2.10 Expression of drought responsive genes

Upon infection with *V. longisporum*, resistant OSR plants respond by increased lignification and accumulation of vascular occlusions (Eynck *et al.,* 2009b). These histological changes in vascular tissue may interfere with water and nutrient transport and might make plants more sensitive to drought stress. In order to verify this hypothesis, expression of drought responsive genes (DR-genes) under optimum watering and severe drought stress as well as VL-infection conditions were investigated.

4.2.10.1 Primer sequence of studied genes

Genes that show contrasting expression levels and encode for functional and regulatory proteins involved in drought stress tolerance and response of *B. napus* were selected from previous studies. Gene specific primers (Table 4.3) which amplify partial sequences of the selected genes were either taken from the literature or designed using primer3 software (http://www.ncbi.nlm.nih.gov/tools/primer-blast).

4.2.10.2 PCR amplification and analysis of PCR products

Optimization of primer annealing temperatures and concentration of PCR components were carried out by running a gradient PCR using a T GRADIENT Thermocycler (Biometra, Göttingen, Germany). After optimization, all genes were amplified in a 25µl total reaction volume composed of 10x PCR buffer, 2.5mM of each dNTPs, 50mM $MgCl_2$, 1 unit of BioTaq Taq DNA polymerase (Bioline GmbH, Luckenwalde, Germany), 25pmol of each primer and 10ng of template DNA. Amplification was performed by heating samples at 95°C for 2 min, followed by 34 cycles each consisting of denaturation at 95°C for 2 min,

annealing at 64°C for 1 min and elongation at 72°C for 1 min. The reaction was terminated with a final extension step of 10 min at 72°C. In order to insure reproducibility, reaction was repeated trice. To verify the amplicon size of each gene, PCR products were electrophoresed in 1.2% (W/V) TBE-agarose gel, stained with ethidium bromide and visualized under UV light as described in section 2.2.7.4. In addition to PCR amplification, specificity of amplification was further confirmed by cloning and sequence analysis of partial DR gene fragments.

Table 4.3 Sequences of primer pairs used in RT-PCR

Gene name	Primer sequence	Source
BnActin	5‘- GCCCAGAAGTCTTGTTCCAG-3‘ (F) 5‘- GAACCACCGATCCAGACACT-3‘ (R)	Lee *et al.,* 2008
BnCBF17	5’- AATGATCATGGCATGAACATGGC-3’ (F) 5’- ACTCCTCGTCCATGTAAAACCC-3’ (R)	Gao *et al.,* 2002
BnCIPK1	5‘- GACCGTGGAAAGGACTCCATGAAGCATC-3‘ (F) 5‘- TCTGGCGGCTTCCAGATGATGT-3‘ (R)	This study
DREB2-23	5‘- GAGCTGTCCGAAGAAACCTG-3‘ (F) 5‘- ATAACTCCAAAGGGACACGTC-3‘ (R)	This study
BnHB6	5'- ATGATGAAGAGATTAAGCAGTTCAGA-3‘ (F) 5'- GTGATCCTCCGTCTGCTCCA-3‘ (R)	Yu *et al.,* 2005
BnP5CS1	5’-CGATTTGGACTTGGTGCTGA-3’ (F) 5’-GCCCATCCTCTCCTAGTCTC-3’ (R)	Xue *et al.,* 2009
BnP5CS2	5’-CCATTATCTTCCTCCTCTCAC-3’ (F) 5’-AACAACTGCTGTCCCAA CC-3’ (R)	Xue *et al.,* 2009
BnLEA4-1	5’-ATGCAGTCGATGAAGGAAACAGC-3’ (F) 5’-CCGGCGGAGGATATACTGGATAA-3’ (R)	This study
ME-leaN4	5’-GGCAAGGACAAGACTTCCCA-3’ (F) 5’-CGGATCAGTGCTCTGAGTAG-3’ (R)	Park *et al.,* 2005

4.2.10.3 Isolation and cloning of drought induced genes

Purification of PCR products from gel

PCR products of DR-gene partial DNA fragments were generated using a high fidelity DNA polymerase (*Pfu* DNA polymerase recombinant, Fermentas) which exhibits 3’ to 5’proof reading activity that enable the correction of nucleotide incorporation errors. PCR was conducted in 25µl reaction volume containing 10ng template DNA, 10X *Pfu* buffer with $MgSO_4$, dNTP mix (0.2mM each), 25 pmol of each primer, and 0.5 unit of recombinant *Pfu* DNA polymerase (Fermentas). A similar PCR program as described in 4.2.10.2 was used for amplification. After examining the PCR products on 1% low melting point agarose gel in 1X TAE buffer, PCR products were purified by extracting DNA fragments from a gel. Briefly, the amplicon was excised from the gel without exposing to UV and immediately weighed and

dissolved in 3 volumes (V/W) of DNA-binding buffer (6M NaCl, 50mM Tris pH 7.6, 10mM EDTA pH 8.0) for 10 min at 50°C. Then, 6µl of glass milk was added and the mixture was incubated for 10 min at RT with constant mixing. The matrix was then pelleted by centrifugation at 16, 060 x g for 30s. After repeating the DNA binding step by adding 500µl DNA binding buffer, the pellet was washed twice by resuspending in 150µl of washing buffer (400mM NaCl, 20mM Tris pH 8.0, 2mM EDTA pH 8.0 and 50% absolute EtOH v/v). Finally, DNA was eluted from glass milk by adding 20µl of ddH_2O water and incubation for 5 min at 50°C.

Preparation of a cloning vector

Pfu DNA polymerase generates a blunt-ended PCR fragment which requires a linearized blunt-ended vector for cloning. Thus, a standard cloning vector pBluescript SK^- was linearized via digestion with a restriction enzyme SmaI (Fermentas) that generates a blunt-ended product. Digestion reaction was set by mixing 5µg of pBluescript SK^-, 1X buffer Tango (Fermentas), 25 unit of SmaI (Fermentas) and ddH_2O to produce a final volume to 50µl. The reaction was performed by incubation at 30°C for 1 h followed by heat inactivation of the enzyme at 65°C for 20 min. To prevent self-ligation (self-circularization), the linearized vector was dephosphorylated prior to ligation with Calf Intestinal Alkaline Phosphatase (CIAP, Invitrogen) following the manufacturer's protocol. Removal of CIAP from linearized dephosphorylated vector was done by phenol extraction.

Ligation

Purified PCR products were cloned to pBluescript SK^- vector by setting up a ligation reaction in 0.5ml microcentrifuge tubes known to have low DNA binding capacity. The ligation reaction cocktail consisted of a 3:1 molar ratio mixture of vector and insert DNA, 2µl of 10x T4 DNA ligase buffer, 2µl 6mM rATP, 2µl of 50% PEG 4000 solution, 5 units T4 DNA ligase (Fermentas) and nuclease-free water to make the final volume 20µl. The reaction was performed by placing reaction tubes in a water bath at RT for 30 min followed by overnight incubation at 4°C.

Preparation of DH5-alpha competent cells

Preparation of DH5-alpha *Escherichia coli* competent cells and transformation procedures were performed according to the modified method of Inoue *et al.,* 1990. Briefly, a starter culture was prepared by growing a single colony bacterial culture in SOB medium for 8 h at 37°C with vigorous shaking at 8.6 x g. Then, 2ml of the starter culture was transferred to 250ml of SOB medium and grown overnight at 18°C with moderate shaking at 2. 1 x g. When the OD_{600} reached 0.6, the culture was transferred to an ice bath for 10 min. Cells were then harvested by centrifugation (1520 x g; 10 min; 4°C)

and gently resuspended in 20ml of ice-cold Inoue transformation buffer (55mM $MnCl_2.4H_2O$, 15mM $CaCl_2.2H_2O$ and 250mM KCl and 10mM PIPES; pH 6.7). Subsequently, 1.5ml of DMSO was added and the bacterial suspension was incubated on ice for 10 min. After incubation, 100µl aliquots of the bacterial suspension were dispensed into chilled sterile 1.5ml microcentrifuge tubes, immediately snap-frozen in liquid nitrogen and stored at -80°C until used for transformation.

Transformation

Transformation was performed by mixing 100µl aliquots of *E. coli* DH5-α competent cell with 2µl of the ligation product. After incubation on ice for 30 min, cells were heat shocked (42°C) for 40 s and immediately placed on ice for 2 min. Afterwards, 900µl of SOC medium was added and cells were incubated at 37°C for 90 min with constant shaking. 200µl volumes of the transformed competent cells were then transferred onto MacConkey agar plates amended with additional lactose (10g/liter) and ampicillin (100µg/ml). After 12-16 h of incubation at 37°C, the transformant cells harboring recombinant plasmids were counter selected from false positives based on colony color and further confirmed via colony PCR. Transformed cells were preserved at -80°C in 10% glycerol.

Plasmid miniprep

Extraction of plasmid DNA from transformant *E. coli* DH5-α competent cells was performed following the alkaline lysis procedure developed by Birnboim & Doly (1979). For this procedure, a bacterial culture was prepared from single colony transformant cells in LB medium supplied with ampicillin (100µg/ml). Two ml of overnight bacterial culture grown at 37°C under constant shaking was transferred to microcentrifuge tubes and cells were harvested by centrifugation (6,082 x g for 5 min). Cells were then resuspended in 200µl ice cold GLC buffer (50mM Tris pH 8.0, 50mM glucose, and 10mM Na-EDTA), 2.4µl lysozyme (40mg/ml) and 3µl RNase A (10mg/ml) and incubated for 15 min at RT. Subsequently, 300µl LYZ buffer (1% SDS and 0.2N NaOH) was added and contents of the mixture were further incubated for 10 min at RT followed by another 30 min incubation after addition of HS buffer (29.4 g of potassium acetate and 3.8 ml of formic acid in 100ml H_2O). Next, cell debris were separated by centrifugation (16,060 x g at 4°C) and the supernatant containing the plasmid DNA was transferred to new tubes, mixed with 0.65 volume of isopropanol and incubated for 10 min at RT. Then, DNA was precipitated by centrifugation (16, 060 x g at 4°C) and washing twice with 70% ethanol. Finally, the DNA pellet was dried in a speed-vac for 10 min at 30°C and resuspended in 50µl of 1X TE buffer. The presence of the correct insert in plasmid DNA was verified by colony PCR and PVUII (Fermentas) restriction digest analysis. Prior

to sequencing, purification of miniprep plasmid DNA samples was performed using SureClean (Bioline GmbH, Germany) kit following manufacture's protocol.

4.2.10.4 DNA sequencing and sequence analysis

Purified plasmid clones were sequenced using the universal T7 promoter forward and reverse primers at Eurofins MWG Operon (Ebersberg, Germany). Resulting sequences were vector-clipped and nucleotide sequence identity was verified by comparison with known gene sequences available in the NCBI GenBank database using Blastn search (http://blast.ncbi.nlm.nih.gov).

4.2.10.5 Real time quantitative PCR analysis

RNA extraction and reverse transcription

RNA was isolated from leaf samples collected 7 days after drought stress treatment (28 DPI), where the effect of drought stress was visible as evidenced by physiological data. Leaf samples collected from normally watered (100% FC) and severely stressed (30% FC) plants were ground in liquid nitrogen and total RNA was isolated from 100mg tissue using Tri-Reagent (Sigma) according to the manufacturer's protocol. Following extraction, RNA integration was analyzed on 1% (w/v) formaldehyde-agarose gel. RNA quality and concentration were measured using an Epoch microplate spectrophotometer (Epoch; BioTek Inc., Winooski, VT, USA). cDNA was synthesized from 1ug of total RNA samples using the QuantiTect Reverse Transcription Kit (QIAGEN) and following the manufacturer's instructions. Before reverse transcription, any genomic DNA contamination was removed with gDNA Wipeout Buffer following the protocol. Furthermore, further check for genomic DNA contamination of RNA samples was done by running a PCR using BnActin gene primer and RNA template.

Real time PCR

Quantitative RT-PCR was performed using ABsolute Blue SYBR Green Fluorescein Mix (ABgene, Hamburg, Germany) following manufacturer's recommendations. PCR reaction was set up in 10µl total reaction volume consisting of 5µl (final 1x) ABsolute Blue SYBR Green Fluorescein Mix, 1µl cDNA (10-fold dilution) and 1µl (10 pmol) of each forward and reverse gene specific primers. PCR was run using CFX384 real-time PCR detection system (Bio-Rad laboratories, Inc) in 384 well plates with three technical replicates. The amplification conditions for all genes were: 95°C for 15 min to activate Thermo-Start™ DNA polymerase; followed by 35 cycles of denaturation (95°C for 1 min), annealing (64°C 30 s) and extension (72°C for 2 min) with a final elongation step of 10 min at 72°C. To ensure amplification specificity, a melting curve analysis (65°C to 95°C with a heating rate of 0.5°C per 5 s and continuous fluorescence measurement) was performed. Furthermore, amplified products were resolved on 1%

agarose gel and size of DR-gene fragments were verified by comparison to a known molecular weight marker (HyperLadder™ 100bp Plus, Bioline, Germany).

Relative gene expression analysis

The relative expression of DR-genes was determined according to the Pfaffl method (Pfaffl, 2001) using the formula: $R=[(E_{target})^{\Delta Ct\ target\ (control-drought\ stressed)}]/[(E_{ref})^{\Delta Ct\ ref\ (control-drought\ stressed)}]$, where R is the relative expression ratio, E_{target} is the real-time PCR efficiency of target genes (DR-genes) transcript, E_{ref} is the real-time PCR efficiency of a reference gene (BnActin) transcript, Ct is the threshold cycle at which relative fluorescence of samples increased above the background fluorescence, and ΔCt is the difference between Ct values of control (100% FC) and treated samples (30% FC). Amplification efficiencies of reference and target genes were determined by using dilutions of DNA template. Relative expression values obtained from three biological replicates were used for statistical analysis.

4.2.11 Data analysis

The whole experiment was repeated twice. Mean data obtained from three replications of two independent experiments were analyzed using SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA). To determine main and interactive effects of genotype, disease and drought stress factors, analysis of variance was performed by the mixed model procedure (PROC MIXED). Means were separated using Tukey's test at 5% level of significance. To analyze the relationships between disease severity, physiological and agronomic parameters, Pearson's linear correlation was performed and correlation coefficients were calculated using the PROC CORR procedure. To determine the relative contribution of variables to the variation in dry matter yield and identify most important variables contributing to the variation in dry matter yield, a stepwise multiple linear regression analysis was performed using the PROC REG procedure. A principal component analysis (PCA) was performed using the Minitab version 14 (Minitab, Coventry, UK).

4.3 Results

4.3.1 *V. longisporum* disease development under drought stress conditions

Typical symptoms of *V. longisporum* infection in *Brassica napus* under controlled environmental conditions are leaf yellowing, stunted growth and senescence of leaves and branches. In the VL-susceptible cultivar Falcon, the initial symptoms of VL infection (yellowing and stunted growth) were clearly observed two weeks after inoculation. In addition to this, most infected leaves showed severe shriveling, deformed shape and curling from the edge (Fig. 4.2). In the resistant genotype SEM however,

these symptoms were distinctly observed relatively late (at 21 DPI) and with lower intensity. VL infection also induced production of excessive side branches in both susceptible and resistant genotypes (See section 4.4.4.2).

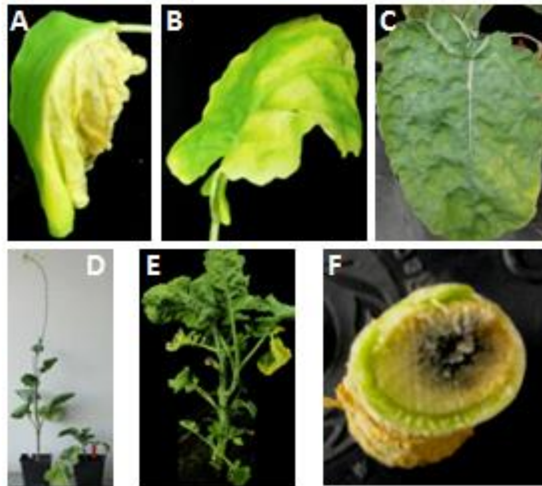


Figure 4.2 Typical symptoms induced by *Verticillium longisporum* infection in *Brassica napus* under greenhouse condition. **A**, One sided chlorosis. **B**, Irregular shaped yellowing on leaf with dark veins. **C**, Leaf shrivelling and deformation. **D**, Stunting: control (left); inoculated (right). **E**, Production of excessive abnormal branches and scabs on main stem showing the point of leaf senescence. **F**, Hypocotyl cross section showing vascular discoloration.

Disease development as measured by AUDPC was significantly affected by genotype and pathogen factors. The interaction of pathogen with genotype had also a significant effect on disease severity. However, drought alone and its interaction with genotype and pathogen had no effect (Table 4.9). Net AUDPC values generated from weekly disease index evaluation showed an increased rate of disease development over time. In cultivar Falcon, at 14 DPI first symptoms were observed and disease levels sharply increased towards 49 DPI. In genotype SEM however, not only that first symptoms were observed relatively late (zero net AUDPC at 14 DPI) but also there was only a very slight change in disease progress over time (Fig. 4.3). Furthermore, addition of drought stress, which begun at 21 DPI, caused no significant change in the response of this genotype to VL infection. At 28 DPI (one week after the beginning of drought stress treatments), similarly low disease levels (net AUDPC = 15) were recorded from 100, 60 and 30% FC treatments. Three weeks later (49 DPI), net AUDPC values of 100, 60 and 30% FC were 36.2, 35.2 and 28.3, respectively, which was again not significantly different (Fig. 4.6). The uniform and significantly low levels of AUDPC values recorded at all watering conditions indicate stable resistance of genotype SEM under either sufficient or limited water supply conditions (Fig. 4.8).

Concerning the VL-susceptible genotype Falcon, significantly high disease levels were recorded at all watering regimes (Fig. 4.8; Fig. 4.6). Respective Net AUDPC values at 100, 60 and 30% FC at 28 DPI were 35.7, 36.9 and 31.4. This is more than twice the values recorded in SEM. At 49 DPI, disease level was further increased to more than double resulting in respective net AUDPC values of 76.0, 83.5 and 71.1.

The lowest net AUDPC values (in contrary to VL DNA) in both resistant and susceptible genotypes were recorded from 30% FC treatments that represent extreme drought conditions (Fig. 4.6).

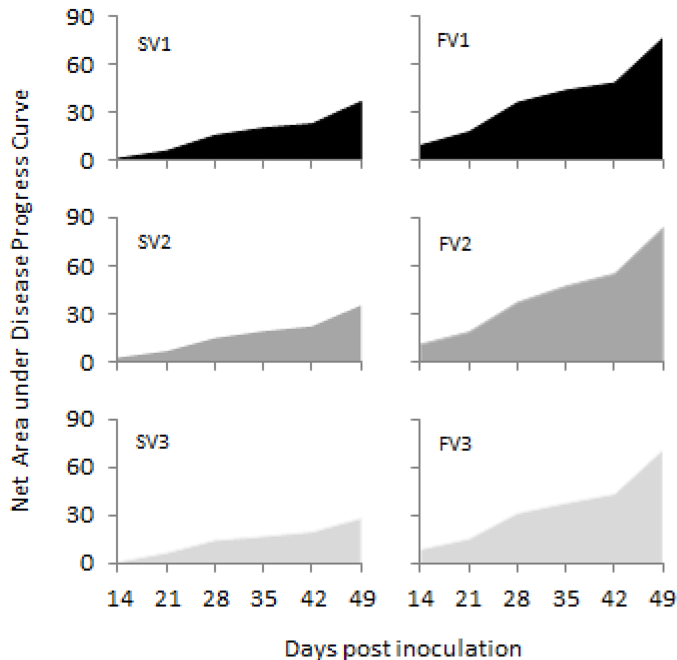


Figure 4.3 Net AUDPC of two *Brassica napus* genotypes infected with *Verticillium longisporum* under optimum watering (100% FC) and different levels of drought stress conditions. AUDPC values are generated from weekly disease index measurements taken from 7 - 49 days post inoculation. **SV**, Tolerant genotype SEM-05-500526 VL-inoculated. **FV**, Susceptible cultivar Falcon VL-inoculated. Treatment names followed by the numbers **1**, **2** and **3** refers to watering at 100, 60 and 30% field capacity, respectively.

Molecular quantification of VL biomass by quantitative PCR also revealed the significant effect of genotype in disease development. The drought factor alone and its interaction with either genotype or disease had no significant effect (Table 4.9). Compared to Falcon, significantly low VL DNA was detected in SEM during early (28 DPI) as well as late (49 DPI) time points. At 28 DPI, fungal biomass in fully irrigated SEM plants was 15-fold lower than that of Falcon. This difference was even bigger (24-fold) when plants were exposed to severe drought. Quantification of VL DNA at 49 DPI also revealed that disease development in the resistant genotype SEM was not significantly altered due to the occurrence of drought. At this time point, the respective amount of VL DNA in this genotype at 100, 60 and 30% FC was 27.1, 29.0 and 36.0 ng/g dry hypocotyl tissue. Nevertheless, this data still showed that the amount of fungal biomass in plant tissue increased as plants get exposed to drought stress (though the difference is very small and statistically insignificant). The situation was more aggravated in the susceptible cultivar Falcon where significantly evevated quantities of VL DNA were detected from severely stressed plants. When we compare fungal DNA at 28 and 49 DPI, at the later time point, quantity of VL DNA either remained constant or was slightly reduced in SEM while it significantly increased (up to 31-fold) in Falcon (Fig. 4.6).

Disease development under different water supply conditions was further studied by assessment of stunting effects. Weekly measurement of plant height from 14 to 49 DPI showed that genotype, VL infection and their interaction have a significant effect on this parameter (Table 4.9). The effect of drought stress on plant height is discussed later in section 4.4.4.1. Regarding the effect of VL, even though infection significantly reduced plant height of both genotypes, the effect was by far stronger in Falcon than in SEM. Furthermore, the stunting effect of VL was more pronounced during early growth stage (28 DPI) compared to later growth periods (49 DPI). As mentioned earlier, in SEM plant height was not affected by VL infection until 14 DPI (Fig. 4.4) and only minor stunting was initially recorded at 21 DPI (data not shown). Significant and severe stunting effect on SEM was first observed at 28 DPI when 50% plant height reduction was recorded at 100% FC. Towards crop maturity again, plants were able to recover and relative stunting at 49 DPI was reduced to 21.4%. Interestingly, the stunting effect of VL under drought stress condition was significantly lower. At 28 DPI, stunting at 60% and 30% FC were 31.2% and 37.9%, respectively, which is by far lower than the effect at 100% FC. Further, at 49 DPI, mock and VL-inoculated plants had similar plant height both at moderate and severe drought stress conditions indicating that VL-infection has no stunting effect during drought stress in genotype SEM (Fig. 4.5; Table 4.7). For Falcon, it was the worst case where severe stunting already occurring at 14 DPI (Fig. 4.4) causing more than 70% reduction of plant height at 28 DPI under all watering conditions. Unlike in SEM, infected Falcon plants were not able to recover during later time points and as a result similarly strong stunting was observed at 49 DPI (Fig. 4.5; Fig. 4.8).

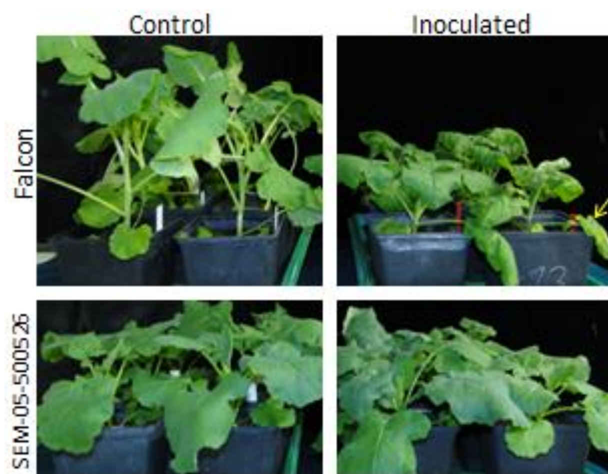


Figure 4.4 Phenotypes of susceptible (Falcon) and resistant (SEM-05-500526) *Brassica napus* plants two weeks after inoculation with *Verticillium longisporum* under greenhouse conditions. Vernalized seedlings were inoculated at BBCH 14. At this time point, leaf yellowing (arrow) and stunting symptoms were only observed in Falcon. SEM plants showed no symptoms.

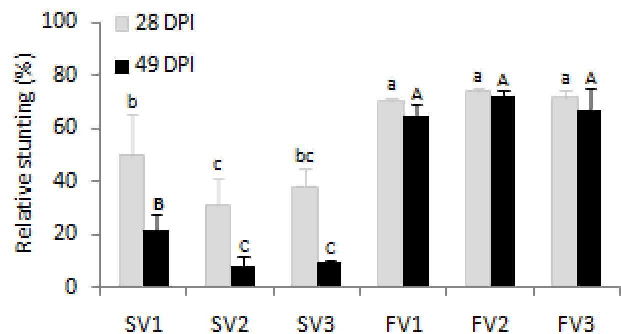


Figure 4.5 Stunting effect of *Verticillium longisporum* in *Brassica napus* under optimum watering and drought stress conditions. Relative stunting refers to percent plant height reduction in inoculated plants relative to the plant height of mock-inoculated plants. Mean data obtained from 30 plants of two independent experiments are presented. Bars indicate standard deviations. Different letters on the bars with the same letter-case indicate significant differences between treatments at P≤0.05. **SV**, Tolerant genotype SEM-05-500526 VL-inoculated. **FV**, Susceptible cultivar Falcon VL-inoculated. Treatment names followed by the numbers **1**, **2** and **3** refer to watering at 100, 60 and 30% field capacity, respectively.

The consistent response of susceptible and resistant *B. napus* genotypes to infection with VL under optimum watering and drought stress conditions was proven by Pearson correlation analysis that showed strong positive relationships between net AUDPC and relative stunting (r=0.95), net AUDPC and VL DNA (r=0.89) and relative stunting and VL DNA (r=0.95) measurements (Table 4.11). This observation was further supported by stepwise regression analysis that showed a significant contribution of the pathogen (r^2=0.74) and genotype (r^2=0.11) factors in determining disease development but not from drought stress (Table 4.12).

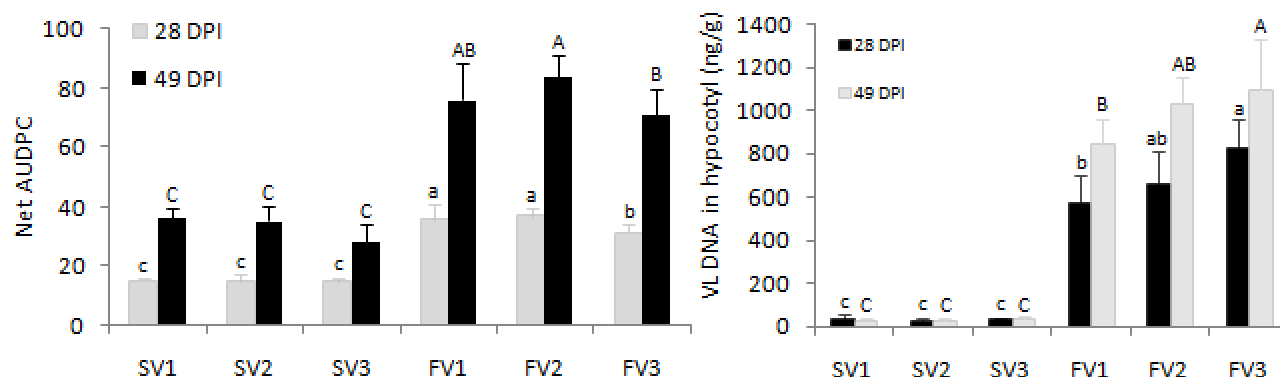


Figure 4.6 Effect of *Verticillium longisporum* in *Brassica napus* under optimum and drought stress conditions as measured by net AUDPC (Left) and quantification of VL DNA from dry hypocotyl tissue by quantitative PCR (right). Mean data obtained from 30 plants of two independent experiments are presented. Bars indicate standard deviations. For each parameter, different letters on the bars with the same letter-case indicate significant differences between treatments at P≤0.05. **SV**, SEM-05-500526 VL-inoculated. **FV**, Falcon VL-inoculated. Treatment names followed by the numbers **1**, **2** and **3** refer to watering at 100, 60 and 30% field capacity, respectively.

4.3.2 Physiological responses to *V. longisporum* infection and drought stress

In order to investigate solitary and combined effects of drought stress and disease factors on plant physiology, several parameters that best explain the response of *B. napus* to water deficit were monitored at 28 and 49 DPI. These include gas exchange measurement, relative water content determination, proline content and gene expression analyses.

4.3.2.1 Gas exchange

Regardless of plant growth stage, the leaf gas exchange parameters [transpiration rate (E), stomatal conductance of CO_2 (gs) and photosynthesis rate (A)] were only significantly affected by drought. Genotype and VL either independently, by interacting with each other, or by interacting with drought did not affect gas exchange (Table 4.9). As a result, no significant difference between genotypes and between mock- and VL-inoculated treatments was observed (Fig. 4.7). For genotype SEM, E at 100% FC in mock-inoculated plants was 1.5 mmol $m^{-2}s^{-2}$. A bit lower (1.3 mmol $m^{-2}s^{-2}$) but insignificantly reduced E was recorded in VL-inoculated and similarly watered plants. Under severe stress conditions however, E

was significantly reduced to 0.3 and 0.4 mmol $m^{-2}s^{-2}$ in mock- and VL-inoculated treatments, respectively. For cultivar Falcon, more or less the same rates of leaf transpiration were recorded. In this cultivar, severe drought reduced E from 1.8 to 0.4 in the absence of VL infection and from 1.6 to 0.5 mmol $m^{-2}s^{-2}$ during infection with VL. Regarding stomatal conductance of CO_2, a similar trend was observed. In SEM, regardless of VL infection, watering at 30% FC reduced gs from 0.13 to 0.02 mol $m^{-2}s^{-2}$. In cultivar Falcon, gs was reduced from 0.1 mol $m^{-2}s^{-2}$ (at 100% FC) to 0.02 and 0.04 mol $m^{-2}s^{-2}$ in mock and VL-inoculated 30% FC treatments, respectively. In the same way, regardless of genotype and VL-infection, the photosynthesis rate was significantly reduced by severe drought stress. In SEM, a similar photosynthesis rate (9.3 µmol $m^{-2}s^{-1}$) was measured from fully watered mock and VL-inoculated treatments. Watering at 30% FC reduced A to 3.3 and 4.2 µmol $m^{-2}s^{-1}$ in mock and VL-inoculated treatments, respectively. For cultivar Falcon, the mean photosynthesis rate at 100% FC regardless of VL infection was 10 µmol $m^{-2}s^{-1}$. Here, watering at 30% FC again significantly reduced the rate to 2.7 and 4.4 µmol $m^{-2}s^{-1}$ under mock and VL- infection conditions, respectively (Fig. 4.7).

Although the analysis of all gas exchange measurements showed that VL infection has no significant effect, the rate by which drought induced gas exchange reduction in mock and VL-inoculated plants varied considerably. Compared to mock-inoculation, the rate of reduction in gas exchange due to drought stress was significantly lower during VL-infection (Table 4.5). For instance, the difference in photosynthesis rate between 100 and 30% FC watered SEM plants in the absence of infection was 6.0 µmol $m^{-2}s^{-1}$. When the pathogen was present, this change was reduced to 5.1 µmol $m^{-2}s^{-1}$. In a similar manner, this effect occurred in the VL-susceptible cultivar and was 7.1 and 5.7 µmol $m^{-2}s^{-1}$ in control and infected treatments, respectively. Considering the transpiration rate, the respective reduction at 30% FC in mock- and VL-infected plants was 3.8 and 2.3-fold in SEM and 4.0 and 2.1-fold in Falcon. Similar but relatively large differences were observed in stomatal conductance of CO_2 where 30% FC watering caused a reduction of 5.8 and 2.9-fold (in SEM) and 5.3 and 2.1-fold (in Falcon) in mock and VL-inoculated treatments, respectively. The above observations suggest that biochemical or histological changes that might be induced by VL infection might help plants to partly mitigate drought stress. A positive role of VL-infection in reducing the impact drought stress under mild stress conditions (60% FC) was not conclusive. Except for the slight reduction in VL-infected susceptible plants, gas exchange measurements at 28 DPI also showed a similar trend (Appendix 4.1; Appendix 4.7).

4.3.2.2 Water use efficiency

Instantaneous water use efficiency (WUE) computed as the ratio of photosynthesis to transpiration rate (Tambuss *et al.,* 2007) is a key indicator of plant economic utilization of soil water. As expected, drought stress generally increased (irrespective of VL-infection) water use efficiency of both genotypes

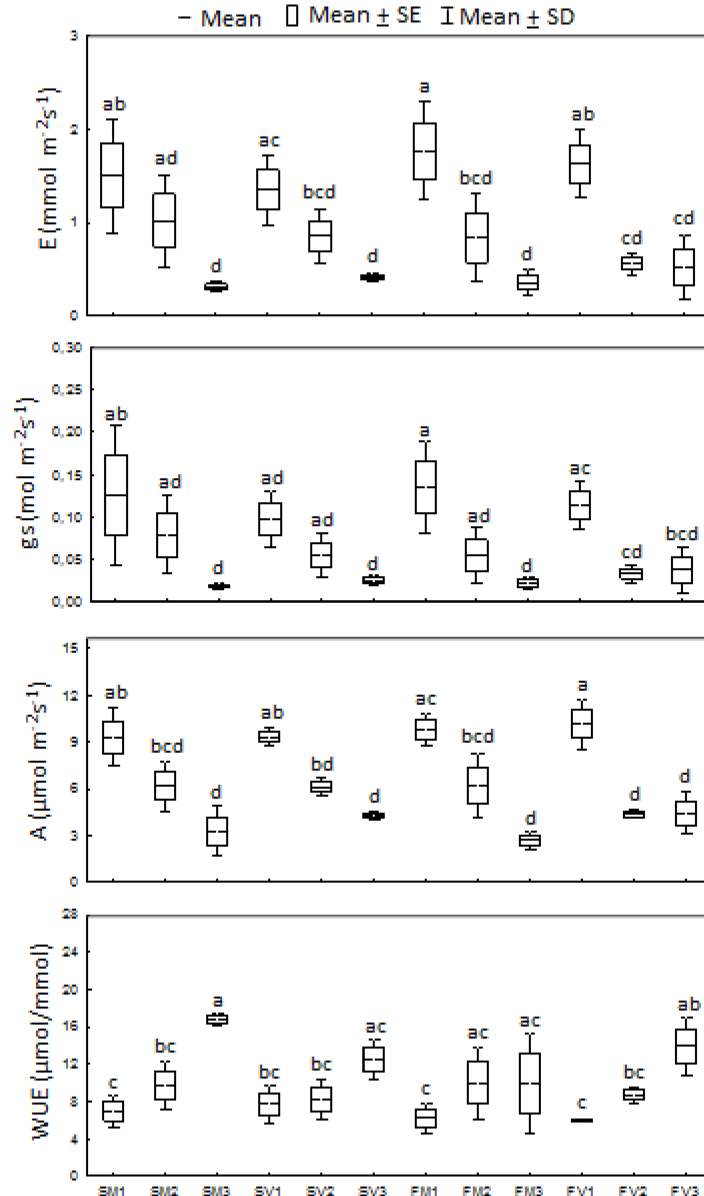


Figure 4.7 Transpiration rate (E), stomatal conductance of CO_2 (gs), photosynthesis rate (A), and water use efficiency (WUE) of two *Brassica napus* genotypes subjected to *Verticillium longisporum* infection and/or drought stress under greenhouse conditions. Mock and/or VL-inoculated plants supplied with water at 100% FC were used as control. Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for four weeks starting from 21 days after inoculation with *Verticillium longisporum*. Mean data obtained from 30 plants of two independent experiments are presented. For each parameter, different letters on the bars indicate significant differences between treatments at P≤0.05. **SM**, SEM-05-500526 mock-inoculated. **FM**, Falcon mock-inoculated. **SV**, SEM-05-500526 VL-inoculated. **FV**, Falcon VL-inoculated. Treatment names followed by the numbers **1**, **2** and **3** refer to watering at 100, 60 and 30% field capacity, respectively.

but significant increments were observed only at 30% FC conditions (Fig. 4.7). In mock-inoculated treatments, due to the shift in water supply from 100% FC to 30% FC, WUE was significantly increased from 6.9 to 16.7 µmol CO_2 mmol H_2O^{-1} in SEM. In Falcon, it was increased from 6.2 to 9.9 µmol CO_2 mmol H_2O^{-1} which is a slight change but still insignificant. The addition of the pathogen factor during drought stress resulted in slightly decreased WUE in SEM. For this genotype, compared to control conditions where 16.7 µmol of CO_2 was fixed per 1 mmol H_2O transpired, during infection with VL, slightly less CO_2

(12.5 µmol) was fixed with the same amount of water transpired. Quite the opposite was observed in VL-susceptible cultivar Falcon. Here, WUE at 30% FC in control plants was 9.9 µmol CO_2 mmol H_2O^{-1}. During VL-infection, the efficiency was slightly increased to 13.9 µmol CO_2 mmol H_2O^{-1} (Table 4.5). Furthermore, although not statistically significant, a genotypic difference in WUE at 30%FC was noticed in the absence of the pathogen as SEM had relatively higher WUE (16.7 µmol CO_2 mmol H_2O^{-1}) compared to Falcon (9.9 µmol CO_2 mmol H_2O^{-1}). During VL-infection, both genotypes had similar WUE (Fig. 4.7). A similar trend was observed from the data measured a week after initiation of drought treatments, 28 DPI (Appendix 4.1; Appendix 4.7).

Single and interactive effects of drought and *V. longisporum* in gas exchange of VL-susceptible and VL-resistant *B. napus* genotypes were further analysed by stepwise multiple regression analysis. The results showed that gas exchange was only significantly affected by drought stress and this stress factor contributed up to 77% of the variation in gas exchange. On the other hand, genotype and disease factors alone or all their possible interactions had no significant effect (Table 4.14). The strong correlations (up to 98%) among gas exchange parameters and between gs exchange and other physiological parameters (Table 4.11) support the finding that major changes in physiological parameters is mainly due to drought stress.

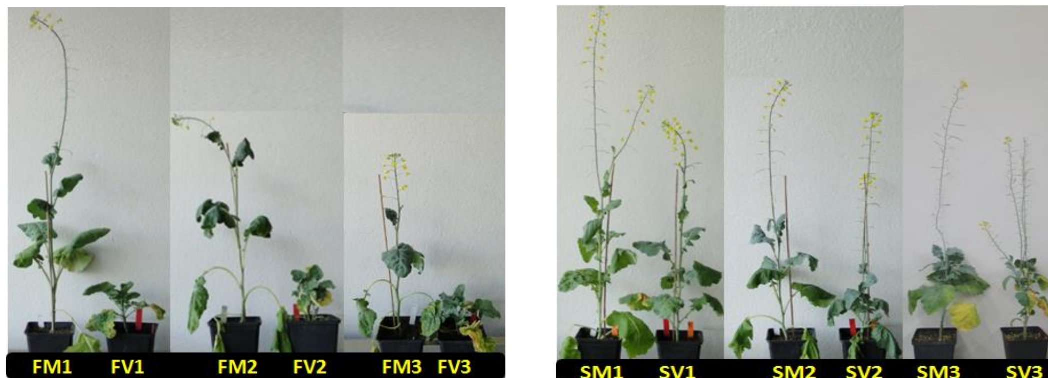


Figure 4.8 Phenotypes of randomly selected *Brassica napus* plants exposed to drought stress and/or *Verticillium longisporum* infection in a greenhouse experiment 28 days after initiation of drought treatments (49 DPI). Vernalized seedlings were inoculated at BBCH14 by submerging injured roots in spore suspension (10^6conidia/ml) for 30 minutes. Mock-inoculated plants were similarly dipped in autoclaved tap water. **SM**, SEM-05-500526 mock-inoculated. **FM**, Falcon mock-inoculated. **SV**, SEM-05-500526 VL-inoculated. **FV**, Falcon VL-inoculated. Treatment names followed by the numbers **1**, **2** and **3** refer to watering at 100, 60 and 30% field capacity, respectively.

4.3.2.3 Leaf relative water content

RWC represents the actual water status of pant tissue and provides the most meaningful index to evaluate tolerance of plants to drought stress. In the present study, measurement of this parameter was also done one (28 DPI) and four weeks (49 DPI) after initiation of drought treatments. According to the

results, leaf RWC was only affected by severe drought stress. Besides, the effect of drought on RWC was dependent on plant genotype and growth stage. One week after initiation of the drought treatments (28 DPI; BBCH 50-60; inflorescence emergence stage), for most treatments, the plant water status was not significantly affected by drought. At this time point, except for VL-infected Falcon plants, a slight and insignificant reduction ranging from 6 to 7% was recorded at 60% and 30 % FC watering treatments of both genotypes. At flowering growth stage (BBCH 60-70; 49 DPI), RWC due to severe drought was only significantly reduced in genotype SEM where RWC was reduced by 13 and 14% in mock and VL-infected treatments, respectively. The respective values for genotype Falcon were 10.3 and 10.1% (Table 4.4).

Table 4.4 Effect of different levels of drought stress and *Verticillium longisporum* infection on leaf relative water content (%) of two Brassica *napus* genotypes 7 and 28 days after initiation of drought treatments, at 28 and 49 DPI, respectively.

Treatments	28 DPI			49 DPI		
	100% FC	60% FC	30% FC	100% FC	60% FC	30% FC
SM	91 ± 2^{a}	83 ± 4^{ab}	84 ± 2^{ab}	87 ± 2^{abc}	83 ± 1^{ad}	74 ± 4^{d}
SVL	90 ± 4^{a}	85 ± 4^{ab}	84 ± 1^{ab}	91 ± 4^{ab}	85 ± 6^{ad}	77 ± 5^{cd}
FM	92 ± 2^{a}	87 ± 3^{ab}	86 ± 1^{ab}	91 ± 2^{a}	86 ± 4^{abc}	81 ± 6^{ad}
FVL	92 ± 4^{a}	84 ± 4^{a}	81 ± 3^{b}	89 ± 2^{ab}	86 ± 1^{abc}	79 ± 4^{bd}

Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for four weeks starting from 21 days after inoculation with *Verticillium longisporum*. Mock and/or VL-inoculated plants supplied with water at 100% FC were used as control. Data shown is mean values ± standard deviations obtained from 30 plants of two independent experiments. Mean values containing the same superscript are not significant at P=0.05. **FC**, Field capacity. **SM**, SEM-05-500526 mock- inoculated. **FM**, Falcon mock-inoculated. **SVL**, SEM-05-500526 VL- inoculated. **FVL**, Falcon VL-inoculated.

4.3.2.4 Proline content

Another drought stress response indicator exploited in this study was proline content, a universal biomarker for stress analysis in plants (Ernst and Peterson, 1994). Proline was extracted from leaf and hypocotyl tissue and concentrations were quantified by referring to the standard curve produced from known concentrations of proline (Fig. 4.9). The results showed significant effects of all experimental factors (Table 4.10) and a wide range of variation (3 to 19 fold) in proline levels in response to drought stress or VL infection, and dependent on the plant part and also to some extent to the plant genotype. The amount of proline also increased with increasing severity of drought stress (Fig. 4.10). To begin with the genotypic differences, the two genotypes not only showed differences in the initial free proline content, but also in the quantity of drought induced proline accumulation. Although this was not statistically significant, the initial proline content seems to be higher in Falcon than in SEM. However,

the drought induced proline accumulation was significantly higher in SEM than in Falcon (Fig. 4.10; Table 4.5).

Drought stress was the most crucial factor that caused pronounced accumulation of free proline in leaf and hypocotyl tissues. Under normal growth conditions where there was no stress from disease or drought, the proline content in SEM plants was 3.9 µmoles/g in leaf and 2.2 µmoles/g in hypocotyl tissues. At 30% FC, these values increased 12fold in leaf and 19fold in hypocotyl tissue. In cultivar Falcon, the proline content in control treatments was 6.9 µmoles/g in leaves and 1.5 µmoles/g in hypocotyls. Similarly, drought stress at 30% FC induced an increase of 4 and 11fold in leaf and hypocotyl tissue, respectively. As in non-infected control treatments, a significant increase in free proline accumulation due to drought stress was observed in VL-inoculated treatments. Furthermore, the comparison of drought induced proline synthesis in mock- and VL-inoculated treatments showed that VL infection caused a significant reduction in proline synthesis in genotype SEM but had no such impact in Falcon (Fig. 4.10).

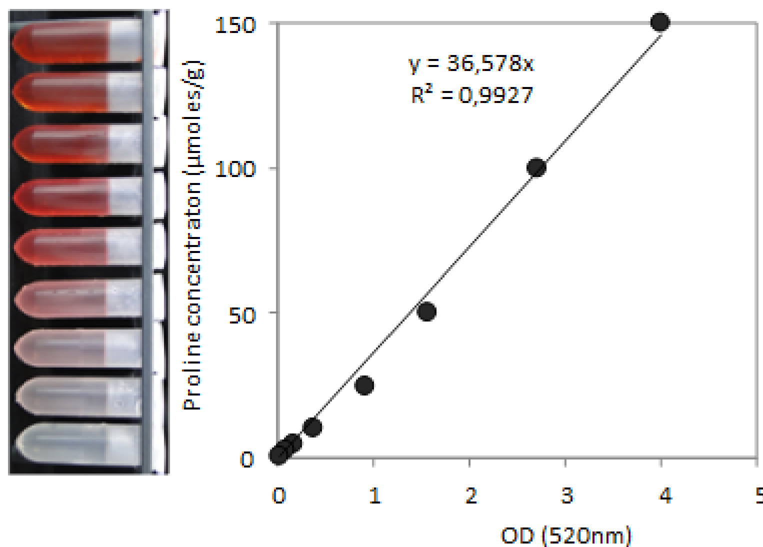


Figure 4.9 Standard curve for quantification of proline concentration in leaf and hypocotyl plant tissues of *B. napus*. Data points represent means of triplicates. Known concentrations of L-proline ranging between 150 and 0.5 µg/ml were prepared in 3% aqueous sulfosalicylic acid.

The forth important factor that showed a difference in proline contents was the plant part. Generally, the initial proline content was significantly higher in leaves than in hypocotyl tissue (Fig. 4.10). However, the drought induced proline synthesis in the presence and absence of the pathogen showed contrasting pattern in different plant parts. On the one hand, drought (30% FC) induced proline synthesis in mock-inoculated plants was significantly higher in hypocotyl than in leaf tissue. On the other hand, during VL-infection, increase in proline content due to watering at 30% FC became higher in leaf tissue compared to the hypocotyl, meaning that upon infection with VL, drought induced proline synthesis increased in leaves while it decreased in the hypocotyl (Table 4.5). As it was previously shown with other physiological parameters, the relatively high accumulation of proline in hypocotyl tissue during VL-

infection might again indicate a reduced impact of drought stress due to VL infection. This is strongly supported by the close correlation between proline synthesis and the physiological parameters considered in this study (Table 4.11). Analysis of variance using the mixed model procedure also showed that unlike other physiological traits, all the three factors (Genotype, VL infection and drought stress) and their interaction significantly affected proline synthesis (Table 4.10) suggesting that proline may play a role in the *B. napus*-VL interaction under drought stress conditions. Analysis of proline content in leaf and hypocotyl tissue samples collected at 28 DPI, one week after initiation of drought treatments, showed a similar but even stronger impact of plant genotype and plant part (Appendix 4.2; Appendix 4.8).

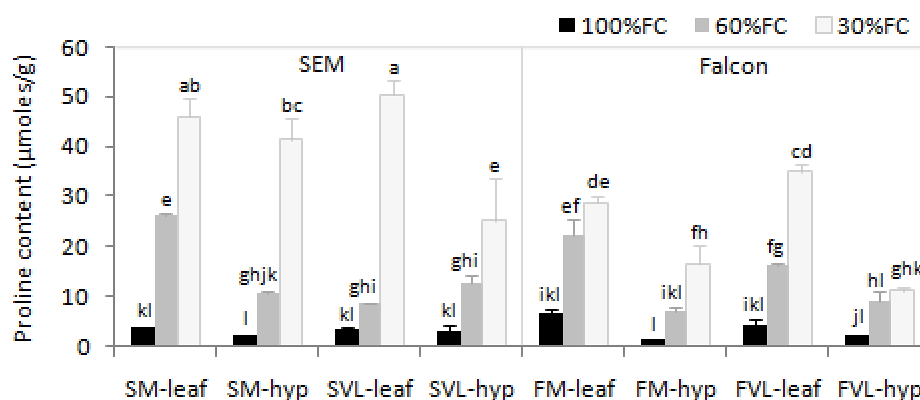


Figure 4.10 Changes in proline content in leaf and hypocotyl tissue of two *Brassica napus* genotypes exposed to different levels of drought stress, and *Verticillium longisporum* infection. Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought for four weeks starting from 21 days after inoculation with *Verticillium longisporum*. Mock and/or VL-inoculated plants supplied with water at 100% FC were used as control. Mean data obtained from 30 plants of two independent experiments are presented. Bars indicate standard deviations. Different letters on the bars indicate significant differences between treatments at P≤0.05. **FC**, Field capacity. **hyp**, Hypocotyl. **SM**, SEM-05-500526 mock- inoculated. **FM**, Falcon mock-inoculated. **SVL**, SEM-05-500526 VL- inoculated. **FVL**, Falcon VL-inoculated.

Table 4.5 Comparison of physiological measurements in fully irrigated (100% FC) and severely stressed (30% FC) *Brassica napus* genotypes under control and *V. longisporum* infection conditions.

Parameters	Relative change				Fold change			
	SM	SVL	FM	FVL	SM	SVL	FM	FVL
E (mmol $m^{-2}s^{-1}$)	-1.19	-0.94	-1.41	-1.11	-3.77	-2.30	-3.95	-2.14
gs (mol m^{-2} s^{-1})	-0.11	-0.07	-0.11	-0.08	-5.80	-2.94	-5.34	-2.05
A (µmol m^{-2} s^{-1})	-6.04	-5.06	-7.13	-5.72	-1.85	-1.19	-2.65	-1.29
WUE (µmol CO_2 mmol H_2O^{-1})	+9.85	+4.75	+3.76	+8.00	+1.43	+0.62	+0.61	+1.34
RWC (%)	-13.0	-14.0	-10.3	-10.1	-0.08	-0.18	-0.13	-0.13
LPC (µmoles/g)	+42.1	+46.8	+21.9	+30.5	+10.70	+12.80	+3.20	+6.70
HPC (µmoles/g)	+39.2	+22.1	+15.3	+9.0	+17.60	+6.60	+10.40	+3.80

Relative change values were obtained by subtracting mean values of severely stressed (30% FC) treatments from respective values of fully irrigated (100% FC) treatments. Fold change was calculated using the formula 1 minus ratio of values at 100% FC to 30% FC treatments. Negative and positive signs indicate increase and decrease in physiological processes, respectively. Data measured 28 days after initiation of drought treatments (49 DPI) were used. **SM**, SEM-05-500526 mock- inoculated. **FM**, Falcon mock-inoculated. **SVL**, SEM-05-500526 VL- inoculated. **FVL**, Falcon VL-inoculated. **E,** transpiration rate. **gs,** stomatal conductance of CO_2. **A,** photosynthetis rate. **WUE,** water use efficiency. **RWC,** leaf relative water content. **LPC,** leaf proline content. **HPC,** hypocotyl proline content.

4.3.3 Effect of drought stress and *V. longisporum* infection on expression of drought responsive genes

The expression of drought inducible genes in mock- and VL- inoculated plants was analyzed by real time quantitative polymerase chain reaction (RT-qPCR). In this procedure, the transcript level of drought responsive genes in severely stressed plants (30% FC) was compared with the level in the respective fully irrigated (100% FC) treatments. Before expression analysis, sequence identity of eight drought responsive (DR) genes was verified by sequencing of cloned PCR products (Table 4.6). Of these, RT-PCR amplification was not successful for the two late embryogenesis abundant (LEA) genes (*BnLEA4-1* and *ME-leaN4*).

Table 4.6 Analysis of nucleotide identity of *Brassica napus* reference and drought inducible genes

Gene symbol	Gene name	Size (bp)	Genbank accession	E-Value	% identity
BnActin	*B. napus* Actin	262	DQ370142.1	2e-106	98%
BnCBF17	*B. napus* C-repeat binding factor	199	AF499034.1	2e-70	94%
CIPK1	*B. napus* Calcineurin B-like (CBL)-interacting protein kinase	314	GU189585.1	6e-35	98%
BnDREB2-23	*B. napus* drought-responsive element binding factor	553	AY444874.1	0.0	99%
BnHB6	*B. napus* Homeodomain Leucine-Zipper	840	AF268422.1	2e-148	97%
BnP5SC1	*B. napus* delta 1-pyrroline-5-carboxylate synthetase A	220	AF314811.1	4e-82	98%
BnP5SC2	*B. napus* delta 1-pyrroline-5-carboxylate synthetase B	200	AF314812.1	1e-69	100%
BnLEA4-1	Brassica napus group 4 late embryogenesis abundant gene	479	AY572958.1	0.0	97%
ME-leaN4	Brassica napus group 3 late embryogenesis abundant gene	552	AB083361.1	0.0	97%

PCR amplification was performed using high fidelity DNA polymerase (*Pfu* DNA polymerase*)* with gene specific primers. PCR products were excised from the gel, cloned into a pBluescript SK^- vector and sequenced. Sequence verification was performed using the NCBI blastn engine.

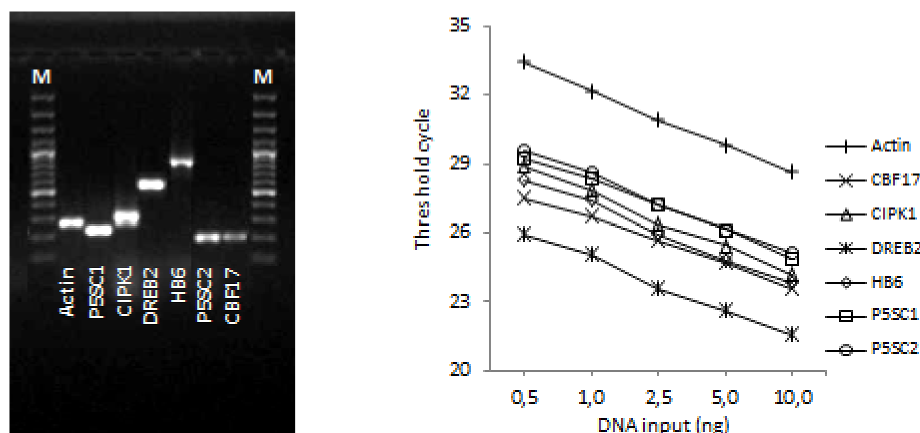


Figure 4.11 Analysis of specificity of RT-PCR products (left) and real time PCR efficiency (right) of reference and drought inducible genes in *Brassica napus*. **M**, 100bP molecular marker. **Actin**, reference gene (R^2 = 0.988; slope -3.474). **P5SC1**, delta 1-pyrroline-5-carboxylate synthetase A (R^2 = 0.994; slope -3.244). **CIPK1**, Calcineurin B-like (CBL)-interacting protein kinase (R^2 = 0.997; slope -3.550). **DREB2**, Drought-responsive element binding factor (R^2 = 0.999; slope -3.351). **HB6**, Homeodomain Leucine-Zipper (R^2 = 0.998; slope -3.431). **P5SC2**, delta 1-pyrroline-5-carboxylate synthetase B (R^2 = 0.999; slope -3.428). **CBF17**, C-repeat binding factor (R^2 = 0.998; slope -3.320). For gene expression analysis, RT-PCR efficiency of each gene was calculated using the formula $E=10^{(-1/slope)}$ (Pfaffl, 2001).

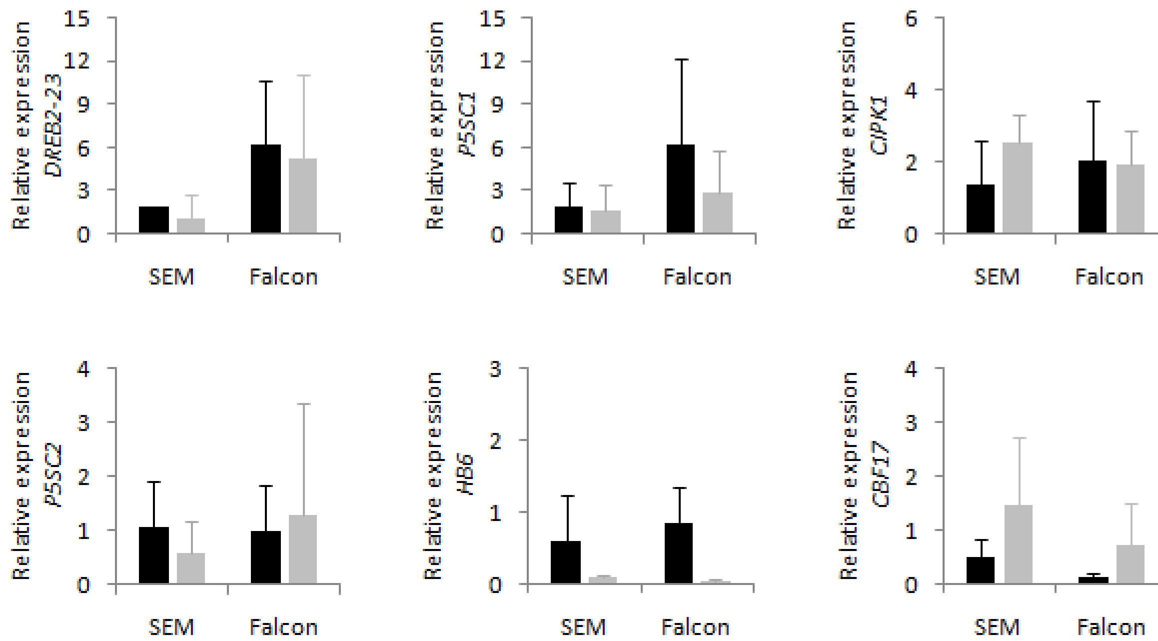


Figure 4.12 RT-PCR analysis of the expression of drought inducible genes in two *Brassica napus* genotypes subjected to drought stress and/or *Verticillium longisporum* infection. Total RNA was extracted from leaf samples collected 7 days after exposure to drought stress (28 days after inoculation with *Verticillium longisporum*). Data show the fold changes in gene expression in drought stressed (30% field capacity) *vs.* optimally watered (100% field capacity) mock (black bars) and *Verticillium longisporum* infected (grey bars) plants. Expression levels were normalized using BnActin gene according to Pfaffl, 2001. Error bars represent standard deviations of means obtained from three independent biological replicates each consisting of 10 plants. **DREB2**, Drought-responsive element binding factor. **P5SC1**, delta 1-pyrroline-5-carboxylate synthetase A. **CIPK1**, Calcineurin B-like (CBL)-interacting protein kinase. **P5SC2**, delta 1-pyrroline-5-carboxylate synthetase B. **HB6**, Homeodomain Leucine-Zipper. **CBF17**, C-repeat binding factor.

Analysis of RT-PCR-products by gel electrophoresis showed the desired single product size of each gene, corroborating specificity of the RT-PCR (Fig. 4.11). Melting curve analysis also showed single product specific melting temperature of 84°C for actin, 84.5°C for CBF17, 82.0°C for CIPK1, 88.5°C for DREB2, 88.5°C for HB6, 82.5°C for P5SC1 and 83.0°C for P5SC2. Relative gene expression was calculated considering RT-PCR efficiency of each gene (Fig. 4.11) and normalized using the BnActin gene transcripts according to Pfaffl, 2001. Results showed that regardless of the plant genotype and VL-infection, up to 6fold increase in gene expression was induced by the severe drought treatment. The P5SC1 gene that regulates proline biosynthesis and the transcription factors DREB2-23 and CIPK1 that control the expression of stress-responsive genes via ABA-independent pathway showed the highest level of expression. Compared to the aforementioned three genes however, the expression of P5SC2, CBF17, and HB6 was relatively lower, with about a one fold increase (Fig. 4.12). Mixed model analysis of variance showed no significant differences between genotypes and between mock and VL-inoculated treatments. Nevertheless, it was observed that the transcript level of P5SC1, DREB2 and CIPK1 genes were relatively higher in Falcon than in SEM. In both genotypes, VL-infection slightly increased

expression of CBF17 while it decreased HB6. In cultivar Falcon, infection slightly reduced expression of P5SC1 and HB6. In SEM, DREB2, P5SC2 and HB6 were slightly reduced in infected treatments while CIPK1 and CBF17 were increasing.

4.3.4 Effect of *V. longisporum* and drought stress on yield and morphological traits

Due to a limited supply of water and mineral nutrients, plant growth and yield can considerably be reduced during drought periods. This condition may be aggravated if a biotic stress factor (e.g. disease) is added. In the present study, in order to assess single and interactive effects of genotype, drought, and *V. longisporum* infection on plant growth and to further estimate the magnitude of yield loss due to these factors, plant height, branch development, stem thickness and dry matter yield accumulation were assessed one and four weeks after the initiation of drought treatments, at 28 and 49 DPI, respectively.

4.3.4.1 Plant height

As expected, VL infection and exposure to drought stress significantly reduced plant height. Mixed model ANOVA showed that not only genotype, disease and drought independently, but also disease*genotype and disease*drought interactions significantly affected plant height (Table 4.9). This is also clearly seen in correlation analysis results that showed strong relationship of this parameter with disease, physiological and other agronomic traits (Table 4.11). Under normal growing conditions, the two genotypes showed comparable growth and attained similar plant height at crop maturity stage (49 DPI). However, when plants were exposed to drought or disease stress factors, they responded differently. The effect of VL-infection on plant growth in the susceptible cultivar Falcon was noticed two weeks after inoculation (Fig. 4.3) and a week later (21 DPI) for the resistant genotype SEM (data not shown). However, the effect of drought stress was only visible after two weeks of exposure to drought (35 DPI), the effect becoming evident and significant in plant height reduction in both genotypes (data not shown). Similar to early time points, four weeks after initiation of drought treatments (49 DPI), the stunting effects of VL and drought were also dependent on the genotype (Table 4.7). In SEM, a reduction of plant height due to VL was only observed at full water supply (100% FC) but not at moderate (60% FC) and severe (30% FC) drought levels. Moreover, it was only under non-inoculated conditions that moderate and severe drought stresses caused significant reductions in plant height, possibly suggesting reduced stunting effect of drought in VL-infected treatments in this genotype. Contrary to SEM and irrespective of water supply, VL infection caused severe plant height reduction in Falcon. Interestingly, only severe drought stress caused stunted growth in mock-inoculated Falcon plants, suggesting less

drought sensitivity of this genotype compared to SEM in which moderate stress also significantly reduced plant height. Due to the extremely severe stunting effect of VL which probably masked the effect of drought stress, the effect of drought on plant height in VL-inoculated Falcon plants was not possible to determine (Table 4.7).

Table 4.7 Plant height (cm) of two *Brassica napus* genotypes exposed to drought stress and/or infection with *Verticillium longisporum*.

	28 DPI			49 DPI		
Treatments	100%FC	60%FC	30%FC	100%FC	60%FC	30%FC
SM	74 ± 20^{a}	50 ± 11^{ae}	51 ± 12^{ad}	140 ± 6^{A}	107 ± 11^{B}	104 ± 7^{B}
SVL	37 ± 11^{cdef}	51 ± 8^{ad}	45 ± 8^{bcdef}	111 ± 8^{B}	98 ± 3^{B}	95 ± 7^{B}
FM	70 ± 9^{ab}	63 ± 5^{ac}	59 ± 8^{ac}	141 ± 4^{A}	138 ± 14^{A}	109 ± 11^{B}
FVL	21 ± 1^{def}	18 ± 1^{f}	20 ± 2^{ef}	50 ± 6^{C}	37 ± 2^{C}	36 ± 7^{C}

Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for four weeks starting from 21 days after inoculation with *Verticillium longisporum*. Mock and/or VL-inoculated plants supplied with water at 100% FC were used as control. Mean ± standard deviation data obtained from 30 plants of two independent experiments are presented. Mean values containing the same letter case superscript are not significantly different at P=0.05. **FC**, field capacity. **SM**, SEM-05-500526 mock-inoculated. **FM**, Falcon mock-inoculated. **SVL**, SEM-05-500526 VL-inoculated. **FVL**, Falcon VL-inoculated.

4.3.4.2 Branching

Unlike other agronomic traits, the unusual production of primary branches (lateral shoots arising from central main stem; Fig. 4.2) was solely affected by VL infection and its interaction with the genotype (Table 4.9). This indicates that the excessive production of lateral shoots in both genotypes was mainly induced by VL infection. This is clearly shown in a correlation analysis that showed a significant positive relationship of branching with disease severity, and a strong negative relationship with agronomic parameters and no correlation with all physiological parameters (Table 4.11). The induction of excessive branching due to VL infection regardless of genotype was observed two weeks after inoculation and remained evident until crop maturity (49 DPI). Compared to genotype SEM however, the effect was much stronger in cultivar Falcon which was also the case in other disease parameters (4.4.1). At 49 DPI and regardless of drought stress, VL-infection induced branching increased on the average by 2.3 and 4.1 fold in SEM and Falcon, respectively. Nevertheless, such a high induction of branching was not reflected in increased biomass yield particularly in the susceptible cultivar Falcon. This could be either due to the very small size of these branches or it might also be due to the fact that they undergo senescence shortly after their emergence. The effect of drought stress on branching either in mock- or VL-inoculated treatments was not significant during both early (Appendix 4.3) and late growth stages (Table 4.8).

4.3.4.3 Hypocotyl thickness

Similar to yield parameters, stem thickness was the most prominent indicator of disease and drought effects, genotypic difference as well as of the interaction of these factors (Table 4.9). It was also the one that showed significant correlation with all other parameters considered in this study (Table 4.11). From the beginning, the two genotypes had genetically different mean stem thickness, SEM being thicker at the hypocotyl (10.4 mm) than Falcon (9.4 mm). Whether infected by VL or not, exposure of both genotypes to drought stress resulted in thinner plant stems, with no significant difference between moderate and severe drought stress treatments (Table 4.8). Compared to 100% FC, hypocotyl thickness at 60% and 30% FC in cultivar Falcon was significantly reduced by 10.8% and 19.1%, respectively. In genotype SEM, the respective reduction percentages were 12.5 and 22.7. Similarly, the respective average stem thickness reduction during VL infection was 11.7 and 14.9% in SEM and 19.0 and 15.6% in Falcon. Even though not statistically significant, these data again tell that SEM is more sensitive to drought stress than cultivar Falcon. Furthermore, it was noticed that the rate of stem thickness reduction due to water stress was less in VL-inoculated treatments of both genotypes, indicating the reduced effect of drought due to VL infection.

On the other hand, the effect of VL infection on stem thickness was only restricted to the susceptible cultivar Falcon causing 13.8, 21.7 and 10.0% significant reduction at 100, 60 and 30% FC, respectively. Interestingly, VL-resistance of genotype SEM was verified without any doubt as stems of infected plants remained as tough and vigorous as their control counterparts both under full irrigation as well as at moderate and severe water stress conditions (Table 4.8).

Table 4.8 Hypocotyl diameter and number of primary branches per plant measured from two *Brassica napus* genotypes exposed to drought stress and/or *Verticillium longisporum* infection at 49 DPI.

	Number of primary branches/plant			Hypocotyl diameter (mm)		
Treatments	100%FC	60%FC	30%FC	100%FC	60%FC	30%FC
SM	3.4 ± 0.5^{b}	2.7 ± 0.8^{b}	2.7 ± 0.3^{b}	10.4 ± 0.1^{a}	9.1 ± 0.4^{bc}	8.1 ± 0.2^{d}
SVL	6.5 ± 0.4^{a}	6.8 ± 1.3^{a}	6.6 ± 0.5^{a}	9.6 ± 0.3^{ab}	8.5 ± 0.2^{cd}	8.2 ± 0.2^{d}
FM	2.0 ± 0.2^{a}	2.0 ± 0.3^{a}	1.6 ± 0.3^{a}	9.4 ± 0.2^{b}	8.4 ± 0.1^{cd}	7.6 ± 0.3^{de}
FVL	7.7 ± 0.3^{b}	7.8 ± 0.8^{b}	7.0 ± 1.2^{b}	8.1 ± 0.3^{d}	6.6 ± 0.7^{f}	6.9 ± 0.2^{ef}

Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for four weeks starting from 21 days after inoculation with *Verticillium longisporum*. Mock and/or VL-inoculated plants supplied with water at 100% FC were used as control. Mean ± standard deviation data obtained from 30 plants of two independent experiments are presented. Mean values containing the same superscript are not significantly different at P=0.05. **FC**, field capacity. **SM**, SEM-05-500526 mock-inoculated. **FM**, Falcon mock-inoculated. **SVL**, SEM-05-500526 VL-inoculated. **FVL**, Falcon VL-inoculated.

4.3.4.4 Phenological growth stage

Assessment of the phenological stages using the BBCH scales (Appendix 4.13) was begun three weeks after inoculation of seedlings with VL (21 DPI) and performed for five consecutive weeks (Fig. 4.13). At the time of inoculation, average growth stage of vernalized seedlings of both genotypes was BBCH 14 (four leaves unfolded). Three weeks after inoculation, control SEM plants had already attained inflorescence emergence stage (BBCH 50) while Falcon plants were at the stage of BBCH 45 (stem elongation and development of lateral shoots). At 49 DPI, SEM plants in control treatments were ending the flowering stage (BBCH 67). At this time, Falcon was just beginning flowering (BBCH 62) (Fig. 4.8; Appendix 4.4). In general, the above results indicated that under normal growing conditions, genotype SEM grows relatively faster than Falcon.

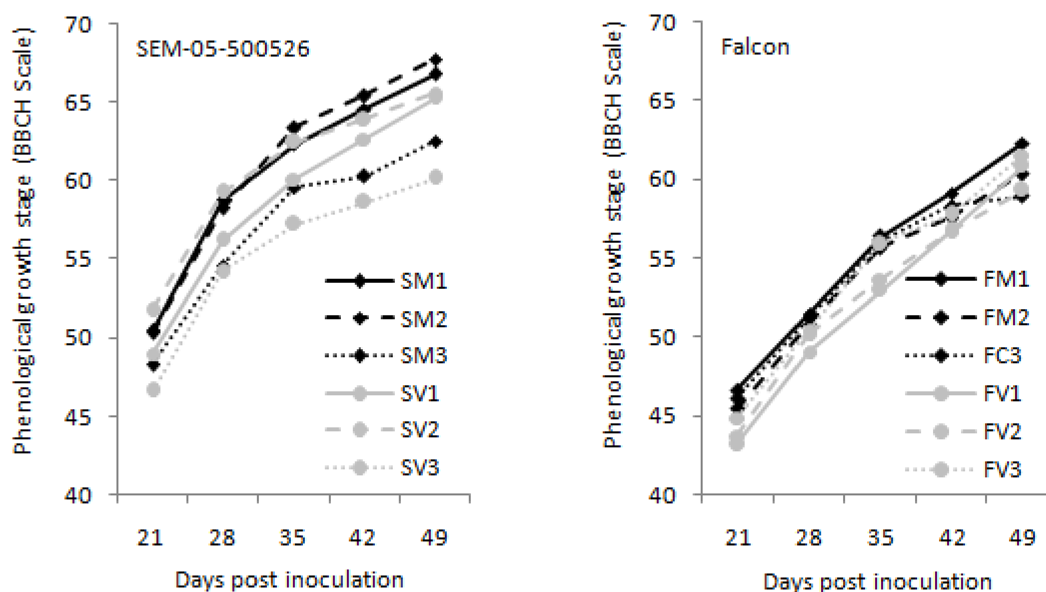


Figure 4.13 Phenological growth stages of two *Brassica napus* genotypes exposed to drought stress and/or *Verticillium longisporum* infection. Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for four weeks starting from 21 days after inoculation with *Verticillium longisporum*. Mock and/or VL-inoculated plants supplied with water at 100% FC were used as control. Mean data obtained from 30 plants of two independent experiments are presented. **FC**, field capacity. **S**, genotype SEM-05-500526. **F**, cultivar Falcon. **M**, mock-inoculated. **V**, infected with *Verticillium longisporum*. **1**, **2** and **3** refer to watering at 100, 60 and 30% field capacity respectively. Assessment was done using the BBCH scale.

The overall results showed that mock- and VL-inoculated had about the same growth stage, indicating no significant impact of VL infection on the phenological stages of both genotypes under greenhouse conditions. Regarding the impact of drought stress, significant differences between genotypes were observed. For cultivar Falcon, whether infected by VL or not, drought stress had no impact at all. Interestingly, as it is previously shown in physiological parameters, drought sensitivity of cultivar SEM was also clearly reflected in growth stage analysis where drought caused delayed flowering. At 49 DPI,

mock-inoculated fully irrigated SEM plants attained BBCH 67, a stage where flowering is declining and the majority of petals are fallen. At this time point, severely drought stressed control SEM plants were still at the beginning stage of flowering (BBCH 63). During infection with VL, a similar delay in flowering was caused by drought stress. Accordingly, VL-infected fully watered SEM plants at 49 DPI attained the full flowering stage (BBCH 65) while severely stressed plants were just at the initial stage of flowering (BBCH 60). No significant impact of moderate drought stress (watering at 60% FC) was observed (Fig. 4.13; Appendix 4.4).

4.3.4.5 Dry matter yield

The potential effect of biotic or abiotic stress factors and the degree of plant resistance towards these stresses is ultimately measured by the plant capability in producing reasonable yield under such growing conditions. In the present study, the asessment of shoot and root dry biomass yield during early and late growth stages revealed significant contributions of genotype, disease, drought and their interactions in determining biomass yield accumulation (Table 4.9). Assessment of plant biomass at early (28 DPI; Appendix 4.5; Appendix 4.6; Appendix 4.7) and late (49 DPI) time points showed more or less similar trends. Hence, biomass yield quantified at the end of the experiement (49 DPI) is presented as follows.

Effect of *Verticillium longisporum* on shoot and root DM yield

Even though the magnitude of yield reduction was by far lower in the tolerant genotype, VL infection caused significant yied loss both in VL-suceptible and VL-tolerant plants (Fig. 4.14). Shoot dry matter (SDM) reduction due to VL infection at 100, 60 and 30% FC in genotype SEM was 25.9, 35.1 and 9.9%, respectively. Interestingly, SDM yield loss in SEM at 30% FC was not significant indicating stable resistance of this genotype even under severe water deficit conditions. Neverthless, the loss due to infection at full irigation and moderate stress conditions was still higher than the one expected from a resistant genotype. From the evaluation of disease parameters, it is obvious that stunted growth (a symptom not induced under field conditions) was the major contributor to yield reduction in this genotype. In cultivar Falcon, susceptible to VL, a high and significant SDM yield reduction was caused by VL-infection resulting in 55.3, 63.2 and 51.4% loss at 100, 60 and 30% FC, respectively (Fig. 4.14; Table 4.13).

Similarly, assessment of root dry matter (RDM) yield showed that the effect of VL was extremely severe in Falcon but had no effect on SEM. Respective RDM accumulation in mock and VL-inculated SEM plants were 4.9 and 4.1 g/plant at 100% FC, 4.0 and 3.7 g/plant at 60% FC and 3.1 and 3.0 g/plant at 30% FC,; indicating that VL infection has no effect during optimum water suply as well as under drought stress.

Whereas in Falcon, substatial root biomass reduction was measured at all watering regimes resulting in 73.9, 79.5 and 62.8% reduction at 100, 60 and 30% FC, respectively (Fig. 4.14; Table 4.13).

Effect of drought stress on shoot and root DM yield

Comparision of biomass formation under optimal growing conditions showed that genotype Falcon and SEM do not significantly differ in yield potential and provided similar quantity of yield. Upon addition of the drought factor however, SEM provided lower yield than Falcon indicating its relative sensetivity towards drought stress (Fig. 4.14). Accordingly, SDM in SEM was significantly reduced both at moderate (26.8%) and severe (48.9%) drought stress situations while it was only at severe drought stress condition (35.5%) that significant shoot yield loss occured in cultivar Falcon. Concering root biomass yield, the effect of drought was noticed only at severe stress conditions. Here, moderate drought stress had no significant effect on root biomass production of both genotypes. Interestigly, enhanced drought sensitivity of genotype SEM was also observed on root biomass accumulation. Here, watering at 30% FC resulted in 36.8% reduction in SEM while it was only 25.2% in cultivar Falcon.

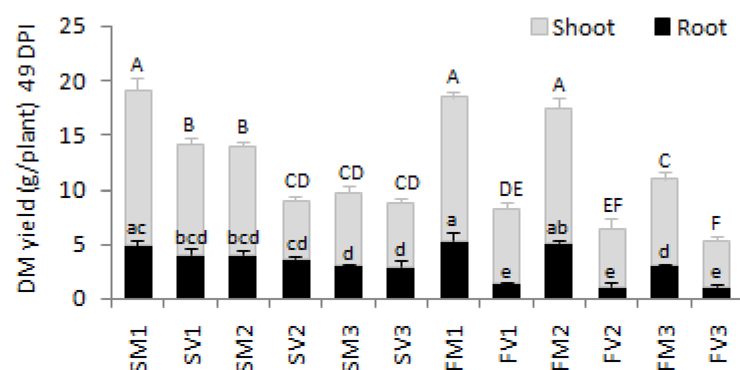


Figure 4.14 Shoot and root dry biomass yield of two *Brassica napus* genotypes subjected to *Verticillium longisporum* infection and/or drought stress conditions in a greenhouse experiment. Mock and/or VL-inoculated plants supplied with water at 100% FC were used as control. Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for four weeks starting from 21 days after inoculation with *Verticillium longisporum*. Mean data obtained from 30 plants of two independent experiments are presented. Bars indicate standard deviation. For each parameter, different letters on the bars with the same letter-case indicate significant differences between treatments at P≤0.05. **DM**, dry matter. **S**, genotype SEM-05-500526. **F**, cultivar Falcon. **M**, mock-inoculated. **V**, infected with *Verticillium longisporum*. Treatment names followed by numbers **1**, **2** and **3** refer to watering at 100, 60 and 30% field capacity, respectively.

During infection with VL as well, shoot DM yield of both genotypes at moderate and severe drought stress conditions was significantly lower than the one obtained at full irrigation (Fig. 4.14). However, when comparing the rate of yield reduction in the presence or absence of VL-infection, the effect of severe drought stress (30% FC) was relatively lower during VL infection compared to control conditions.

In contrast, moderate drought stress (60% FC) caused significantly higher losses with VL-infection compared to mock-inoculated treatments. Regarding accumulation of root biomass during VL infection, drought stress had no significant effect in both genotypes (Fig. 4.14).

Total dry matter yield

Total dry matter (TDM) reflecting the overall yield potential of the genotypes was obtained from the summation of shoot and root biomass yields of each treatment (Fig. 4.15). Mixed model ANOVA showed that all factors independently as well as thier interaction had significant effects on TDM accumulation (Table 4.9). Pearson's correlation analysis also showed a significant correlation of this trait with all disease, physiological and morphological parameters reflecting the strong link between plant stress response and yield accumulation (Table 4.11).

Irrispective of VL infection, TDM was significantly reduced by drought stress. The amount of loss at severe stress was signficantly higher compared to the loss occurring at moderate stress conditions. Genotypic differences with regard to drought stress tolerance were also reflected by TDM yield accumulation. In genotype SEM, TDM yield was significantly reduced both at moderate and severe drought stress conditions but in cultivar Falcon a significant reduction occured only at severe drought stress. Accordingly, the respective TDM yield reduction due to exposure to moderate and severe drought stress was 25.1 and 46.5% in SEM and 5.1 and 41.1% in Falcon, again suggesting better drought trolerance of genotype Falcon.

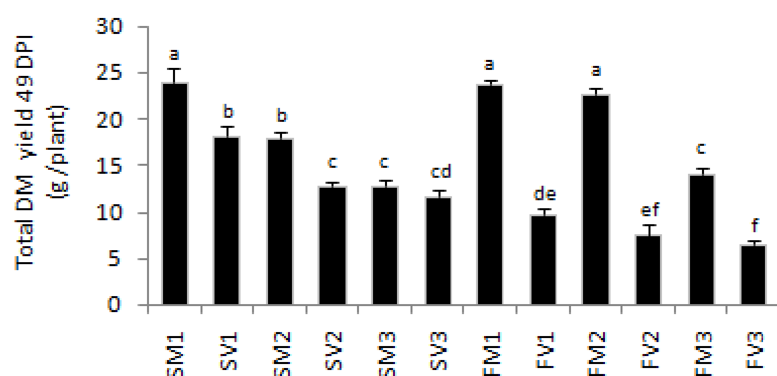


Figure 4.15 Total dry biomass yield of two *Brassica napus* genotypes subjected to *Verticillium longisporum* infection and/or drought stress in a greenhouse experiment. Mock- and/or VL-inoculated plants supplied with water at 100% FC were used as control. Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for four weeks starting from 21 days after inoculation with *Verticillium longisporum*. Mean data obtained from 30 plants of two independent experiments are presented. Bars indicate standard deviation. For each parameter, different letters on the bars indicate significant difference between treatments at P≤0.05. **S**, genotype SEM-05-500526. **F**, cultivar Falcon. **M**, mock inoculated. **V**, infected with *Verticillium longisporum*. Treatment names followed by numbers **1**, **2** and **3** refer to watering at 100, 60 and 30% field capacity, respectively.

During VL infection as well, TDM yield in genotype SEM was significantly reduced both at 60% and 30% FC. In cultivar Falcon however, even though relatively lower yields were obained from drought stress plants, the reduction was not significant (Fig. 4.15). This could be due to the stronger effect of the pathogen in this cultivar which might mask the effect of drought stress. Further comparison of TDM yield loss in mock- and VL-inoculated treatments showed that the impact of severe drought stress (30% FC) was reduced during VL infection. For instance, TDM yield loss at 30% FC in mock inoculated SEM and Falcon plants was 46.5 and 41.1%, respectively. In VL-inoculated plants, these values were reduced to 29.6% in SEM and to 22.4% in Falcon. In contrast, a completely opposite situation was observed at moderate drought stress (60% FC) condition. Here, the occurrence of drought during VL-infection caused more yield loss. As a result, the 25.1% TDM yield loss occurred in mock-inoculated SEM was increased to 29.6% during infection with VL. Similarly, in cultivar Falcon, TDM loss was higher at the time when VL and moderate drought stress occurred simultanously (22.4% loss) compared to only moderate drought stress (5% loss) (Table 4.14).

Regarding the impact of VL infection, an obvious significant reduction of TDM yield occurred in both genotypes. However, compared to the suceptible cultivar Falcon, VL-infected SEM plants grown at all watering regimes provided consisently high yields. Accordingly, TDM yield in mock-inoculated SEM plants at 100, 60 and 30% FC was 24.0, 18.0 and 12.8 g/plant, respectively. The respective yield during VL infection was 18.2, 12.8 and 11.7 g/plant. Interestingly, the yield obtained from mock- and VL-inculated plants at 30% FC was almost the same indicating the consistency of disease resistance of SEM under drought conditions. In Falcon, the respective TDM yied in mock- and VL-incoculated plants was 23.8 and 9.7 g/plant at 100% FC, 22.6 and 7.5 g/plant at 60% FC and 14.0 and 6.5 g/plant at 30% FC, with significant reduction at all watering regimes (Fig. 4.15).

In general, the effect of VL and drought stress on TDM accumulation of genotype SEM seems to be comparable. However when we look at the yield reduction due to VL infection, the contribution of stunting induced by VL-infection VL was more significant than any other disease symptom of VL. In Falcon, the suffering from VL infection was significantly higher than the effect of drought stress. Further comparison of the individual effects of either drought or *V. longisporum* with the combined effects, (i.e. comparison of mock-inoculated fully irrigated treatments with the rest of the treatments), the combined effect of VL and drought was by far stronger than any stress factor alone (Table 4.15).

Table 4.9 F-values of analysis of variance for the effects of genotype, *V. longisporum* infection, drought stress and interaction factors on disease development, plant physiolgy and agronomic trtaits.

	Parameters	G	VL	D	G*VL	G*D	VL*D	G*VL*D
	DF	1	1	2	1	2	2	2
Disease	AUDPC	53.5^{*}	398.5^{***}	7.3^{ns}	62.3^{**}	1.4^{ns}	7.3^{**}	1.4^{ns}
	VL DNA	286.0^{***}	323.8^{***}	2.0^{ns}	286.0^{***}	1.8^{ns}	2.0^{ns}	1.8^{ns}
	RS	568.6^{***}	1227.5^{***}	1.5^{ns}	568.6^{***}	7.4^{**}	1.5^{ns}	7.4^{**}
	PB	0.3^{ns}	428.5^{***}	1.4^{ns}	19.8^{***}	0.4^{ns}	0.5^{ns}	0.5^{ns}
Physiological	E	0.1^{ns}	0.7^{ns}	52.1^{***}	0.0^{ns}	2.5^{ns}	1.3^{ns}	0.1^{ns}
	gs	0.0^{ns}	1.5^{ns}	30.3^{***}	0.1^{ns}	1.3^{ns}	1.4^{ns}	0.0^{ns}
	A	0.1^{ns}	0.2^{ns}	73.0^{***}	0.1^{ns}	1.2^{ns}	2.6^{ns}	1.0^{ns}
	WUE	2.4^{ns}	0.3^{ns}	24.7^{***}	2.6^{ns}	1.3^{ns}	0.4^{ns}	3.6^{*}
	RWC	3.5^{ns}	0.8^{ns}	28.4^{***}	3.1^{ns}	0.6^{ns}	0.0^{ns}	0.3^{ns}
Agronomic	PH	59.4^{***}	273.5^{***}	30.4^{***}	134.0^{***}	2.8^{ns}	5.2^{*}	3.6^{ns}
	HD	123.3^{***}	72.8^{***}	100.9^{***}	17.5^{***}	1.7^{ns}	7.6^{**}	1.0^{ns}
	RDM	41.3^{**}	158.3^{***}	42.3^{***}	96.9^{***}	0.5^{ns}	11.6^{**}	5.7^{*}
	SDM	19.9^{**}	472.5^{***}	86.2^{***}	301.4^{***}	26.4^{***}	51.5^{***}	1.1^{ns}
	TDM	30.9^{**}	409.1^{***}	334.5^{***}	105.5^{***}	23.3^{***}	62.5^{***}	4.4^{*}

Drought treatments were applied by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for four weeks starting from 21 days after inoculation with *Verticillium longisporum* (49 days post inoculation). ANOVA was performed using the PROC MIXED procedure (SAS 9.3, SAS Inst. 2002). P-values are indicated in superscripts and the values less than 0.05 are considered a significant effect. *****, significant at P=0.05. ******, significant at P=0.01. *******, significant at P=0.001. **ns**, not significant. **DF**, degree of freedom. **G**, genotype. **VL**, *Verticillium longisporum*. **D**, drought. **AUDPC**, net area under disease progress curve, **VL DNA**, *Verticillium longisporum* DNA in hypocotyl. **RS**, relative stunting. **PB**, number of primary branches per plant. **E,** transpiration rate. **gs,** stomatal conductance of CO_2. **A,** photosynthesis rate. **WUE,** water use efficiency. **LPC,** leeaf proline content. **HPC,** hypocotyl proline content. **RWC,** leaf relative water content. **PH,** plant height. **HD,** hypocotyl diameter. **SDM,** shoot dry matter. **RDM,** root dry matter. **TDM,** total dry matter.

Table 4.10 F-value of analysis of variance for the effects of genotype, *Verticillium longisporum* infection, drought stress, and interaction factors on acumulation of free proline in leaf and hypocotyl tissue of *Brassica napus*.

Factors	G	VL	D	PP	G*VL	G*D	G*PP	VL*D	VL*PP	D*PP	G*VL*D	G*VL*PP	G*D*PP	VL*D*PP	G*VL*D*PP
DF	1	1	2	1	1	2	1	2	1	2	2	1	2	2	2
F	57.2^{***}	10.3^{*}	543.0^{***}	432.4^{***}	5.4^{*}	69.6^{***}	18.7^{***}	3.6^{ns}	0.0^{ns}	85.0^{***}	3.0^{ns}	0.0^{ns}	1.0^{ns}	97.6^{***}	11.5^{***}

Drought treatments were applied by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for four weeks starting from 21 days after inoculation with *Verticillium longisporum* (49 days post inoculation). ANOVA was performed using the PROC MIXED procedure (SAS 9.3, SAS Inst. 2002). P-values are indicated in superscripts and the values less than 0.05 are considered a significant effect. *****, significant at P=0.05. ******, significant at P=0.01. *******, significant at P=0.001. **ns**, not significant. **DF**, degree of freedom. **F**, F-value. **G**, genotype. **VL**, *Verticillium longisporum*. **D**, drought. **PP**, plant part.

4.3.5 Correlation and regression analyses

Pearson linear correlation analysis was conducted to analyse relationships within and between the different data categories. Among disease (AUDPC, VL DNA, stunting and branching) and agronomic parameters (plant height, hypocotyl diameter and DM yield), there were significant and strong positive correlations (r=0.74 to 0.99). Regarding the relationship between disease and agronomic traits, again significant and strong, but negative correlations were found. Among these, the strongest relationship (r=0.83 to 0.91) was observed between plant height and disease variables. In the same way, the relationship among physiological parameters (gas exchange, WUE, RWC and proline content) was strong and significant (r=0.52 to 0.98). Depending on the type of parameters, the relationships were either positive or negative. The significance and strength of the relationships between physiological and agronomic variables was also variable depending on the type of the parameters. Of all the agronomic parameters, the one that showed the strongest significant correlation with physiological parameters was hypocotyl diameter. The positive correlation found between gas exchange and SDM was reasonably strong as well. On the other hand, the correlations between physiological parameters and plant height/RDM were either weak or insignificant. Interestingly, none of the disease parameters showed a significant correlation with any of the physiological measurements (Table 4.11). Similar results were obtained from data collected at 28 DPI (Appendix 4.9).

Stepwise regression analysis is one of the most commonly used statistical tool which is applied to identify independent variables that significantly affect or most effectively predict the variation of a dependent variable (Gomez and Gomez, 1984). In the present study, this analysis was conducted to determine the relative contributions of genotype, disease and drought in affecting disease development, plant physiology and agronomic performance. Besides, a separate analysis was also performed to identify the most appropriate variables responsible for variations in TDM yield accumulation under drought and VL-infection conditions.

To determine the effects of the three experimental factors (genotype, disease and drought), stepwise regression was applied to 16 dependent variables representing disease, physiological and agronomic traits (Table 4.12). Before analysis, the three qualitative independent variables were coded as 0 and 1 (Gomez and Gomez, 1984). For convenience of analysis, data from fully watered and severely stressed treatments were used. Results showed that disease parameters were only significantly affected by VL-infection. Considering physiological parameters, except for the slight genotypic differences in proline synthesis, all physiological traits were only significantly affected by drought stress and no effect of VL on

Table 4.11 Coefficients of Pearson's correlation (r) describing relationships within and between disease, physiological and agronomic parameters measured from two *Brassica napus* genotypes exposed to drought stress and/or *Verticillium longisporum* infection 28 days after initiation of drought treatments (49 DPI).

	Parameters	Disease				Physiological							Agronomic			
		AUDPC	VL DNA	RS	PB	E	gs	A	WUE	RWC	LPC	HPC	PH	HD	SDM	RDM
Disease	VL DNA	0,89***														
	RS	0,95***	0,95***													
	PB	0,88***	0,66***	0,76***												
Physiological	E	-0,05^{ns}	-0,11^{ns}	-0,02^{ns}	-0,05^{ns}											
	gs	-0,10^{ns}	-0,12^{ns}	-0,05^{ns}	-0,10^{ns}	0,98***										
	A	0,02^{ns}	-0,08^{ns}	0,03^{ns}	0,05^{ns}	0,85***	0,79***									
	WUE	-0,07^{ns}	0,04^{ns}	-0,05^{ns}	-0,06^{ns}	-0,76***	-0,70***	-0,63***								
	RWC	0,14^{ns}	0,05^{ns}	0,12^{ns}	0,07^{ns}	0,58***	0,52**	0,69***	0,68***							
	LPC	-0,15^{ns}	-0,04^{ns}	-0,12^{ns}	-0,13^{ns}	-0,71***	-0,64***	-0,75***	0,75***	-0,79***						
	HPC	-0,22^{ns}	-0,19^{ns}	-0,24^{ns}	-0,13^{ns}	-0,63***	-0,58***	-0,67***	0,70***	-0,80***	0,83***					
Agronomic	PH	-0,89***	-0,88***	-0,91***	-0,73***	0,29*	0,30*	0,24^{ns}	-0,21^{ns}	0,13^{ns}	-0,16^{ns}	0,07^{ns}				
	HD	-0,58***	-0,67***	-0,61***	-0,39*	0,59***	0,59***	0,65***	-0,37*	0,38*	-0,46**	-0,34*	0,74***			
	SDM	-0,73***	-0,66***	-0,68***	-0,67***	0,50**	0,51**	0,48**	-0,35*	0,40*	-0,41*	-0,40*	0,88***	0,82***		
	RDM	-0,81***	-0,82***	-0,84***	-0,65***	0,35*	0,36*	0,33*	-0,20^{ns}	0,26^{ns}	-0,26^{ns}	-0,17^{ns}	0,94***	0,77***	0,90***	
	TDM	-0,76***	-0,72***	-0,73***	-0,68***	0,43**	0,48**	0,46**	-0,32*	0,37*	-0,38*	-0,35*	0,91***	0,82***	0,99***	0,94***

Correlation analysis was performed using PROC CORR procedure (SAS 9.3, SAS Inst. 2002). Minus signs indicate negative correlations. **ns**, not significant. ***,** significant at P=0.05. ****,** significant at P=0.01. *****,** significant at P=0.001. **DPI**, Days post inoculation with *Verticillium longisporum*. **AUDPC**, net area under disease progress curve, **VL DNA**, *Verticillium longisporum* DNA in hypocotyl. **RS**, relative stunting. **PB**, number of primary branches per plant. **E,** transpiration rate. **gs,** stomatal conductance of CO_2. **A,** photosynthesis rate. **WUE,** water use efficiency. **LPC,** leaf proline content. **HPC,** hypocotyl proline content. **RWC,** leaf relative water content. **PH,** plant height. **HD,** hypocotyl diameter. **SDM,** shoot dry matter. **RDM,** root dry matter. **TDM,** total dry matter.

plant physiology was found. As expected, agronomic and yield traits were significantly influenced by all the three factors. Regarding plant height, most variation (49%) was induced by VL infection, followed by genotype (16%) and drought stress (11%). Conversely, compared to VL (partial r^2=0.10), drought (partial r^2=0.59) and genotype (partial r^2=0.22) had stronger effects on hypocotyl diameter. Regarding yield parameters, the effect of VL and drought seems to be dependent on genotype and plant part (Table 4.12). Analysis of data collected at 28 DPI showed similar trends (Appendix 4.10).

Table 4. 12 Coefficients of determination (b), partial regression coefficients (r^2), and p-values of stepwise regression analysis indicating the effects of genotype, *V. longisporum* infection and drought stress on disease, physiological and agronomic traits of two *B. napus* genotypes.

	Factors	Genotype		*V. longisporum*		Drought	
	Parameters	b	Partial r^2	b	Partial r^2	b	Partial r^2
Disease	AUDPC	20.6	0.11***	52.9	0.74***	ns	ns
	VL DNA	468.1	0.30***	499.7	0.34**	ns	ns
	Stunting	25.1	0.21***	40.5	0.55***	ns	ns
	PB	ns	ns	4.5	0.90***	ns	ns
Physiological	E	ns	ns	ns	ns	-1.2	0.77***
	gs	ns	ns	ns	ns	0.09	0.65***
	A	ns	ns	ns	ns	-6.0	0.87***
	WUE	ns	ns	ns	ns	6.6	0.58***
	RWC	ns	ns	ns	ns	-11.9	0.69***
	LPC	-7.2	0.04**	ns	ns	35.3	0.89***
	HPC	-10.1	0.14**	ns	ns	21.4	0.61***
Agronomic	PH	-28.3	0.16**	-50.1	0.49***	-24.2	0.11**
	HD	-1.1	0.22***	-0.7	0.10***	-1.7	0.59***
	SDM	-2.2	0.05*	-5.4	0.34***	-6.3	0.45***
	RDM	-1.0	0.12**	-1.7	0.35**	-1.4	0.22**
	TDM	-3.2	0.07*	-7.2	0.35***	-7.6	0.40***

Mean data obtained from fully irrigated and severely stressed treatments of two independent experiments were used. Drought treatments (30% FC) were applied for four weeks starting from 21 days after inoculation with *Verticillium longisporum*. **AUDPC,** net area under disease progress curve. **VL DNA,** *Verticillium longisporum* DNA in hypocotyl. **RS,** relative stunting. **PB,** number of primary branches per plant. **E,** transpiration rate. **gs,** stomatal conductance of CO_2. **A,** photosynthesis rate. **WUE,** Water use efficiency. **RWC,** Leaf relative water content. **LPC,** leaf proline content. **HPC,** hypocotyl proline content. **PH,** plant height. **HD,** hypocotyl diameter. **SDM,** shoot dry matter. **RDM,** root dry matter. **TDM,** total dry matter; **ns,** not significant. ***,** significant at P≤0.05. ****,** significant at P≤0.01; *****,** significant at P≤0.001.

To determine the functional relationships between yield contributing parameters and TDM yield, further stepwise regression analysis was performed. In this procedure, TDM was considered as the dependent variable against the rest of all disease, physiological and agronomic parameters. During each step of analysis, predicting variables with a partial regression coefficient ≥5% were retained in the model. Those predicting variables that show no or insignificant (P≥0.05) relationships were used in following steps of analysis (Fig. 4. 16).

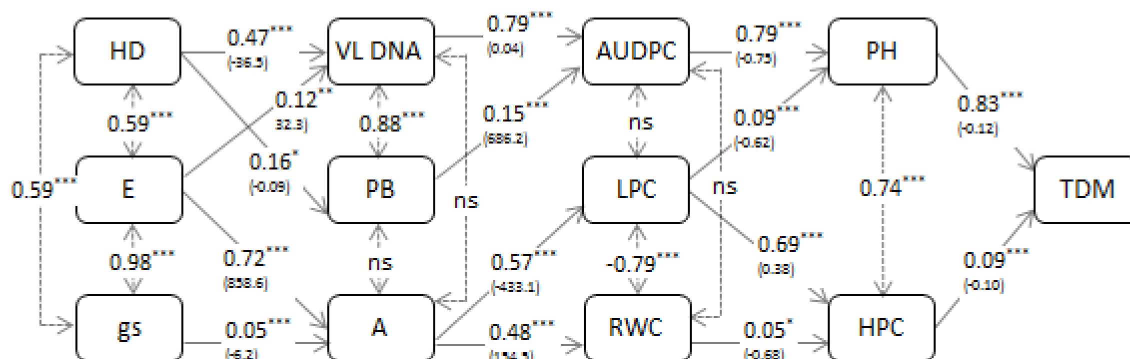


Figure 4.16 Sequential path model indicating stepwise regression coefficients of determination (b), partial regression coefficients (r^2), Pearson's correlation coefficients (r) and probability values of variables predicting total dry matter yield in *Brassica napus* genotypes subjected to drought stress and *Verticillium longisporum* infection. Mean data obtained from 30 plants of two independent experiments were used for analysis. Drought treatments were applied for four weeks starting from 21 days after inoculation with *Verticillium longisporum* (49 DPI). Solid arrows show a stepwise regression analysis with partial regression coefficients. Coefficients of determination are indicated in brackets. Dotted arrows show coefficients of Pearson's correlation indicating relationships between the predicting variables. **AUDPC,** net area under disease progress curve. **VL DNA,** *Verticillium longisporum* DNA in hypocotyl. **PB,** number of primary branches per plant. **E,** transpiration rate. **gs,** stomatal conductance of CO_2. **A,** photosynthesis rate. **RWC,** leaf relative water content. **LPC,** leaf proline content. **HPC,** hypocotyl proline content. **PH,** plant height. **HD,** hypocotyl diameter. **TDM,** total dry matter; **ns,** not significant; ***,** significant at P=0.05; ****,** significant at P=0.01; *****,** significant at P=0.001.

In the first order analysis, PH and HPC were kept in the model. Compared to the physiological parameter, HPC, which had little effect (9%), and the disease parameter PH were responsible for most of the variation (83%) in TDM indicating the stronger yield effect of VL infection compared to drought stress. Interestingly, the two parameters had a very strong significant positive correlation (r=0.74). In the second order analysis, PH and HPC were considered as dependent variables and the stepwise regression was conducted with the rest of the predicting variables. Accordingly, AUDPC, LPC, and RWC that have shown significant relationships with PH and HPC were retained in the model. Here, the disease parameter AUDPC accounted for the largest variation (79%) in PH but had no direct effect on HPC. The direct effect of LPC on PH was relatively small (9%) but it contributed for the largest share (69%) of variation in HPC (69%). RWC had a minor effect (5%) on HPC. Pearson correlation analysis showed no correlation between AUDPC and LPC or RWC whilst LPC had a strong negative correlation with RWC (r=

-0.79). In the third order analysis which considered AUDPC, LPC and RWC as dependent variables, VL DNA, PB and A were retained in the model. VL DNA and PB justified 94% of the variation in AUDPC. 57% of the variation in LPC and 48% in RWC were associated with variation in photosynthesis rate. Except for the strong correlation found between VL DNA and PB (r=88), no correlation was observed between the rest of disease and physiological parameters. In the fourth order analysis, a similar procedure was followed. The last three predicting variables remaining in the model were HD, E and gs. There were very strong positive correlations among these parameters. HD accounted for 47 and 16% of the variation in VL DNA and PB, respectively. E contributed for 72 and 12% of the variation in VL DNA and Photosynthesis. gs had very little direct effect (5%) on photosynthesis rate (Fig. 4.16). The results from a similar analysis of data collected at 28 DPI can be found in Appendix 4.11.

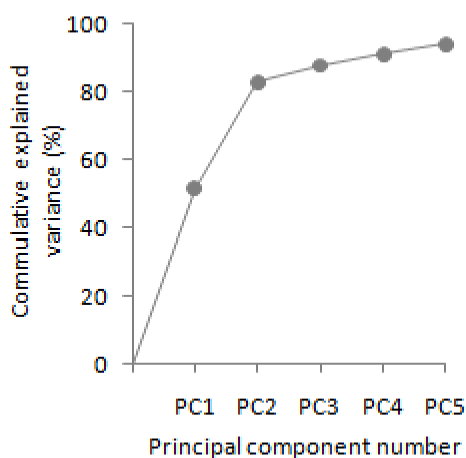


Figure 4.17 Scree plot of principal component analysis showing percentage of explained cumulative variance by principal components. Sixteen parameters measured from two *B. napus* genotypes subjected to drought stress and VL-infection were used in the analysis. Parameters included in the PCA: AUDPC, VL DNA, relative stunting, primary branches per plant, transpiration rate, stomatal conductance of CO_2, photosynthesis rate, water use efficiency, leaf relative water content, leaf proline content, hypocotyl proline content, plant height, hypocotyl diameter, shoot dry matter, root dry matter, total dry matter.

4.3.6 Principal component analysis

Principal component analysis (PCA) is one of the multivariate statistical procedures used to compress data size, examine data structure and identify variables that best explain sample variability (Ringnér, 2008). In this study, PCA was employed to analyze the pattern of sixteen parameters and identify those that best explain variability in VL-resistant and VL-susceptible *B. napus* genotypes exposed to drought stress and VL-infection. Figure 4.18 shows the contribution of each principal component to total variance. A scores scatter plot (Fig. 4.18A) of the first two principal components that explain 83% of the total variation (Fig. 4.17) shows a clear separation of six groups of treatments arranged along principal component one (PC1) and two (PC2). The first three groups located along PC1 were separated due to disease and genotype factors (mock-inoculated, VL-inoculated resistant and VL-inoculated susceptible, Fig. 4.18A, circled). The separation of the second three groups situated along PC2 (shown in different colours) was mainly due to the drought factor (non-stressed, moderately-stressed and severely-stressed), with further separation between control and infected treatments (Fig. 4.18A). The loading

scatter plot that illustrates separation of the 16 parameters into four distinct groups (Fig. 4.18B). Physiological parameters were split into two distinct groups, the first comprising gas exchange and RWC and the second one proline and WUE. Agronomic (PH, HD, SDM, RDM and TDM) and disease (PB, VL DNA, RS and PB) parameters were grouped separately. Strong positive correlations within disease, physiological and agronomic variables, negative correlation between disease and agronomy parameters and another negative correlation between the two groups of physiological parameters are also shown (Fig. 4.18B). Furthermore, association of scores *vs.* loading plot indicates the contribution of all disease variables to distinct separation of infected treatments of the VL-susceptible cultivar. Proline content and WUE parameters seem to be the best variables to explain the variation due to severe drought stress in mock and VL-infected treatments of both genotypes.

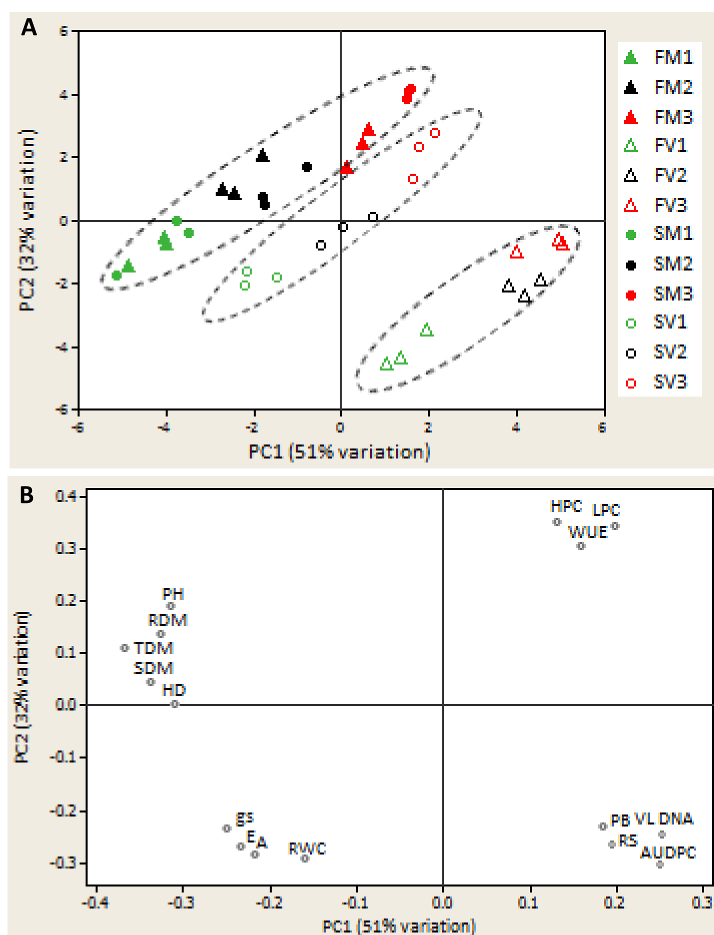


Figure 4.18 (A) Principal component analysis scores scatter plot of sixteen traits (disease, physiological and agronomic) measured in two *B. napus* genotypes grown under drought stress and *Verticillium longisporum* infection conditions. **PC**, principal component, **S**, genotype SEM-05-500526. **F**, cultivar Falcon. **M**, mock-inoculated. **V**, infected with *Verticillium longisporum*. Treatment names followed by the numbers **1**, **2** and **3** refer to watering at 100, 60 and 30% field capacity, respectively.
(B) Principal component analysis loading scatter plot of disease, physiological and agronomic parameters measured in two *B. napus* genotypes grown under drought stress and *VL* infection conditions. Parameters included in the PCA: Area under disease progress curve (AUDPC),VL DNA in hypocotyl (VL DNA), relative stunting (RS), Primary branches per plant (PB), transpiration rate (E), stomatal conductance of CO_2 (gs), photosynthesis rate (A), water use efficiency (WUE), leaf relative water content (RWC)), leaf proline content (LPC), hypocotyl proline content (HPC), plant height (PH), hypocotyl diameter (HD), shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM).

4.4 Discussion

4.4.1 *B. napus* infection against *V. longisporum* under drought stress

AUDPC and *V. longisporum* DNA quantification by qPCR

Verticillium longisporum is a narrow host range pathogen mainly specialized on crucifers (Zeise and Tiedemann, 2002). A previous study on mechanisms of VL resistance in *B. napus* demonstrated that among other factors, accumulation of vascular occlusions is one of the key components contributing to VL resistance (Eynck *et al.,* 2009b). Initially, it was assumed that despite serving as a mechanical barrier against fungal growth and dissemination, increased accumulation of vascular occlusions in hypocotyl xylem tissue of resistant *B. napus* genotypes might limit the translocation of water and mineral nutrients and thereby make plants more vulnerable to disease and drought stress. Disease evaluation results of the present comprehensive study however proved that the rate and level of Verticillium disease development in resistant *B. napus* plants is not affected by water supply. Hence, consistency of VL-resistance under optimum watering and drought conditions not only proves that VL-resistance mechanisms have no additive negative consequence on plant performance during drought stress but also demonstrates effective operation of the quantitative VL- resistance mechanisms even under conditions of severe drought stress.

A weekly disease severity evaluation has shown that in addition to the late appearance of initial symptoms (21 DPI) in the resistant genotype SEM, the rate and level of symptom development over time and across all watering regimes was uniformly very low. For this genotype, average net AUDPC values at 28 and 49 DPI were 15 and 36, respectively. These are less than half of the values recorded from the susceptible cultivar Falcon. Quantification of VL DNA by quantitative real time PCR also revealed a significant suppression of VL growth in SEM, irrespective of water supply. Further comparison of fungal biomass between early (28 DPI) and late (49 DPI) time points showed reduced fungal biomass also at the later time point. With optimum water supply, the average VL DNA quantified at 28 DPI was 39.1 ng/g of dry hypocotyl tissues. When the plant reached the flowering stage (49 DPI), the quantity decreased to 27.1 ng/g. In the presence of drought stress, fungal biomass during early and late growth stages remained similar. Even though in relatively small quantity, the detection of fungal DNA in hypocotyl tissue of the resistant genotype showed the occurrence of successful infection and indicates that resistance is not due to complete defense against infection. Eynck *et al.* (2009) have shown that VL-resistance in *B. napus* is achieved by impaired shoot fungal spread caused by resistance factors induced after root penetration. In the present study, the reduction of fungal biomass at the later growth stage (49 DPI) might have also been due to the sufficient accumulation of resistance factors which halted

fungal growth or even degrade some fungal structures while the plant continues growing and attaining increased size. In tomato plants infected with the wilt causing vascular pathogen *Pseudomonas solanacearum*, extensive and faster accumulation of the physical defence structure tyloses was observed in resistant varieties compared to susceptible plants (Grimault *et al.,* 1994).

In contrast to SEM, successful infection in cultivar Falcon occurred shortly after infection and the typical symptoms of yellowing and stunted growth were distinctly observed relatively early, at 14 DPI. In addition to this, disease levels as measured by net AUDPC and VL DNA showed a trend of sharp increase over time. At 28 DPI, a net AUDPC value of 36.3, which is more than twice the values in SEM, was recorded. At 49 DPI, disease severity was increased by more than one fold (net AUDPC = 76.0), which is again significantly higher than the values recorded from genotype SEM. In the presence of drought, a similarly high disease level as in fully watered treatments was recorded. Absolute quantification of fungal biomass by qPCR provided a better picture of the difference between susceptible and resistant genotypes. For instance, at optimal watering, 15-fold more VL DNA was quantified at 28 DPI in Falcon compared to SEM. Later at 49 DPI, this difference increased to 31-fold, indicating a sharp increase in fungal growth in cultivar Falcon *vs*. a decreasing tendency in SEM. Furthermore, qPCR results showed that levels of fungal DNA were positively correlated with the intensity of drought stress. A week after application of drought treatments (28 DPI), the respective average fungal DNA in dry hypocotyl tissue at 100, 60, and 30% FC were 39.1, 30.4 and 35.2ng/g in SEM and 574.5, 659.9 and 826.6ng/g in Falcon. Three weeks later (49 DPI), the respective average fungal DNA at 100, 60, and 30% FC was 27.1, 29.0 and 36.0 ng/g in SEM and 839.1, 1,032.4 and 1,096.4 ng/g in Falcon; indicating more pronounced effect of VL during drought stress particularly on the susceptible cultivar. The consistent inhibition of fungal growth across all watering levels in genotype SEM *vs*. the significant increased growth in cultivar Falcon during drought stress has led to a greater difference between the two genotypes. Significant impact of cultivar on the rate of VL colonization and symptom development has already been reported (Eynck *et al.,* 2009a). Very thin and weak Falcon plants resulting from simultaneous stress from VL-infection and drought might favor VL to invade vascular tissue of susceptible cultivars and suggests a potential additive effect of VL-infection on susceptible *B. napus* varieties grown in drought-prone regions.

Stunting effect

One of the most peculiar greenhouse symptoms of VL infection in *B. napus* is stunted growth (Eynck *et al.,* 2009a). Unlike the previously discussed disease parameters, significant stunting effect due to VL infection was not restricted only to the susceptible genotype. The indiscriminate stunting effect of VL-

infection suggests an infection induced change in plant metabolism that might cause hormonal imbalance leading to stunted growth and other abnormal phenotypes such as leaf shriveling and excessive production of side shoots (Fig. 4.2). Study on ethylene-mediated resistance in Arabidopsis showed the level of ethylene (a hormone known for inhibition of vegetative growth; promoting ripening and senescence) found to be increased in response to VL-infection (Johansson *et al.,* 2006). Another study on the effect of VL-infection on plant nutrient relations in *B. napus* (Floerl *et al.,* 2008) and Arabidopsis (Floerl *et al.,* 2010) showed that severe stunting caused by VL-infection is not the consequence of nutrient limitation caused by xylem obstruction. Interestingly, significant stunting by VL-infection in the resistant genotype SEM was observed only in fully irrigated plants. Even the strong stunting effect (50%) observed at 28 DPI was further significantly reduced to 21% with further plant growth (49 DPI) indicating the recovery of plants over time. Failure of VL to induce stunting in the resistant genotype during drought stress provides another supportive evidence for stability of VL-resistance under drought stress. On the other hand, this result might be also explained by the relatively high drought sensitivity of genotype SEM which showed strong stunting due to drought in mock-inoculated plants compared to VL-infected treatments (Table 4.7). Concerning the VL-susceptible cultivar Falcon, the stunting effect of VL was extremely severe and not comparable with the drought effect. After all, VL-induced stunting was observed relatively early (14 DPI) and the degree or extent of stunting remained high over time. As a result, stunting effect of VL remained consistently significant until 49 DPI causing up to 74% reduction of plant height. Due to the severe stunting effect of VL-infection in this cultivar which probably masked the impact of drought stress, the clear determination of the stunting effect due to VL-infection under drought conditions was not possible.

Considering plant height as agronomic parameter, during the first week of exposure to drought stress (21-28 DPI), the effect on plant length was not significant in both genotypes. A significant reduction was first observed two weeks after the initiation of drought treatments (35 DPI) and remained evident until 49 DPI. Previous studies have shown that *B. napus* plants exposed to drought stress respond with shorter plant length (Qaderi *et al.,* 2006; Shirani Rad and Zandi, 2012; Khalili *et al.,* 2012). Interestingly, irrespective of genotype and growth stage, drought stress had no significant effect on plant height in VL-inoculated plants. This could either be due to the relative strong stunting effect of VL, which masked the effect of drought stress, or due to a reduced impact of drought stress during VL-infection. It was also noticed that the impact of drought was relatively stronger on genotype SEM. Accordingly, moderate and severe drought stress significantly reduced plant height of SEM while it was only severe drought that

caused significant reduction of plant height in Falcon. This is in strong agreement with the physiological measurements that revealed higher drought sensitivity of genotype SEM.

The consistent reaction of susceptible and resistant *B. napus* genotypes to VL-infection under different moisture conditions was reflected by correlation analysis that showed significant and strong positive relationships among phenotypic and molecular disease evaluation parameters (Table 4.11). The mixed model analysis of variance also showed that genotype, VL infection and their interaction were the major responsible factors that significantly affected all disease parameters (Table 4.9). According to stepwise multiple regression analysis, VL-infection and genotype were the most important factors that significantly contributed to the variation of all disease variables. For instance, 74% and 11% of the change in AUDPC was by VL and genotype, respectively. The respective contribution of these factors to the variation in VL DNA was 30 and 34%. 55% and 21% of the variation in stunting was explained by VL-infection and genotype factors, respectively. 90% of the variation in branching was explained by VL infection. Drought alone and its interaction with VL or genotype and the combination of all the three factors have no significant impact on Verticillium disease development in *B. napus,* particularly in resistant genotypes (Table 4.12). Any of the disease evaluation variables showed significant correlation with physiological measurements (Table 4 .11). To date, there is no comprehensive study that looked at the impact of drought stress on the *B. napus - V. longisporum* interaction in general and the effect of drought stress on VL disease development in particular. Even the very few studies in Arabidopsis (Reusche *et al.,* 2012; Reusche *et al.,* 2014) were focusing only on the impact of infection on plant responses to drought stress. Nevertheless, related studies on other Verticillium species have shown that the impact of drought stress on disease development seems to be dependent on the crop and Verticillium species combination. According to Arbogast *et al.* (1999) the effect of moisture deficit stress on increased foliar senescence due to *V. dahliae* depends on potato cultivars. Pennypacker *et al.* (1991) showed reduced disease symptoms of *Verticillium albo-atrum* during drought stress in alfalfa. Bletsos *et al.* (1999) have reported an increased *V. dahliae* disease severity with decrease in irrigation frequency in eggplant.

4.4.2 *B. napus* physiology during drought stress and infection with *V. longisporum*

Physiological parameters were monitored in order to address the questions concerning *B. napus*-VL interaction under drought stress conditions. The first and foremost objective was to investigate whether VL-infection and the resulting accumulation of vascular occlusions have a negative impact on plant fitness under conditions of water deficit stress. In addition, the role of genotypic difference in

determining physiological responses under disease and drought stress conditions was studied. To answer the above questions, several physiological parameters in plants grown under optimal conditions were compared to those exposed to either drought, VL-infection or both stress factors. Results obtained from the different physiological measurements as well as gene expression analysis consistently showed that VL infection in *B. napus* did not cause a significant change to most of the physiological variables considered. That is, except for proline synthesis and water use efficiency, all other physiological parameters measured in this study were only significantly affected by drought stress. Neither VL alone nor its interaction with drought or the genotype had a significant effect on expression of drought inducible genes (Fig. 4.12), gas exchange and RWC parameters (Table 4.9).

Gas exchange and water use efficiency

In general, regardless of genotype and VL infection, stepwise decrease in gas exchange (transpiration rate, stomatal conductance, photosynthesis rate) and increase in WUE was observed as water supply dropped from full watering (100% FC) to 60% and 30% FC. Mixed model ANOVA showed that a significant reduction in gas exchange was only induced by drought. The remaining two factors and their interaction had no impact at all. Regarding WUE, in addition to the strong impact of drought, genotype x VL x drought interaction had a significant effect (Table 4.9). It was also noticed that the genotype SEM had a slightly higher WUE than Falcon. Besides, unlike VL-inoculated plants, 30% FC watering in mock-inoculated SEM plants significantly increased WUE (Fig. 4.7). Since increased WUE is a common evolutionary designed response of plants to drought stress (Blum, 2005), the above observations suggest two key points, namely the drought sensitivity of genotype SEM and the reduced impact of drought stress during infection with VL. In contrast to SEM, severe drought stress significantly increased WUE in VL-inoculated Falcon plants but not in mock-inoculated treatments, again suggesting a better reaction of this genotype to drought and the severe impact of VL-infection in this genotype as well. As it is shown for gas exchange parameters, VL infection has no significant impact on WUE of both genotypes (Table 4.9; Table 4.12).The impact of drought stress on *B. napus* gas exchange has been extensively investigated in various field and greenhouse studies. According to these studies, photosynthesis, transpiration and stomatal conductance are the major gas exchange parameters that are significantly reduced due to plant exposure to drought stress (Hashem *et al.,* 1998; Naderikharaji *et al.,* 2008; Sangtarash *et al.,* 2009).

Regarding the impact of VL, except for the slight reduction in transpiration rate and stomatal conductance in fully irrigated (100% FC) treatments, VL-infection alone and its interaction with drought

and genotype had no significant effect on gas exchange (Table 4.9; Table 4.12). Similar results have been reported by Floerl *et al.* (2008) who have shown no impact of VL-infection on photosynthesis and transpiration rates in *B. napus*. In another study with Arabidopsis, they also have shown that VL-infection has no negative impact on plant water and nutrient relation (Floerl *et al.,* 2010). Similar observations have also been reported elsewhere in several crop species infected with other *Verticillium* species. Haverkort *et al.,* 1990 found that *V. dahliae* alone and its interaction with drought had no impact on transpiration rate and stomatal conductance in early developmental stages of potato. In sunflower, stomatal conductance was unaffected by infection with *V. dahliae* (Sadras *et al.,* 2000). *Verticillium albo-atrum* infection in alfalfa caused no significant reduction in stomatal conductance and photosynthesis (Pennypacker *et al.,* 1991). Besides drought stress, different concomitant factors such as fungal toxins are suggested as causes of stomatal closure that lead to depression in photosynthetic activity in *Verticillium albo-atrum* infected tomato plants (Lorenzini *et al.,* 1997). In another pathosystem, Thorne *et al.* (2006) have shown that grapevine infection by the xylem-limited bacterium *Xylella fastidiosa* caused no effect on stomatal conductance and transpiration rate. In the *B. napus*-VL interaction, it is evident that vascular colonisation of VL and accumulation of occlusions is restricted to individual xylem vessels and adjacent vessels remained completely free and unaffected (Eynck *et al,* 2007). The presence of unaffected and fully functional vessels could provide adequate capacity for sufficient uptake of water and mineral nutrients. This could be one of the possible explanations why gas exchange is not affected by VL-infection and also why VL is not causing wilt symptoms in *B. napus*. Floerl *et al.* (2008) suggested that chlorotic and stunting symptoms of VL-infection in *B. napus* are not the result of limited water supply due to pathogen induced xylem obstruction. Induced accumulation of vessel occlusions due to infection with the bacterium *Xylella fastidiosa* was not causal for the water deficit and plant wilting symptoms in grapevine (Thorne *et al.,* 2006). In contrast, a significant reduction of gas exchange due to drought stress induced by *V. dahliae* infection is reported in several crop species including pepper (Pascual *et al.,* 2010), tomato (Bowden *et al.,* 1990) and cotton (Hampton *et al.,* 1990).

Leaf relative water content

As previously described, reduction in leaf relative water content (RWC) was observed due to water deficit and the effect was particularly significant at severe drought stress and at the later time point (Table 4.4). A week after initiation of drought treatments (28 DPI), RWC of stressed plants was slightly decreased but the reduction was not statistically significant except in infected Falcon plants. Since drought treatments began at 21 DPI and plants at this time point were still small (BBCH 50; lower bud development stage), this might have led to low water demand and consequently to a low rate of

transpiration. As a result, the soil moisture difference between normally watered and drought stressed treatments at 28 DPI might have not been large enough to show significant differences. Kumar and Elson (1992) have shown a significant effect of plant growth stage on leaf RWC of drought-stressed *B. napus* plants. At 49 DPI, however, due to large biomass production, applied water might be used quickly and reduction in leaf RWC was observed shortly after re-watering. Accordingly, 13% and 14% reduction in RWC was observed in mock- and VL-inoculated SEM plants, respectively. Interestingly, even at this time point, the reduction in cultivar Falcon was lower and insignificant, resulting in 10.3 and 10.1% loss at moderate and severe drought stress treatments, respectively. Depending on the plant cultivar or intensity of drought, up to 40% reduction of leaf RWC in *B. napus* have been reported in several studies (Ullah *et al.,* 2012; Khalili *et al.,* 2012; Sepehri and Golparvar, 2011; Good and Zaplachinski, 1994; Khalili *et al.,* 2012). Regarding the disease factor, irrespective of plant age and genotype, no impact of VL-infection on RWC was observed (Table 4.9). Similarly, Reusche *et al.* (2014) recently reported no effect of the wilt inducing vascular pathogen *V. dahliae* on leaf water content of Arabidopsis. In pepper as well, leaf RWC remained unchanged until four weeks after inoculation with *Verticillium dahliae* (Goicoechea, *et al,* 2000). In contrast, Reusche *et al.* (2012) showed a rather positive impact of V*. longisporum* in Arabidopsis where infection provided improved leaf water content under drought stress condition.

Proline content

Accumulation of substantial amounts of free proline as a response to a wide range of biotic and abiotic stress is a common phenomenon in different groups of organisms (Delauney and Verma, 1993). In plants, stress-induced accumulation of proline has multiple positive roles in stress adaptation, recovery and signaling. It is involved in intracellular osmotic adjustment between cytoplasm and vacuole, protects photosynthetic organelles, stabilizes redox balance and influences programmed cell death which triggers HR during infection with avirulent pathogens, and regulates plant growth and development during stress conditions (Szabados and Savouré, 2010). In the present study, exposure of *B. napus* plants to drought stress caused up to 18-fold increase of proline accumulation. Drought-induced synthesis of free proline was positively correlated with the intensity of drought in plants in this study, with the highest amount quantified in plants supplied with water at 30% FC. Similarly, a gradual pattern of increase in proline concentration with increase in intensity and duration of drought (Ghaffari *et al.,* 2011; Omidi, 2010) and salinity stress (Saadia, *et al.,* 2012) has previously been reported in *B. napus*.

Other factors responsible for variations in drought-induced proline synthesis were genotype and plant part. The initial leaf proline content was slightly higher in cultivar Falcon than in SEM. Drought stress

however induced significantly higher proline synthesis in SEM, regardless of VL-infection and plant part (Fig. 4.10). It is possible that due to its sensitivity to drought, this genotype is responding with higher accumulation of proline. High proline accumulation does not necessarily reflect the level of drought tolerance in plants since it is rather a stress sensor and an indicator of the plant water status (Sundaresan and Sudhakaran, 1995; Hanson *et al.,* 1977). This is in strong agreement with physiological, agronomic and disease evaluation results that clearly showed drought sensitivity and VL-resistance of this genotype. Expression analysis of the specific proline metabolism gene Δ1-pyrroline-5-carboxylate synthase1 (*P5CS1*) also revealed relatively increased expression of this gene in Falcon compared to SEM (Fig. 4.12). Saadia, *et al.* (2012) noted a maximum expression of the *P5CS1* gene in a drought sensitive *B. napus* line. On the other hand, several studies showed strong association of high proline concentration and improved drought tolerance in several crop species including *B. napus* (Ghaffari *et al.,* 2011; Saadia, *et al.,* 2012), soybean (Silvente *et al.,* 2012), alfalfa (Kang *et al.,* 2011) and rice (Bunnag and Pongthai, 2013). Considering the impact of VL-infection in proline synthesis, it was noticed that drought induced proline accumulation in leaf and hypocotyl tissue was significantly higher in the VL-resistant genotype SEM (Fig. 4.10). In pepper, Goicoechea, *et al*. (2000) suggested an increased proline accumulation as a sensor of wilt damage caused by *V. dahliae* infection. In other pathosystems, high proline concentration is associated with resistance of Arabidopsis (Fabro *et al.,* 2004) and tobacco (Senthil-Kumar and Mysore, 2012) against avirulent strains of *Pseudomonas syringae* via triggering of HR. In our *B. napus*-VL interaction however, since VL resistance does not involve induction of HR, the involvement of proline (as a scavenger of ROS) in plant resistance is not likely. The second interesting factor that showed significant differences in the amount of drought-induced proline accumulation was the plant part. Irrespective of any other factor considered in this experiment, it was found that the drought-induced total amount of proline was significantly higher in leaf than in hypocotyl tissue (Table 4.5; Fig. 4.10). As a key regulator of drought stress adaptation and signaling, it is not surprising that proline is present in high concentration in leaf tissues. Higher production of drought induced proline in the leaf than in stem and root tissue has been previously reported in potato (Ghorbanli *et al.,* 2012) and in the ornamental plant *Matthiola incana* (El-Quesni *et al.,* 2012).

Comparison of drought induced changes in rate of physiological processes in the presence and absence of VL infection

The most interesting and important observation regarding physiological parameters was the difference in physiological changes between mock- and VL-inoculated plants. Comparison of changes in gas exchange, hypocotyl proline and WUE due to drought stress under mock- and VL-inoculation conditions

indicated that the impact of drought stress seems to be reduced during infection with VL. For instance, in mock-inculcated treatments, the rate of transpiration, stomatal conductance and photosynthesis at 30% FC was reduced 4-, 6- and 2-folds in SEM and 4-, 5- and 3-fold in Falcon, respectively. During infection with VL, the respective reduction of the three gas exchange parameters was 2-, 3-, and 1-fold in SEM and 2-, 2-, and 1-fold in Falcon. In case of hypocotyl proline content, 10- and 18-fold increase due to severe drought stress was observed in mock-inoculated SEM and Falcon plants, respectively. Whenever the disease factor was added, proline accumulation was increased only by 7-fold in SEM and 4-fold in Falcon. Analysis of WUE also showed a similar trend for genotype SEM (Table 4.5). The reduced impact of drought stress during VL-infection in both VL-resistant and VL-susceptible *B. napus* genotypes may be associated with infection induced anatomical changes in xylem tissue. According to Eynck *et al.* (2007) and Eynck *et al.* (2009b), colonization of xylem vessels with VL and accumulation of VL-induced vascular occlusions in *B. napus* are restricted to individual vessels and other adjacent vessels remain completely uninfected and free of obstructions. The presence of a sufficient number fully functional vessel might provide efficient uptake and transport of water and this may explain why plants are not suffering from drought stress during VL-infection. Yadeta and Thomma *et al.* (2013) also suggested that if less numbers of vessels are closed by occlusions, the host plant will not suffer from drought stress. Furthermore, a recent study in Arabidopsis has shown enhanced drought tolerance of infected plants due to VL-induced *de novo* xylem formation (Reusche *et al.,* 2014). Xu *et al.* (2008) showed viral infection induced an increase in osmoprotectant and antioxidant substances providing better drought tolerance to several crop species. Enhanced drought tolerance associated with mycorrhizal fungi has been reported in many crops such as wheat (Ellis *et al.,* 1985; Abdel-Fattah and Abdul-Wasea, 2012), lettuce (Ruiz-Lozano *et al.,* 1995), onion (Nelsen and Safir, 1982), common bean (Aroca *et al.,* 2007), rosemary (Sánchez-Blanco *et al.,* 2003), and pigeon pea (Qiao *et al.,* 2011). Stimulation of increased accumulation of osmolites and sugars, improved nutrient uptake and root growth, reduced plant surface area of water loss etc. are among the mechanisms of mycorrhizal induced drought tolerance reported previously. It is also well known that an increase in xylem vessel density and diameter provides improved water absorption during drought which is closely associated with drought tolerance in tree (Qian and Ning, 2012) and annual crop species (Kulkarni *et al.,* 2008).

4.4.3 Expression of drought responsive genes during drought stress and infection with *V. longisporum*

The main intention of this study was to investigate the expression level of drought inducible genes in two *B. napus* genotypes that show contrasting resistance to VL-infection and to further analyze whether the pattern of gene expression is changing due to the formation of infection induced histological

changes in xylem tissue. RT-PCR analysis of six different drought inducible genes showed up to 6-fold increase in gene expression caused by severe drought stress. This is in agreement with the results of most physiological measurements that showed a significant impact of drought on major physiological characters in mock- and VL- inoculated plants. The transcription factors DREB2 and CIPK1 that control the expression of stress-responsive genes via an ABA-independent pathway (Yang *et al.,* 2010) and the P5SC1 gene which regulates proline biosynthesis in cytosol and chloroplast (Szabados and Savouré, 2010) showed the highest levels of expression. Previous studies in *B. napus* have shown that dehydration induced increased expression of DREB (Kagale *et al.,* 2007) and P5SC1 (Saadia, *et al.,* 2012; Xue *et al.,* 2009) genes. Chen *et al.* (2012) reported increased expression of CIPK genes in *B. napus* due to salt stress and overexpression of these genes in Arabidopsis provided enhanced tolerance to salt stress. On the other hand, the expression of P5SC2, CBF17 and HB6 was relatively lower. P5CS2 is one of the key regulators of proline biosynthesis in mitochondria (Szabados and Savouré, 2010). CBF17 is a family of DREB1 transcription factors which is known to be mainly induced by cold stress (Gao *et al.,* 2002; Savitch *et al.,* 2005). Regarding HB6 which is a single copy gene in *B. napus* (Yu *et al.,* 2005), not only that its expression was slightly increased by drought stress, but also its expression seems to be unaffected by drought stress when plants are infected with VL. According to Yu *et al.* (2005), this gene is constitutively induced by several abiotic factors in a time dependent manner. Genotypic comparisons also suggest that the accumulation of P5SC1, DREB2 and CIPK1 gene transcripts were relatively higher in Falcon than SEM. This is in agreement with physiological and yield assessment results that showed better performance of Falcon under drought conditions. Furthermore, the slightly reduced expression of HB6, P5SC1, DREB2, and P5SC2 genes observed at least in one of the genotypes might also suggest a reduced impact of drought stress during infection with VL.

4.4.4 Single and combined effects of *V. longisporum* infection and drought stress on yield and agronomic traits

Branching

Regarding production of primary branches, drought stress had very slight and insignificant effects (Table 4.8). Hashem et al. (1998) have showed similar results. Since plants were grown in a very confined root environment (limited to pot size), this condition might have physically limited root growth. Consequently, in order to balance the root-shoot ratio, plants might be forced to limit shoot vegetative growth such as branching. This may explain why numbers of branches in fully irrigated plants were not different from drought stressed treatments. In contrast, studies have shown that drought induced a significant reduction of primary branches in *B. napus* (Bilibio *et al.,* 2011; Shirani Rad and Zandi, 2012).

Sadaqat *et al.,* (2003) found a high correlation between branching and seed yield of drought tolerant *B. napus* genotypes under drought stress conditions and suggested branching as best parameter for selection of high yielding canola varieties under drought. When we look at the effect of VL-infection, it induced excessive production of abnormal side branches both in susceptible and resistant genotypes. Unfortunately, due to the small size and short life span characteristics of these unusual branches, increased branching (>3-fold) observed in infected plants was not reflected as improved biomass yield.
A similar branching effect of VL infection in *B. napus* has been reported previously (Zeise, 1992). Häffner *et al.,* (2010) have also shown two QTLs that control excessive branching effect of VL infection in Arabidopsis.

Hypocotyl diameter

Stem diameter, apart from genotypic differences, was remarkably influenced by drought, VL infection and all interaction factors (Table 4.8). Besides, stem diameter was the only trait (other than SDM) that showed a very strong correlation with the disease, physiological and other agronomic and yield measurements (Table 4.11). Genetically, the two genotypes had a significantly different stem thickness, SEM ticker than Falcon. VL-infection alone only reduced the hypocotyl diameter in the susceptible cultivar across all watering regimes. This may have been due to the utilization of xylem sap nutrients and the degradation of vessel walls by the fungus which might have led to reduced xylem diameter growth. Previous studies have shown that VL spends the largest part of its life cycle in the vascular environment (Eynck *et al.,* 2007) using xylem sap constituents as nutrient source (see chapter 3 on xylem sap experiment; Floerl *et al.,* 2010). Vascular phytopathogens including Verticillium species acquire their nutritional demand either directly from xylem sap, by degrading xylem vessel walls or inducing nutrient leakage from surrounding tissues (Yadeta and Thomma, 2013). In contrast, the hypocotyl of VL-infected SEM plants remained as thicker as in the control plants (Table 4.8), indicating consistency of VL-resistance regardless of exposure to water stress. Under field conditions however, Gladders, (2009) showed no relationship between stem diameter and VL disease severity in UK. Considering the impact of drought stress, irrespective of genotype and VL-infection, the stem diameter of plants exposed to moderate and severe drought treatments was significantly reduced. It is obvious that drought exposed *B. napus* plants produce thinner stems (Bilibio *et al.,* 2011; Qaderi *et al.,* 2006). Principal component analysis also showed that stem diameter is mainly related to PC1 which contributed for much of the total variation (Appendix 4.12). In general, the above results suggest that among other things, the hypocotyl diameter can be considered as a potential trait for screening (greenhouse) *B. napus* varieties for *V. longisporum* resistance and possibly for drought tolerance as well.

Phenological growth stage

The overall agronomic performance of the two genotypes under optimum and stressed growing conditions was further evaluated by weekly monitoring the phenological growth stages using the BBCH scale (Appendix 4.13). Interestingly, the results obtained from growth stage assessment are in line with the differential reactions of the two genotypes towards VL and drought stress. The comparison of growth stages of the two *B. napus* genotypes revealed that irrespective of infection or drought stress, SEM grows slightly faster than Falcon. At the time of inoculation, vernalized seedlings of both genotypes had the similar growth stage (BBCH 14, four leaves unfolded). Three weeks later (21 DPI), SEM plants grown under optimum conditions began the inflorescence emergence stage (BBCH 50) while Falcon plants were completing the stem elongation stage (BBCH 47). One week later (28 DPI), SEM plants almost began flowering (BBCH 59) while Falcon plants just started the inflorescence emergence stage (BBCH 51). Assessment of growth stages until the 7th week (49 DPI) regardless of infection and drought stress showed similar trends (Fig. 4.13; Appendix 4.4). The fast growth and development of genotype SEM, may contribute for early and rapid accumulation of resistance factors. This condition in turn might have made life very difficult for the pathogen particularly for the systemic dissemination in the shoot. Therefore, faster growth and accumulation of resistance factors, among other things, may be one of the factors contributing to VL-resistance in oilseed rape. In cotton, *V. dahliae* induced syntheses of antibiotic secondary metabolites (flavonoids) occur sooner and in greater concentration in resistant species than in susceptible materials (Bell and Stipanovic, 1978). More and rapid accumulation of the plant defence phytoalexin rishitin was shown in resistant tomato cultivars infected with *Verticillium albo-atrum* (Hutson and Smith, 1980) and *Fusarium oxysporum* (Elgersma and Liem, 1989). In another vascular pathosystem, Pritsch *et al.* (2000) have shown that PR genes were expressed earlier and stronger in a *Fusarium graminearum* resistant wheat cultivar than in a susceptible cultivar.

Considering the independent impact of drought stress, a clear genotypic difference which exactly reflects the differential physiological response of the two genotypes, was observed. Whether infected or not, drought stress had no visible impact on phenological growth stages of cultivar Falcon. This is in agreement with most physiological measurements where this cultivar has showed less sensitivity to drought stress. On the other hand, exposure of SEM plants to severe drought caused a reduced growth rate and finally resulted in delayed or inhibited flowering. Accordingly, the average growth stage at 49 DPI in mock inoculated fully-watered and severely-stressed SEM plants was BBCH 67 and BBCH 63, respectively. In the same way, the respective average growth stage of the VL-inoculated plants was BBCH 65 and BBCH 60 (Fig. 4.13; Appendix 4.4). Flowering stage is the most drought sensitive stage in

oilseed rape and leads to high reduction in yield and oil content (Ahmadi and Bahrani, 2009; Hashem *et al.,* 1998). Genotypic variations in drought resistance and drought sensitivity in the flowering stage of *B. napus* are reported in several studies (Naderikharaji *et al.,* 2008; Champolivier and Merrien, 1996; Bouchereau *et al.,* 1996; Good and Zaplachinski, 1994).

Regarding the pathogen factor, except for the slight delayed growth observed in VL-inoculated Falcon plants at 21 DPI (Appendix 4.4), VL-infection in general had no observable impact on plant growth rate until the flowering stage (49 DPI). Nevertheless, early flowering is linked to a susceptible response to *V. dahliae* infection in Arabidopsis (Veronese *et al.,* 2003; Steventon *et al.,* 2001). It is also known that the switch from vegetative to flowering stage is a critical time for systemic spread of VL in *B. napus* (Zhou *et al.,* 2006).

***V. longisporum* infection and dry matter production**

The ultimate objective of this study was to examine the status of VL-resistance in *B. napus* during drought and to further find out whether there are costs associated with VL-resistance under drought condition and *vice versa*. As expected, DM yields were significantly reduced by VL-infection and drought stress. The severity of plant biomass loss was variable depending on plant genotype, type of stress and level of drought. Interestingly, compared to Falcon, VL-infection caused significantly lower shoot biomass yield in the VL-resistant genotype SEM with no impact on root biomass at all. Furthermore, VL-infection at severe drought stress did not affect shoot biomass yield of genotype SEM. Accordingly, infection-related SDM reduction under optimum watering was 25.9%. At moderate drought stress, a slightly higher and again significant reduction (35.1 %) occurred. At severe stress level however, an extremely low (9.9%) and insignificant reduction was recorded. Regarding RDM, the reduction at 100, 60 and 30% FC was 16.8, 6.4, and 4.2%, respectively (Table 4.13), which were insignificant at all watering conditions (Fig. 4.14). The consistently low level of disease severity across all watering conditions (Fig. 4.6) and in contrast the strong plant height reduction due to VL infection (Table 4.6; Fig. 4.7) indicate that the significant SDM reduction in the VL-resistant genotype SEM is mainly attributable to the severe stunting effect of VL. As VL does not induce stunting under field conditions (Dunker *et al.,* 2008), field grown resistant plants apparently perform much better and provide sufficient yield despite the prevalence of VL disease epidemics. In general, the reasonable SDM obtained from VL-infected fully and moderately irrigated plants, the lack of impact of infection on RDM and the SDM of severely stressed plants clearly show a stable and effective functioning of VL-resistance mechanisms regardless of the amount of water supply.

Table 4.13 Dry matter yield reduction (%) in two *B. napus* genotypes caused by *Verticillium longisporum* infection under optimum water supply and drought stress conditions.

Yield	SEM			Falcon		
parameters	100% FC	60 % FC	30 % FC	100% FC	60 % FC	30 % FC
SDM	25.9	35.1	9.9	55.3	63.2	51.4
RDM	16.8	6.4	4.2	73.9	79.5	62.8
TDM	24.1	28.7	8.6	59.5	66.9	53.9

Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for four weeks starting from 21 days after inoculation with *Verticillium longisporum*. Percent yield reduction due to VL-infection at 100, 60 and 30% FC was calculated by comparing with the yield obtained from respective mock-inoculated plants. Mean data obtained from 30 plants of two independent experiments are presented. **FC**, field capacity. **SDM**, shoot dry matter yield. **RDM**, root dry matter yield. **TDM**, total dry matter yield.

In contrast to SEM, DM reduction due to VL-infection in the VL-susceptible cultivar Falcon was significantly higher (Fig. 4.14). SDM reduction at 100, 60 and 30% FC was 55.3, 63.2, and 51.2%, respectively. Further high losses regardless of soil moisture conditions occurred in root biomass, resulting in 73.9, 79.5, and 62.8% significant reduction of RDM at 100, 60 and 30% FC, respectively (Table 4.13). The significant yield impact of VL in cultivar Falcon was positively correlated with high disease levels across all watering levels. A yield loss assessment study has shown a significant yield impact of VL and a negative correlation between yield and VL disease severity in *B. napus* (Dunker *et al.,* 2008). The relatively low rate of pathogen induced yield reduction occurred in severely-stressed tolerant and susceptible plants (Table 4.13) which also correlated with slightly lower AUDPC values (Fig. 4.6) suggesting that VL-drought interaction may favor the host plant. Possibly severe drought might impair VL growth whilst infection provides plants with better tolerance towards drought stress. As most fungi require free water or high moisture conditions for reproduction and dispersal, which is more crucial particularly for vascular pathogens like VL, drought usually has a negative direct impact on fungal growth (Desprez-Loustau, *et al.,* 2006). On the other hand, it is also possible that the already induced significant yield reduction due to severe drought stress might have masked the effect of VL.

Drought stress and dry matter production

For the effect of drought stress on biomass accumulation, both moderate and severe drought conditions caused substantial yield loss, with more loss occurring at severe stress levels. Obviously, biomass or grain yield in oilseed rape is significantly affected under drought stress (Ashraf *et al.,* 2013; Khalili *et al.,* 2012; Bilibio *et al.,* 2011; Siddiqui *et al.,* 2008; Qaderi *et al.,* 2006). Despite the significant biomass yield reduction in both genotypes, the effect of drought seems to be stronger in SEM than in Falcon. For genotype SEM, moderate and severe drought stress caused 26.8 and 48.9% significant loss of SDM, respectively. In cultivar Falcon however, SDM loss due to moderate drought stress was not significant

(only 5%). Nevertheless, SDM loss under severe stress conditions was significant (40.7%) but still lower than in SEM. The above figures indicate that compared to Falcon, 22 and 8% more biomass yield loss occurred in SEM at 60 and 30% FC treatments, respectively. Similar results have been observed during infection with VL as well. The respective percentage of SDM loss at moderate and severe drought in infected SEM plants was 35.9 and 37.9%, while for Falcon it was 22.2 and 35.5% (Table 4.14). This is in agreement with the physiological measurements that showed less drought sensitivity of cultivar Falcon. Several studies have shown a differential genetic potential of *B. napus* cultivars to the accumulation of biomass yield under drought stress conditions (Ashraf *et al.,* 2013; Hosseini *et al.,* 2011; Dunker *et al.,* 2008). Among the possible mechanisms associated with drought stress tolerance in *B. napus,* improved uptake and maintenance of increased concentration of essential nutrients (Ashraf *et al.,* 2013; Siddiqui *et al.*, 2008; Moradshai *et al.,* 2004), stomatal resistance (Sadaqat *et al.,* 2003), greater capacity in accumulation of proline along with other compatible solutes (Ghaffari *et al.,* 2011; Saadia *et al.,* 2012; Moradshai *et al.,* 2004) and increased activity of ROS scavenging antioxidant enzymes (Abedi and Pakniyat, 2010) can be mentioned.

Further comparison of drought induced yield reduction in mock- *vs*. VL-infected SEM plants showed that severe drought caused 48.9% and 37.9% reduction in SDM in the absence and presence of the pathogen, respectively (Table 4.14). This means compared to mock inoculated plants, 11% less yield reduction occurred in plants infected with VL. For cultivar Falcon as well, 5% less SDM yield reduction occurred when plants were infected with VL. These results may suggest a reduced impact of severe drought stress during VL infection which is in agreement with physiological data measurements. Furthermore, drought possibly limited growth and systemic spread of the fungus and this might have led to a slight contribution of infection for yield loss occurring during severe drought stress. In contrast, compared to optimum watering conditions, relatively high biomass yield reduction was recorded from VL-inoculated moderately stressed plants. In SEM, a moderate drought caused 9% more SDM loss in VL-infected plants than in mock-inoculated treatments. Similarly in Falcon, 17% more SDM loss occurred during infection with VL (Table 4.14). This is possibly because 60% FC, that has caused significant effects on the host plant performance, might still be sufficient for growth and systemic spread of VL. Even though it is not known for VL, an earlier study on *V. dahliae* showed that infection on Brussels sprouts is more severe in wet soils than in dry conditions, with dry conditions delaying the onset of symptom development and reducing disease severity (Isaac, 1957).

Compared to its large impact on shoot development, the influence of drought on root growth seems to be minimal. In mock inoculated treatments, 60% FC drought stress had no impact at all. At 30% FC however, a significant RDM reduction was recorded from both genotypes. Interestingly, drought in VL-inoculated plants had no impact at all (Fig. 4.14). For cultivar Falcon, the severe impact of VL-infection that caused up to 80% root biomass loss (Table 4.13) might have masked the effect of drought stress and may perhaps explain why no significant difference was observed among VL-infected differentially watered treatments. In genotype SEM, root biomass remained unaffected by both drought and VL-infection. Since this genotype is resistant to VL, it is not surprising that root growth was not significantly affected by infection. However, it is interesting that the relative drought sensitivity of this genotype did not show in root growth. A recent study showed that irrespective of drought tolerance, root growth in *B. napus* cultivars increases with an increase in duration and intensity of drought stress (Ashraf *et al.,* 2013). As observed in SDM yield analysis, a lower impact of severe drought stress on root biomass reduction in VL-infected treatments was noticed (Table 4.14).

Table 4.14 Dry matter yield reduction (%) in two *B. napus* genotypes caused by moderate (60% FC) and severe (30%FC) drought stress in the presence and absence of *Verticillium longisporum* infection.

Genotype	Yield parameters	Mock-inoculated		VL-inoculated	
		60 % FC	30 % FC	60 % FC	30 % FC
SEM	SDM	26.8	48.9	35.9	37.9
	RDM	18.3	36.8	8.1	27.3
	TDM	25.1	46.5	29.6	35.5
Falcon	SDM	5.1	40.7	22.0	35.5
	RDM	4.8	25.2	42.4	18.0
	TDM	5.1	41.1	22.4	33.0

Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for four weeks starting from 21 days after inoculation with *Verticillium longisporum*. Percent yield reduction at 60% FC and 30% FC was calculated by comparing with the yields obtained from mock- or VL-inoculated 100% FC treatments. Mean data obtained from 30 plants of two independent experiments are presented. **FC**, field capacity. **SDM**, shoot dry matter yield. **RDM**, root dry matter yield. **TDM**, total dry matter yield.

Overall, dry matter yield analysis indicated that severe drought may not favor VL-growth while at the same time VL-infection is possibly triggering changes in plant metabolism or architecture that provide plants with some level of tolerance against drought stress. However, this does not mean that simultaneous exposure of *B. napus* to drought stress and VL-infection has less impact than the independent effects of each stress factor. When either shoot, root or total DM yield of fully irrigated

mock-inoculated plants (plants grown under normal or optimum conditions) were compared with the yields obtained from those subjected to either VL, drought or VL*drought, it is definitely true that the simultaneous occurrence of disease and drought stress causes significantly more yield reduction than what either of the stress factors are causing alone (Fig. 4.14 and 4.15). For instance, in genotype SEM, VL alone caused 24.1% of total biomass yield reduction. Moderate and severe drought independently caused 25.1 and 46.5% of total yield loss, respectively. Whenever both stress factors were applied simultaneously, the total yield loss due to the combined effect becomes 46.6% due to VL and moderate stress and 51.0% due to VL and severe drought stress. Similarly, in cultivar Falcon total yield reduction due to VL infection alone was 59.5%, due to moderate drought it was 5.1% and to severe drought 40.7%. VL and moderate drought caused 68.6% loss. VL and severe stress caused 72.8% loss. Separate comparison of shoot and root biomass yields of both genotypes showed exactly the same trend (Table 4.15). From the above observations therefore, it may be concluded that despite the consistency of VL-resistance under drought conditions and better drought resistance of VL-infected plants, the combined effect of VL and drought was by far stronger than any stress factor alone. This indicates the high significance of VL epidemics during drought seasons which can lead to a more pronounced yield loss.

Table 4.15 Single and combined effects of *Verticillium longisporum* infection and drought stress on dry matter yield reduction (%) of two *Brassica napus* genotypes.

Yield	SEM					Falcon				
parameters	SM2	SM3	SV1	SV2	SV3	FM2	FM3	FV1	FV2	FV3
SDM	26.8	48.9	25.9	52.5	54.0	5.1	40.7	55.3	65.1	71.2
RDM	18.3	36.8	16.8	23.6	39.5	4.8	25.2	73.9	80.5	78.6
TDW	25.1	46.5	24.1	46.6	51.0	5.1	41.1	59.5	68.6	72.8

Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for four weeks starting from 21 days after inoculation with *Verticillium longisporum*. All yield reduction percent values were calculated by comparing with the yield obtained from mock-inoculated fully watered (100% FC) treatments. Mean data obtained from 30 plants of two independent experiments are presented. **FC**, field capacity. **SDM**, shoot dry matter yield. **RDM**, root dry matter yield. **TDM**, total dry matter yield. **SM**, SEM-05-500526 mock-inoculated. **FM**, Falcon mock-inoculated. **SV**, SEM-05-500526 VL-inoculated. **FV**, Falcon VL-inoculated. Treatment names followed by the numbers **1**, **2** and **3** refers to watering at 100, 60 and 30% field capacity, respectively.

4.4.5 Correlation, regression and principal component analyses

The outcome from correlation, regression and PCA analyses were considerably interconnected and provided strong evidence for consistent and differential responses of the two *B. napus* genotypes to VL infection and drought stress. With correlation analysis, the very strong relationships within disease, physiological and agronomic parameters, between disease and agronomic traits, and between physiological and agronomic traits were shown. Furthermore, the absence of association between VL-

infection and changes in most physiological variables was also verified. PCA analysis in turn illustrated separate patterns of the three groups of variables and how strong they are linked to drought and disease treatments. With regression analysis, it was possible to confirm the significant but generally independent effect of pathogen and drought factors on disease and physiological traits, respectively. Apart from this, the significant effect of disease and drought on yield and agronomic traits as well as the significant contribution of the plant genotype in determining the variation in most variables were shown. With this analysis, it was also possible to identify key parameters (plant height and proline content) which are directly linked to most variations in dry mater biomass yield accumulation. Overall, the results of the three analyses suggest that VL and drought may affect DM biomass yield of oilseed rape under greenhouse conditions by influencing plant systems that follow independent routes.

4.5 References

Abdel-Fattah G. M. and Abdul-Wasea A. A. 2012. Arbuscular mycorrhizal fungal application to improve growthand tolerance of wheat (*Triticum aestivum* L.) plants grown in saline soil. *Acta Physiologiae Plantarum*, 34:267-277.

Abedi T. and Pankiyat H. 2010. Antioxidant enzyme changes in response to drought stress in ten cultivars of oilseed rape (*Brassica napus* L.). *Czech Journal of Genetics and Plant Breeding*, 46(1): 27-34.

Achuo E. A., Prinsen E. and Höfte M. 2006. Influence of drought, salt stress and abscisic acid onthe resistance of tomato to *Botrytis cinerea* and *Oidium Neolycopersici*. *Plant Pathology*, 55:178-186.

Ahmadi M. and Bahrani M. J. 2009. Yield and yield components of rapeseed as influenced by water stress at different growth stages and nitrogen levels. *American-Eurasian Journal of Agricultural & Environmental Sciences*, 5(6): 755-61.

Anjum S. A., Xie X., Wang L., Saleem M. F., Man C. and Lei W. 2011. Morphological, physiological and biochemical responses of plants to drought stress. *African Journal of Agricultural Research*, 6(9): 2026-2032.

Arbogast M., Powelson M. L., Cappaert M. R., and Watrud L. S. 1999. Response of six potato cultivars to amount of applied water and *Verticillium dahliae*. *Phytopathology*, 89:782-788.

Aroca R., Porcel R. and Ruiz-Lozano J. M. 2007. How does arbuscular mycorrhizal symbiosis regulate root hydraulic properties and plasma membrane aquaporins in *Phaseolus vulgaris* under drought, cold or salinity stresses? *New Phytologist,* 173: 808-816.

Ashraf M. and Mehmood S. 1990. Response od four *Brassica* species to drought stress. *Environmental and Experimental Botany,* 30, (1):93-100.

Ashraf M., Shahbaz M. and Ali Q. 2013. Drought-induced modulation in growth and mineral nutrients in Canola (*Brassica napus* L.). *Pakistan Journal of Botany*, 45(1): 93-98.

Babadoost M., Chen W., Bratsch A. D. and Eastman C. E. 2004. *Verticillium longisporum* and *Fusarium solani*: two new species in the complex of internal discoloration of horseradish roots. *Plant Pathology*, 53:669 -676.

Bates L. S. 1973. Rapid determination of free proline for water-stress studies. *Plant and Soil*, 39:205-207.

Bell A. A., and Stipanovic R. D. 1978. Biochemistry of disease and pest resistance in cotton. *Mycopathologia*, 65:91-106.

Berg G., Frankowski J. and Bahl H. 1999. Biocontrol of Verticillium wilt in oilseed rape by chitinolytic *Serratia Plymuthica*. Proceedings of 10th International Rapeseed Congress. Canberra, Australia.

Bilibio C., J. Carvalho de A., Hensel O., Richter U. 2011. Effect of different levels of water deficit on rapeseed (*Brassica napus* L.) crop. *Ciencia e Agrotecnologia,* 35(4):672-684.

Birnboim H.C. and Doly J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research*, 7(6):1513-1523.

Bletsos F. A., Thanassoulopoulos C. C. and Roupakias D. G. 1999. Water stress and Verticillium wilt severity on eggplant (*Solanum melongena* L.). *Journal of Phytopathology*, 147(4):243-248.

Blum A. 2005. Drought resistance, water-use efficiency, and yield potential - are they compatible, dissonant, or mutually exclusive?. *Australian Journal of Agricultural Research*, 56:1159-1168.

Bouchereau A., Clossais-Besnard N., Bensaoud A., Leport L., and Renard M. 1996. Water stress effects on rapeseed quality. *European Journal of Agronomy*, 5:19-30.

Bowden R. L., Rouse D. I., and Sharkey T. D. 1990. Mechanism of photosynthesis decrease by *Verticillium dahliae* in potato. *Plant Physiology,* 94:1048-1055.

Brandfass, C., and Karlovsky, P. 2008. Upscaled CTAB-based DNA extraction and real-time PCR assays for *Fusarium culmorum* and *F. graminearum* DNA in plant material with reduced sampling error. *International Journal of Molecular Sciences, 9*: 2306-2321.

Bunnag S. and Pongthai P. 2013. Selection of rice (*Oryza sativa* L.) cultivars tolerant to drought stress at the vegetative stage under field conditions. *American Journal of Plant Sciences*, 4:1701-1708.

CABI/EPPO. 2011. *Verticillium longisporum*. Distribution maps of plant diseases, No. 1117. CAB International, Wallingford, UK.

Campbell C. L. and Madden L. V. 1990. Introduction to plant disease epidemiology. John Wiley and Sons, New York City, USA.

Carré P. and Pouzet A. 2014. Rapeseed market, worldwide and in Europe. Oilseeds and fats crops lipids, 21 (1) D102.

Champolivier L. and Merrien A. 1996. Effects of water stress applied at different growth stages to *Brassica napus* L. var. *oleifera* on yield, yield components and seed quality. *European Journal of Agronomy*, 5:153-160.

Chen L., Ren F, Zhou L, Wang Q. Q., Zhong H. and Li X. B. 2012. The *Brassica napus* Calcineurin B-Like 1/CBL-interacting protein kinase 6 (CBL1/CIPK6) component is involved in the plant response to abiotic stress and ABA signalling. *Journal of Experimental Botany*, 63(2):695-709.

Cong L., Chai T. Y and Zhang Y. X. 2008. Characterization of the novel gene BjDREB1B encoding a DRE-binding transcription factor from *Brassica juncea* L. *Biochemical and Biophysical Research Communications*, 371:702-706.

Dalal M., Tayal D., Chinnusamy V. and Bansal K. C. 2009. Abiotic stress and ABA-inducible Group 4 LEA from *Brassica napus* plays a key role in salt and drought tolerance. *Journal of Biotechnology,* 139:137-145.

Delauney A. J. and Verma D. P. S. 1993. Proline biosynthesis and osmoregulation in plants. *The Plant Journal,* 4(2):215-223.

Desprez-Loustau M. L., Marçais B., Nageleisen L. M., Piou D. and Vannini A. 2006. Interactive effects of drought and pathogens in forest trees . *Annals of Forest Science,* 63:597-612.

Din J., Khan S. U., Ali I. and Gurmani A. R. 2011. Physiological and agronomic responses of canola varieties to drought stress. *The Journal of Animal & Plant Sciences*,21(1):78-82.

Dunker S., Keunecke H., Steinbach P. and von Tiedemann A. 2008. Impact of *Verticillium longisporum* on yield and morphology of winter oilseed rape (*Brassica napus*) in relation to systemic spread in the plant. *Journal of Phytopathology,* 156: 698-707.

Earl H. J. 2003. A precise gravimetric method for simulating drought stress in pot experiments. *Crop Science,* 43:1868-1873.

Elgesma D. M. and Liem J. I . 1989. Accumulation of phytoalexins in susceptible and resistant near-isogenic lines of tomato infected with *Verticillium albo-atrum* or *Fusarium oxysporum* f.sp. *lycopersici*. *Physiological and Molecular Plant Pathology*, 34: 545-555.

Ellis J. R., Larsen H. J. and Boosalis M. G. 1985. Drought resistance of wheat plants inoculated with vesicular-arbuscular mycorrhizae. *Plant and Soil*, 86(3):369-378.

El-Quesni F., Azza Mazhar A. M., Abd El Aziz N. G. and Metwally S. A. 2012. Effect of compost on growth and chemical composition of *Matthiola incana* (L.)R.Br. under different water intervals. *Journal of Applied Sciences Research*, 8(3): 1510-1516.

Ernst W.H. and Peterson P.J. 1994. The role of biomarkers in environmental assessment (4). Terrestrial plants. *Ecotoxicology*, 3:180-92.

Eynck C. 2008. Identification of resistance sources and characterization of resistance factors in *Brassica* species to *Verticillium longisporum*. PhD thesis. Georg-August-Universität Göttingen, Göttingen, Germany.

Eynck C., Koopmann B. and von Tiedemann A. 2009a. Identification of *Brassica* accessions with enhanced resistance to *Verticillium longisporum* under controlled and field conditions. *Journal of Plant Diseases and Protection*, 116(2), 63-72.

Eynck C., Koopmann B., Grunewaldt-Stoecker G., Karlovsky P. and von Tiedemann A. 2007. Differential interactions of *Verticillium longisporum* and *V. dahliae* with *Brassica napus* detected with molecular and histological techniques. *European Journal of Plant Pathology*, 118(3): 259-274.

Eynck C., Koopmann B., Karlovsky P. and von Tiedemann A. 2009b.Internal resistance in winter oilseed rape inhibits systemic spread of the vascular pathogen *Verticillium longisporum*. *Phytopathology*, 99:802-811.

Fabro G., Kovács I., Pavet V., Szabados L. and Alvarez M. E. 2004. Proline accumulation and AtP5CS2 gene activation are induced by plant-pathogen incompatible interactions in Arabidopsis. *Molecular Plant-Microbe Interactions*, 17(4):343-50.

Farooq M., Wahid A., Kobayashi N., Fujita D. and Basra S. M. A. 2009. Plant drought stress: effects, mechanisms and management. *Agronomy for Sustainable Development*, 29(1): 185-212.

Fernandez-Garcia N., Hernandez M., Casado-Vela J., Bru R., Elortza F., Hedden P. and Olmos E. 2011. Changes to the proteome and targeted metabolites of xylem sap in *Brassica oleracea* in response to salt stress. *Plant, Cell and* Environment, 34:821-836.

Floerl S., Druebert C., Aroud H. I., Karlovsky P. and Polle A. 2010. Disease symptoms and mineral nutrition in *Arabidopsis thaliana* in response to *Verticillium longisporum* Vl43 infection. *Journal of Plant Pathology*,92 (3), 693-700.

Floerl S**,** Druebert C.**,** Majcherczyk A.**,** Karlovsky P.**,** Kües U. and Polle A. 2008. Defence reactions in the apoplastic proteome of oilseed rape (*Brassica napus* var. *napus*) attenuate *Verticillium longisporum* growth but not disease symptoms. *BMC Plant Biology,* 8:129.

Gao M. J., Allard G., Byass L., Flanagan A. M. and Singh J. 2002. Regulation and characterization of four CBF transcription factors from *Brassica napus*. *Plant Molecular Biology,* 49: 459-471.

Garrett K. A., Dendy S. P., Frank E. E., Rouse M. N., and Travers S. E. 2006. Climate change effects on plant disease: Genomes to ecosystems. *Annual Review of Phytopathology*, 44:489-509.

Ghaffari G., Toorchi M., Aharizad S. and Shakiba M. R. 2011. evaluation of traits related to water deficit stress in winter rapeseed cultivars. *Universal Journal of Environmental Research and Technology*, 1(3):338-350.

Ghorbanli M., Gafarabad M., Amirkian T. and Mamaghani B. A. 2012. Investigation of proline, total protein, chlorophyll, ascorbate and dehydroascorbate changes under drought stress in Akria and Mobil tomato cultivars. *Iranian Journal of Plant Physiology*, 3(2):651-658.

Goicoechea N., Aguirreolea J., Cenoz S. and Garcia-Mina J. M. 2000. *Verticillium dahliae* modifies the concentrations of proline, soluble sugars, starch, soluble protein and abscisic acid in pepper plants. *European Journal of Plant Pathology,* 106:19-25.

Gomez K. A. and Gomez A. A. 1984. Statistical procedures for agricultural research, 2nd ed., John Wiley and Sons, New York.

Good A. G. and Zaplachinski S. T. 1994. The effects of drought stress on free amino acid accumulation and protein synthesis in *Brassca napus*. *Physiologia Plantarum*, 90: 9-14.

Grimault V., Gélie B., Lemattre M., Prior P. and Schmit J. 1994. Comparative histology of resistant and susceptible tomato cultivars infected by *Pseudomonas solanacearum*. *Physiological and Molecular Plant Pathology*, 44:105-123.

Grzesiak M. T., Waligórski P., Janowiak F., Marcińska I., Hura K., Szczyrek P. and Głąb T. 2013. The relations between drought susceptibility index based on grain yield (DSI_{GY}) and key physiological seedling traits in maize and triticale genotypes. *Acta Physiologiae Plantarum*, 35(2):549-565.

Hampton R. E., Wullschleger S. D. and Oosterhuis D. M. 1990. Impact of Verticillium wilt on net photosynthesis, respiration and photorespiration in field-grown cotton (*Gossypium hirsutum* L.). *Physiological and Molecular Plant Pathology*, 37:271-280.

Hanson A. D., Nelsen C. E. and Everson E. H. 1977. Evaluation of free proline accumulation as an index of drought resistance using two contrasting barley cultivars. *Crop Science*.17 (5):720-726.

Hartung W., Peuke A. D. and Davies W. J. 1999. Abscisic acid - a hormonal long distance stress signal in plants under drought and salt stress. In: Pessarakali M. ed. *Handbook of crop stress*, 2nd ed. New York, Marcel Dekker, 731-747.

Hashem A., Majumdar M. N. A., Hamid A. and Hossain M. M. 1998. Drought stress effects on seed yield, yield attributes, growth, cell membrane stability and gas exchange of synthesized *Brassica napus* L. *Journal of Agronomy and Crop Science*, 180(3): 129-136.

Haverkort A. J., Rouse D. I. and Turkensteen L. J. 1990. The influence of *Verticillium dahliae* and drought on potato crop growth. 1. Effects on gas exchange and stomatal behavior of individual leaves and crop canopies. *Netherlands Journal of Plant Pathology*, 96(5): 273-289.

Heale J. B. and Karapapa K. V. 1999. The *Verticillium* threat to Canada's major oilseed crop: Canola. *Canadian Journal of Plant Pathology*, 21(1):1-7.

Hosseini S. M. and Hassibi P. 2011. Effects of water deficit stress on several quantitative and qualitative characteristics of canola (*Brassica napus* L.) cultivars. *Notulae Scientia Biologicae*, 3(3):120-125.

Hutson R. A. and Smith I. M. 1980. Phytoalexins and tyloses in tomato cultivars infected with *Fusarium oxysporum* f.sp. *lycopersici* or *Verlicillium albo-atrum*. *Physiological Plant Pathology*, 17:245-257.

Ikeda K., Banno S., Watanabe K., Fujinaga M., Ogiso H., Sakai H., Tanaka H., Miki S., Shibata S. , Shiraishi T. and Fujimur M. 2012. Association of *Verticillium dahliae* and *Verticillium longisporum* with Chinese cabbage yellows and their distribution in the main production areas of Japan. *Journal of General Plant Pathology*, 78(5): 331-333.

Inderbitzin P., Davis R. M., Bostock R. M. and Subbarao K. V. 2011a. The Ascomycete *Verticillium longisporum* is a hybrid and a plant pathogen with an expanded host range. *PLoS ONE*, 6(3): e18260.

Inoue H., Nojima H. and Okayama H. 1990. High efficiency transformation of *Escherichia coli* with plasmids. *Gene*, 96(1):23-28.

Isaac I. 1957. Verticillium wilt of Brussels sprout. *Annals of Applied Biology*, 45(2): 276-283.

Jensen C. R., Mogensen V. O., Mortensen G., Fieldsend J. K., Milford G. F. J., Andersen M. N., and Thage J. H. 1996. Seed glucosinolate, oil and protein contents of field-grown rape *(Brassica napus* L.) affected by soil drying and evaporative demand. *Field Crops Research*, 47:93-105.

Johansson A., Goud J. C. and Dixelius C. 2006. Plant host range of *Verticillium longisporum* and microsclerotia density in Swedish soils. *European Journal of Plant Pathology*, 114:139-149.

Jun-Wei W., Feng-Ping Y., Xu-Qing C., Rong-Qi L., Li-Quan Z., Dong-Mei G., Xiao-Dong Z., Ya-Zhen S. and Gai-Sheng Z. 2006. Induced expression of DREB transcriptional factor and study on its physiological effects of drought tolerance in transgenic wheat. *Acta Genetica Sinica*, 33(5):468-476.

Kagale S., Divi U. K., Krochko J. E., Keller W. A. and P. Krishna. 2007. Brassinosteroid confers tolerance in *Arabidopsis thaliana* and *Brassica napus* to a range of abiotic stresses. *Planta*, 225:353-364.

Kang Y., Han Y., Torres-Jerez I., Wang M., Tang Y., Monteros M. and Udvardi M. 2011. System responses to long-term drought and re-watering of two contrasting alfalfa varieties. *The Plant Journal*, 68:871-889.

Karapapa V. K., Bainbridge B. W. and Heale J. B. 1997. Morphological and molecular characterization of *Verticillium longisporum* comb. nov., pathogenic to oilseed rape. *Mycological Research*, 101:1281-1294.

Khalili M., Aboughadareh A. P., Naghavi M. R. and Talebzadeh S. J. 2012. Response of spring canola (*Brassica napus* L.) genotypes to water deficit stress. *International Journal of Agriculture and Crop Sciences,* 4 (21):1579-1586.

Knüfer J. 2013. Improvement of winter oilseed rape resistance to *Verticillium longisporum* – Assessment of field resistance and characterization of ultrastructural plant responses. PhD thesis. Georg-August-Universität Göttingen, Göttingen, Germany.Kulkarni M., Borse T. and Chaphalkar S. 2008. Mining anatomical traits: A novel modelling approach for increased water use efficiency under drought conditions in plants. *Czech Journal of Genetics and Plant Breeding*, 44(1):11-21.

Kumar A. and Singh D. P. 1998. Use of physiological indices as a screening technique for drought tolerance in oilseed *Brassica* species. *Annals of Botany,* 81:413-420.

Lee S. C., Lim M. H., Kim J. A., Lee S. I., Kim J. S., Jin M., Kwon S. J., Mun J. H., Kim Y. K., Kim H. U., Hur Y. and Park B. S.2008. Transcriptome analysis in *Brassica rapa* under the abiotic stresses using Brassica 24K oligo microarray. *Molecules and Cells,* 26(6):595-605.

Lehner B. and Ďoll P. 2001. Europe's droughts today and in the future. In Eurowasser – Model-based Assessment of European Water Resources and Hydrology in the Face of Global Change, Kassel World Water Series Report No 5, Lehner B., Henrichs T., Ďoll P and Alcamo J. (eds). Kassel, Germany. Leino M. 2006. Fungal diseases on oilseed rape and turnip rape. Norrköping, Sweden.

Lindemose S., O'Shea C., Jensen M. K. and Skriver K. 2013. Structure, function and networks of transcription factors involved in abiotic stress responses. *International Journal of Molecular Sciences*, 14:5842-5878.

Lorenzini G., Guidi L., Nali C., Ciompi S. and Soldatini G. F. 1997. Photosynthetic response of tomato plants to vascular wilt Diseases. *Plant Science*, 124:143-I52.

Mahmood S., A. Hussain, Z. Tabassum and F. Kanwal. 2004. Comparative performance of *Brassica napus* and *Eruca sativa* under water deficit conditions: An *assessment* of *selection criteria*. *Journal of Research (Science),* 14(4): 439-446.

Mithen R.. 1992 . Leaf glucosinolate profiles and their relationship to pest and disease resistance in oilseed rape. *Euphytica*, 63:71-83.

Moghadam H. R. T., Zahedi H., Ghooshchi F. 2011. Oil quality of canola cultivars in response to water stress and super absorbent polymer application. *Pesquisa Agropecuária Tropical (Agricultural Research in the Tropics),* 41: 579-586.

Moradshahi A., Eskandari S. B. and Kholdebarin B. 2004. Some physiological responses of canola (*Brassica napus* L.) to water deficit stress under laboratory conditions. *Iranian Journal of Science & Technology, Transaction A*, 28(A1):43-50.

Müller T., Lüttschwager D. and Lentzsch P. 2010. Recovery from drought stress at the shooting stage in oilseed rape (*Brassica napus*). *Journal of Agronomy and Crop Science*, 196(2): 81-89.

Naderikharaji R., Pakniyat H. and Biabani A. R. 2008. Effect of drought stress on photosynthetic rate of four rapeseed (*Brassica napus*) cultivars. *Journal of Applied Sciences*, 8(23): 4460-4463.

Nelsen C. E. and Safir G. R. 1982. Increased drought tolerance of mycorrhizal onion plants caused by improved phosphorus nutrition. *Planta*, 154:407-413.

Neupane S., Andersson B., Högberg N., Ihrmark K. and Alström S. 2013. Fungal communities associated with field grown oilseed rape (*Brassica napus* L.) - their possible role in early crop establishment. *Acta Agriculturae Scandinavica, Section B- Soil & Plant Science*, 63(3):241-252.

Newton A. C. and Young l. M. 1996. Temporary partial breakdown of Mlo-resistance in spring barley by the sudden relief of soil water stress. *Plant pathology*, 45:973-977.

Obermeier C., Hossain M. A., Snowdon R., Knüfer J., von Tiedemann A. and Friedt W. 2013. Genetic analysis of phenylpropanoid metabolites associated with resistance against *Verticillium longisporum* in *Brassica napus*. *Molecular Breeding*, 31:347-361.

Omidi H., 2010. Changes of proline content and activity of antioxidative enzymes in two canola genotype under drought stress. *American Journal of Plant Physiology*, 5: 338-349.

Park B. J., Liu Z., Kanno A., Kameya T. 2005. Genetic improvement of Chinese cabbage for salt and drought tolerance by constitutive expression of a *B. napus* LEA gene. *Plant Science*, 169:553-558.

Pascual I., Azcona I., Morales F., Aguirreolea J. and Sanchez-Dıaz M. 2010. Photosynthetic response of pepper plants to wilt induced by *Verticillium dahliae* and soil water deficit. *Journal of Plant Physiology*, 167:701-708.

Pennypacker B. W., Leath K. T. and Hill R. R. Jr. 1991. Impact of drought stress on the expression of resistance to *Verticillium albo-atrum* in alfalfa. *Phytopathology*, 81:1014-1024.

Pertot I. and Elad Y.. 2012. Climate change impact on plant pathogens and plant diseases, Envirochange project booklet. Fondazione Edmund Mach. San Michele, Italy.

Pfaffl M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, 29(9):e45.

Pritsch C., Muehlbauer G. J., Bushnell W. R., Somers D. A. and Vance C. P. 2000. Fungal development and induction of defense response genes during early infection of wheat spikes by *Fusarium graminearum. Molecular Plant-Microbe Interactions,* 13(2):159-169.

Qaderi M. M., Kurepin L. V. and Reid D. M. 2006. Growth and physiological responses of canola (*Brassica napus*) to three components of global climate change: temperature, carbon dioxide and drought. *Physiologia Plantarum*, 128:710-721.

Qian X. and Ya-Ning C. 2012. Response of anatomy and hydraulic characteristics of xylem stem of *Populus euphratica* Oliv. to drought stress. *Chinese Journal of Eco-Agriculture*, 20(8):1059-1065.

Qiao G., Wen X. P., Yu L. F. and Ji X. B. 2011. The enhancement of drought tolerance for pigeon pea inoculated by arbuscular mycorrhizae fungi. *Plant*, *Soil* and *Environment*, 57 (12): 541–546.

Ratzinger A., Riediger N., von Tiedemann A. and P. Karlovsky. 2009. Salicylic acid and salicylic acid glucoside in xylem sap of *Brassica napus* infected with *Verticillium longisporum*. *Journal* of *Plant Research*, 122:571-579.

Reusche M., Thole K., Janz D., Truskina J., Rindfleisch S., Drübert C., Polle A., Lipka V. and Teichmann T. 2012. Verticillium infection triggers VASCULAR-RELATED NACDOMAIN7–dependent de novo xylem formation and enhances drought tolerance in Arabidopsis. *The Plant Cell*, 24: 3823-3837.

Reusche M., Truskina J., Thole K., Nagel L., Rindfleisch S., Tran Van T. and Braus-Stromeyer S. A. 2014. Infections with the vascular pathogens *Verticillium longisporum* and *Verticillium dahliae* induce distinct disease symptoms and differentially affect drought stress tolerance of *Arabidopsis thaliana*. *Environmental and Experimental Botany,* in press, Accepted manuscript.

Ringnér M. 2008. What is principal component analysis? *Nature Biotechnology*, 26(3):303-304.

Ristaino J. B. and Duniway J. M. 1989. Effect of preinoculation and postinoculation water stress on the severity of Phytophthora root rot in processing tomatoes. *Plant disease*, 73(4): 349-352.

Ruiz-Lozano J. M., Azcon R. and Gomez M. 1995. Effects of arbuscular-mycorrhizal *Glomus* species on drought tolerance: Physiological and nutritional plant responses. *Applied and Environmental Microbiology*, 61(2):456-460.

Rygulla W., Friedt W., Seyis F., Lühs W., Eynck C., von Tiedemann A. and R. J. Snowdon. 2007a. Combination of resistance to *Verticillium longisporum* from zero erucic acid *Brassica oleracea* and oilseed *Brassica rapa* genotypes in resynthesized rapeseed (*Brassica napus*) lines. *Plant Breeding*, 126:596-602.

Rygulla W., Snowdon R. J., Eynck C., Koopmann B., von Tiedemann A., Lühs W. and Friedt W. 2007b. Broadening the genetic basis of *Verticillium longisporum* resistance in *Brassica napus* by interspecific hybridization. *Phytopathology*, 97:1391-1396.

Saadia M., Jamil A., Akram N. A. and M. Ashraf. 2012. A study of proline metabolism in canola (*Brassica napus* L.) seedlings under salt stress. *Molecules*, 17: 5803-5815.

Sadaqat H. A., Tahir M. H. N. and Hussain M. T. 2003. Physiogenetic aspects of drought tolerance in canola (*Brassica napus*). *International Journal of Agriculture and Biology*, 5(4):611-614.

Sadras V. O., Quiroz F., Echarte L., Escande A. and Pereyra V. R. 2000. Effect of *Verticillium dahliae* on photosynthesis, leaf expansion and senescence of field-grown sunflower. *Annals of Botany*, 86: 1007-1015.

Sánchez-Blanco M. J., Ferrández T., Morales M. A., Morte A. and Alarcón J. J. 2004. Variations in water status, gas exchange, and growth in *Rosmarinus officinalis* plants infected with *Glomus deserticola* under drought conditions. *Journal* of *Plant Physiology*, 161:675-682.

Sangtarash M. H., Qaderi M. M., Chinnappa C. C. and Reid D. M. 2009. Differential sensitivity of canola (*Brassica napus*) seedlings to ultraviolet-B radiation, water stress and abscisic acid. *Environmental and Experimental Botany*, 66:212-219.

Savitch L. V., Allard G., Seki M., Robert L. S., Tinker N. A., Huner N. P. A., Shinozaki K. and Singh J. 2005. The effect of overexpression of two Brassica CBF/DREB1-like transcription factors on

photosynthetic capacity and freezing tolerance in *Brassica napus*. *Plant* and *Cell Physiology*, 46(9):1525-1539.

Senthil-Kumar M. and Mysore K. S. 2012. Ornithine-delta-aminotransferase and proline dehydrogenase genes play a role in non-host disease resistance by regulating pyrroline-5-carboxylatemetabolism-induced hypersensitive response. *Plant, Cell and Environment*, 35:1329-1343.

Sepehri A. and Golparvar A. R. 2011. The effect of drought stress on water relations, chlorophyll content and leaf area in canola cultivars (*Brassica napus L.*). *Electronic Journal of Biology,* 7(3): 49-53.

Shirani Rad A. H. and Zandi P. 2012. The effect of drought stress on qualitative and quantitative traits of spring rapeseed (*Brassica napus* L.) cultivars. *Žemdirbystė-Agriculture*, 99(1):47-54.

Siddiqui Z. S., Khan M. A., Kim B. G., Huang J. S. and Kwon T. R. 2008. Physiological responses of *Brassica napus* genotypes to combined drought and salt stress. *Plant Stress*, 2 (1):78-83.

Silvente S., Sobolev A. P. and Lara M. 2012. Metabolite adjustments in drought tolerant and sensitive soybean genotypes in response to water stress. *PLoS ONE*, 7(6): e38554.

Sinaki J. M., ESlam M. H., Shirani Rad A. H., Noormohammadi G. and Tarei G. 2007. The effects of water deficit during growth stages of canola (*Brassica napus* L.). *American-Eurasian Journal of Agricultural & Environmental Science*, 2(4):417-422.

Singh M., Chauhan J. S. and Meena S. S. 2009. Drought induced changes in water use efficiency and other morpho-physiological characters in Indian mustard (*Brassica juncea* L.). 16th Australian Research Assembly on Brassicas, Ballarat Victoria, Australia.

Somasegaran, P. and H.J. Hoben. 1985. Methods in legume-rhizobium technology. Nitrogen fixation in tropical agricultural legumes and microbiological resources center (NifTAL), Paia, Hawaii.

Stanke C., Kerac M., Prudhomme C., Medlock J. and Murray V. 2013. Health effects of drought: a systematic review of the evidence. *PLOS Currents Disasters*. Edition 1.

Steventon L. A., Okori P. and Dixelius C. 2001. An investigation of the susceptibility of *Arabidopsis thaliana* to isolates of two species of *Verticillium*. *Journal of Phytopathology*, 149:395-401.

Sundaresan S. and P. Sudhakaran R. 1995. Water stress-induced alterations in the proline metabolism of drought-susceptible and -tolerant cassava (*Manihot esculenta*) cultivars. *Physiologia Plantarum*, 94:635-642.

Szabados L. and Savouré A. 2010. Proline: a multifunctional amino acid. *Trends in Plant Science*, 15(2):89-97.

Tambussi E. A., Bort J. and J. Araus L. 2007. Water use efficiency in C3 cereals under Mediterranean conditions: a review of physiological aspects. *Annals of Applied Biology*, 150:307-321.

Tang D., Qian H., Zhao L., Huang D. and Tang K. 2005. Transgenic tobacco plants expressing *BoRS1* gene from *Brassica oleracea* var. *acephala* show enhanced tolerance to water stress. *Journal of Biosciences*, 30(5):647-655.

Thorne E. T., Stevenson J. F., Rost T. L., Labavitch J. M. and Matthews M. A. 2006. Pierce's disease symptoms: Comparison with symptoms of water deficit and the impact of water deficits. *American Journal of Enology and Viticulture*, 57:1-11.

Tisserat N. A. 2004. Canker diseases of trees. Kansas State University. October 2004, Kansas, USA.

Ullah F. Bano A. and Nosheen A.. 2012. Effects of plant growth regulators on growth and oil quality of canola (*Brassica napus* L.) under drought stress. *Pakistan Journal of Botany*, 44(6): 1873-1880.

Valliyodan B. and Nguyen H. T. 2006. Understanding regulatory networks and engineering for enhanced drought tolerance in plants. *Current Opinion in Plant Biology*, 9:189-195.

Wiese J, Kranz T. and Schubert S. 2004. Induction of pathogen resistance in barley by abiotic stress. *Plant Biology,* 6(5):529-36.

Xiang Y., Huang Y. and Xiong L. 2007. Characterization of stress-responsive CIPK genes in rice for stress tolerance improvement. *Plant Physiology*, 144:1416-1428.

Xu P., Chen F., Mannas J. P., Feldman T., Sumner L. W. and Roossinck M. J. 2008. Virus infection improves drought tolerance. *New Phytologist*, 180: 911-921.

Xue X., Liu A. and Hua X. 2009. Proline accumulation and transcriptional regulation of proline biothesynthesis and degradation in *Brassica napus*. *Biochemistry and Molecular Biology Reports*, 42(1):28-34.

Yadeta K. A. and Thomma B. P. H. J. 2013. The xylem as battleground for plant hosts and vascular wilt pathogens. *Frontiers in Plant* Science, 4(97):1-12.

Yang S., Vanderbeld B., Wan J. and Huang Y. 2010. Narrowing down the targets: Towards successful genetic engineering of drought-tolerant crops. *Molecular Plant*, 3(3):469-490.

Yu S. W., Zhang L. D., Zuo K. J., Tang D. Q., Sun X. F. and Tang K. X. 2005. *Brassica napus* L. homeodomain leucine-zipper gene *BnHB6* responds to abiotic and biotic stresses. *Journal of Integrative Plant Biology*, 47(10):1236-1248.

Zakirullah, Swati Z. A., Ahmad A. and Raziuddin. 2000. Morpho-physiological response of selected Brassica lines to moisture stress. *Pakistan Journal of Biological Sciences,* 3(1):130-132.

Zeise K. and von Tiedemann A. 2001. Morphological and physiological differentiation among vegetative compatibility groups of *Verticillium dahliae* in relation to *V. longisporum*. *Journal of Phytopathology*, 149:469-475.

Zeise K. and von Tiedemann A. 2002. Host specialization among vegetative compatibility groups of *Verticillium dahliae* in relation to *Verticillium longisporum*. *Journal of Phytopathology,* 150, 112-119.

Zeise, K. 1992. Gewächshaustest zur Resistenzprüfung von Winterraps (*Brassica napus* L. var. *oleifera* Metzger) gegen den Erreger der Rapswelke *Verticillium dahliae* Kleb. *Nachrichtenblatt DeutscherPflanzenschutzdienst*, 44:125-128.

Zentková I. and Cvengrošová F. 2013. The utilization of rapeseed for biofuels production in the EU. *Visegrad Journal on Bioeconomy and Sustainable Development*, 2(1)11-14.

Zhou L., Hu Q., Johansson A. and Dixelius C. 2006. *Verticillium longisporum* and *V. dahliae*: infection and disease in *Brassica napus*. *Plant Pathology*, 55:137-144.

Zhou M. L., Ma J. T., Pang J. F., Zhang Z. L., Tang Y. X. and Wu Y. M. 2010. Regulation of plant stress response by dehydration responsive element binding (DREB) transcription factors. *African Journal of Biotechnology*, 9(54):9255-9279.

5. General discussion

Since 2000, the global OSR production has surpassed cottonseed and became the world's second most important source of vegetable oil (Carré and Pouzet, 2014). The recently originated vascular pathogen of OSR, *V. longisporum* (Inderbitzin *et al.,* 2011a), is becoming a potential threat to OSR production particularly in the northern European countries where this crop has become the major oilseed due to the expansion of biodiesel industry (Carré and Pouzet, 2014). Under conditions of high disease severity, *V. longisporum* can cause severe yield losses in OSR (Dunker *et al.,* 2008; Gladders, 2009) and its recent report from UK and Czech Republic (CABI and EPPO, 2011) may also suggest its great potential for rapid expansion to new geographic regions. As no other management options, other than the use of plant resistance, are effective or available against this pathogen, the efforts on exploration of control options so far were mainly focusing on identification or development of OSR varieties with enhanced resistance. The present study was one of the efforts towards improving the resistance of OSR against VL with a particular focus on understanding mechanisms of disease resistance and investigation of the physiological and agronomic significance of VL resistance mechanisms under drought stress conditions. The work encompassed a range of studies from identification of resistant genotypes (Chapter 2) towards searching (Chapter 3) and functional analysis (Chapter 4) of VL- resistance factors in winter OSR.

5.1 Identification of *B. napus* genotypes with enhanced resistance to *V. longisporum*

With a general objective of identifying VL-resistant winter OSR genotypes, screening experiments were conducted under different experimental condition, namely in a climate controlled greenhouse, under field condition with different edaphic, climatic and disease pressure conditions, and under outdoor condition which represent an intermediate situation between the greenhouse and field experiments.

In the greenhouse experiments, the resistance response of more than 230 winter OSR genotypes (DH lines and other accessions) was evaluated using an efficient screening method (Eynck, 2008) that involves assessment of disease severity and stunting effect. From the analysis of the disease severity parameter NNA, it was found that more than 38% of the tested lines had less disease severity than the resistant standard Express (NNA=0.58). On the other hand, only very few lines (3%) showed severe susceptibility with greater NNA values than the susceptible standard Falcon (NNA=1.42). For the remaining larger proportion (58%) of the tested lines, a wide range of disease severity, ranging between the NNA value of the susceptible and resistant standards, was observed (Appendix 2.3). With few exceptions, comparable results (with more or less similar proportion compared to the one observed in disease severity results) were obtained from assessment of stunting effect (Appendix 2.3). The

consistency of disease evaluation results obtained from stunting and disease index measurements were further verified by correlation analysis that showed significantly strong positive relationship of these parameters (r=0.66), indicating that both parameters are most appropriate and reliable phenotypic traits for evaluation of VL resistance in oilseed rape under controlled conditions. Moreover, since greenhouse screening involves the use of non-vernalized few weeks old seedlings, this method allows the evaluation of quite a large number of plants within a relatively small space and short period. In general, since the genetic background of the tested genotypes is confidential, it was not possible to explain the possible source and mechanisms of resistance in this set of lines. Nevertheless, the present greenhouse disease screening results obtained from a large number of plant materials not only showed the availability of VL-resistance in Brassica species, but also demonstrated the presence of a wide range of variations in level of resistance. This is in strong agreement with previous reports that showed the presence of various levels of VL-resistance in different species of the *Brassica* genus (Rygulla *et al.,* 2007a; Rygulla *et al.,* 2007b; Eynck *et al.,* 2009a; Happstadius *et al.,* 2003). In conclusion, of the 235 *B. napus* accessions screened under greenhouse conditions, 23 genotypes that showed very strong resistance (with NNA and NRS values less than 0.40; Appendix 2.3) are recommendable for further evaluation under field conditions.

Field experiments were conducted in three locations and for three consecutive seasons (2010/11, 2011/12 and 2012/13). During each season trial, 40 genotypes were evaluated. Of these, fourteen best performing lines (See section 2.4.2.1) were selected based on previous greenhouse screening results. These lines and the four reference varieties were evaluated in all season trials across all locations. The remaining 22 lines were also evaluated in all locations but only for one season (See section 2.4.2.2). The results obtained from both sets of lines showed the significant influence of genotype, seasonal variations and locational differences in disease development. The following discussions based on the results of the first set of 18 genotypes (13 DH lines, 1 accession and 4 reference varieties) which reasonably reflect the interaction effects among the aforementioned three factors.

Disease assessment under field conditions was performed using two methods, disease index scoring (SDI and RDI) and quantification of fungal DNA by qPCR. Due to the loss of plants by hard winter in 2010/11 and 2011/12 in Göttingen and in 2012/13 in Svalöv, disease index data from these trials were unavailable. In Fehmarn, disease index data was recorded from all season trials. For the rest six different trials conducted in different locations or seasons, analysis of variance showed that except in 2010/11 in Fehmarn where SDI showed significant differences among the reference varieties and some DH lines

(Table 2.3), both disease index measurements showed no significant difference among either the reference standards or the DH lines. Similarly, none of the disease index measurements in **Svalöv** showed significant differences among any of the genotypes (Table 2.4). In **Göttingen**, the only disease index data obtained from 2012/13 also showed a similar trend. Here, with the exception of Oase and SW 08-190002-8 that had significantly lower disease index compared to the susceptible standard Laser, no significant difference was found among the rest of the 13 DH lines and the remaining 3 reference varieties (Table 2.5).

Table 5.1 Seasonal and locational comparisons of *Verticillium longisporum* disease index measured from stubbles of 18 *B. napus* genotypes at BBCH growth stage 97 (after grain harvest).

Location	Growing season					
	2010/11		2011/12		2012/13	
	SDI	RDI	SDI	RDI	SDI	RDI
Fehmarn	2.73	1.67	2.16	2.14	1.95	2.11
Svalöv	2.52	2.42	1.44	1.56	ND	ND
Göttingen	ND	ND	ND	ND	1.17	1.18

Data shown are average disease index values of 18 genotypes (13 DH lines, 1 accession and 4 reference varieties). Disease index was measured using a key with 1-4 scales (Figure 2.1). **SDI**, shoot disease index. **RDI**, root disease index. **ND**, no data due to loss of plants by hard winter.

Regarding comparison of disease index across locations, except RDI in 2010/11, during the first two seasons (2010/11 and 2011/12), there was relatively lower disease severity in Svalöv than Fehmarn (Table 5.1). In 2012/13, compared to Göttingen where the trial was conducted using artificial inoculation, again relatively higher disease severity was measured in Fehmarn. Comparison of disease index across years was done for Fehmarn and Svalöv trials where data were available at least from two seasons. Accordingly, again with the exception of Fehmarn RDI, in 2010/11, compared to the other two years, relatively higher disease index in both locations was recorded in 2010/11 (Table 5.1). In Göttingen, since the trials in 2010/11 and 2011/12 were completely destroyed by hard winter, comparison of infestation across years was not possible (Table 5.1). In general, since there was no qPCR data in 2010/11 from where relatively high disease index was recorded in both Fehmarn and Svalöv, it was not possible to verify phenotypic disease evaluation results with molecular disease assessment. Nevertheless, the slightly lower average temperatures and higher moisture conditions in both locations might be among the possible factors that could explain the slightly increased disease index recordings. Further correlation analysis across locations showed no significant relationship among any of the disease index results (Table 2.7). Within the same location however, some significant correlations between SDI

and RDI and between disease index and greenhouse/outdoor results were found (Table 2.6). The presence of high natural VL infestation in Svalöv and Fehmarn and little/no infestation in Göttingen is already known from previous studies (Knüffer, 2013; Eynck, 2008; Johansson *et al.,* 2006). Thus, in addition to the differences in amounts of inoculum in the soil and variations in climatic and edaphic factors (Table 2.2), possible locational differences in terms of composition and diversity of virulent VL isolates might explain the variations in disease levels across locations.

Regarding evaluation of disease severity by molecular (qPCR) method, assessment was done in 2011/12 and 2012/13. In this report, qPCR data from Fehmarn and Svalöv in 2011/12 and from Göttingen in 2012/13 were included. For the remaining experiments, qPCR data were not available either due to the loss of plants by the hard winter or because sample analysis is not finalized yet. Unlike the disease index results however, qPCR results revealed significant differences not only among the reference standards but also among the DH lines. In **Fehmarn** for example, in 2011/12, the amount of VL DNA quantified from the susceptible cultivars Falcon and Laser was 9.8 and 11.4 ng/gram of stem dry matter, respectively. From the resistant genotypes Express and Oase, significantly lower amounts, 0.8 and 1.8ng/g, respectively, were quantified. Furthermore, significant differences among the DH lines were observed. The least quantity of fungal DNA (0.13ng/g) which is significantly lower than both resistant controls was detected from accession SEM 05-500256. It is so interesting that this genotype, which is used as a resistant reference in the other greenhouse studies focusing on resistance mechanisms (chapter 3 and 4), showed quite remarkable resistance under field conditions (Table 2.3). During the same season (2011/12), qPCR results in **Svalöv** also showed a similar trend. Compared to the resistant standards, about two fold higher fungal DNA was quantified from susceptible standards. However, this difference was not statistically significant, possibly due to the high variability between replicates. Regarding the DH lines, seven had significantly lower amounts of VL DNA compared to the susceptible controls. Of these lines, three of them including SEM05-500256 had significantly lower VL DNA than both resistant controls (Table 2.4). As observed in previous year experiments in Fehmarn and Svalöv, the qPCR data in 2012/13 in **Göttingen** also revealed significant difference among genotypes. Accordingly, the highest quantity of VL DNA was recorded for the susceptible standard Laser (10.4ng/g) flowed by the other susceptible standard Falcon (1.5ng/g). These quantities were significantly different from the amounts detected in the resistance standards Oase (0.37ng/g) and Express (0.08ng/g). Regarding the remaining DH lines, eight of them had significantly lower VL DNA than both susceptible standards. In this location as well, genotype SEM05-500256, from which the third least amount of fungal DNA (0.12ng/g) was quantified, displayed very high level of resistance response (Table 2.5). As is the case of disease

index assessment, since there were no complete data sets from the different season trials of the same location available, comparison of disease levels across years by qPCR data was not possible. With regard to variations across locations however, it was found that unlike in Göttingen (where the experiment was conducted with artificial application of additional inoculum), disease pressure was significantly higher in Svalöv and Fehmarn (Table 5.2). This is in strong agreement with the results of disease index evaluations. Regarding correlation of results, it was so interesting that qPCR was the only field disease evaluation parameter that showed very strong positive correlation (r=0.86) across locations (Table 2.7). Furthermore, unlike disease index measurements, qPCR results were more significantly correlated to field disease index recordings in different locations and years. It also showed significant correlations with NNA and NRS results of greenhouse and outdoor experiments (Table 2.6). Overall, based on the results of the present multi-site field trial conducted for three seasons, the following winter OSR lines SEM05-500256, SW08-190001-12, SW08-190001-7, SW08-190001-25, SW08-190002-9, SW08-190002-8 and SW08-190002-11 are highly recommended for use as parent materials in future breeding programs that aim at integration of *Verticillium longisporum* resistance traits into elite winter oilseed rape varieties.

Table 5.2 Comparisons of *Verticillium longisporum* disease severity across seasons and locations at BBCH growth stage 80.

Location	VL DNA in dry stem tissue (ng/g)		
	2010/11	2011/12	2012/13
Fehmarn	ND	2.23	ND
Svalöv	ND	2.35	ND
Göttingen	ND	ND	0.91

Data shown is average VL DNA quantified from 18 genotypes (13 DH lines, 1 accession and 4 reference varieties) by qPCR. **VL,** *Verticillium longisporum***. ND**, no data either due to loss of plants by hard winter or because sample analysis is not yet completed.

The locational variation in disease development shown in the above experiments was verified in another parallel experiment that involved qPCR analysis of disease severity in field grown different aged reference varieties. This experiment was conducted in 2012/13 in all the three locations and disease assessment by qPCR was done at three different growth stages, 50% flowering (BBCH65), fruit development (BBCH75) and ripening (BBCH80). The core objective of this study was to identify the critical crop growth stage for the detection of VL-infection and further differentiation of resistant and susceptible genotypes. Furthermore, the applicability of this method for general assessment of disease epidemics was validated.

Results showed that regardless of disease resistance, detection of the VL in OSR stem tissues was possible as early as BBCH65. However, distinct separation of resistance and susceptible varieties and differentiation of infestation levels across locations was achieved at BBCH80; verifying the long biotrophic latent phase of VL disease cycle in OSR in the field (Dunker *et al.,* 2008; Gladders, 2009). Göttingen was the site where the least level of infestation was observed (Fig. 2.6A). In this location, the average VL DNA quantified from cultivars Oase, Express, Laser and Falcon were 0.083, 0.37, 1.47 and 10.4ng/g, respectively. Interestingly, even under this very low infestation level, the amount of VL DNA quantified from the susceptible varieties was generally higher than the resistance genotypes. The second highest level of infestation was recorded in Fehmarn. Here, compared to resistant standards, high amounts of VL DNA were detected from the susceptible varieties with the highest quantity which is significantly higher than all the remaining three reference standards (79.9ng/g) was detected from cultivar Falcon (Fig. 2.6B). This quantity is almost 8 fold higher compared to the highest amount in Göttingen. Again considering qPCR data, compared to all locations, the highest infestation was found in Svalöv (Fig. 2.6C). Here, compared to the resistant cultivars, more than 300 fold VL DNA was quantified from the susceptible varieties. The highest significant infestation (3,252.6ng/g) was found in Laser and this amount was 141 and 314 fold higher compared to the infestation in Fehmarn and Göttingen, respectively. Similar trend was observed on the other susceptible cultivar Falcon where infestation in Svalöv was 7 and 377 fold higher compared to Fehmarn and Göttingen, respectively. The observed locational variations were not only in terms of infestation levels. It was also noticed that, unlike in Fehmarn and Göttingen, results from Svalöv were poorly correlated with each other and with greenhouse and outdoor results as well. And this is in strong agreement with the correlation analysis results of disease index measurements (Table 2.6). Furthermore, when the two susceptible genotypes were compared, the highest VL DNA in Svalöv and Göttingen was found in Laser. In Fehmarn however, the highest infestation occurred in Falcon. As mentioned earlier, the possible reasons for locational variation in terms of levels of disease infestation and degree of correlation between field trials and experiments in controlled conditions could possibly be due to the climatic, edaphic and pathogen factors that potentially influence host-pathogen interaction and thereby host resistance or pathogen aggressiveness (Agrios, 1995). The above described variations in VL disease infestations across locations was comparable with disease index assessment results of previous studies conducted with other sets of OSR genotypes (Eynck, 2008; Knüfer, 2013). On the other hand, it somehow contradicts with the 2010/11 and 2011/12 disease index results of the present study that showed relatively high levels of infestation in Fehmarn (Table 5.1).

In general, the results of the present study demonstrated that with the sole use of phenotypic methods (SDI and RDI), it is not always possible to differentiate field performance of resistance and susceptible genotypes. Besides, results obtained from this method show high variability across different locations and seasons. Differences in growth habits among cultivar (late *vs* late ripening) also significantly determine microsclerotia development and thus disease index (Knüfer, 2013). In addition, in most cases, stubble disease index results were hardly and only occasionally correlated with field qPCR as well as with NNA and NRS results of greenhouse and outdoor experiments. Therefore, as this parameter is influenced by many factors, decisions on OSR resistance to *V. longisporum* based on field disease index should either be considered with caution or should be supported with additional evidence such as molecular data. Unlike the field phenotypic parameters (SDI and RDI) however, greenhouse and outdoor phenotypic disease evaluation parameters (NNA and NRS) distinctly differentiated resistant and susceptible genotypes (Figure 2.3; Figure 2.8). This could partly be because of the possibility of controlling inoculum and climatic conditions in these experiments. Compared to NRS however, NNA was more correlated with field data (Table 2.6A). This is somehow suggesting the effects of genotype on the extent of VL-induced stunting which is in strong agreement with the observations in greenhouse screening experiments where some genotypes with resistant reactions in AUDPC measurements showed severe stunting (Appendix 2.3).

The other important issue regarding stubble disease index is the time point at which assessment is carried out. After all, understanding disease threshold at or after crop maturity stage (after grain harvest) is not useful, particularly with regard to taking immediate control action. Because, at this time point, plants have already attained the maximum stage of development and therefore application of any possible control measure may not be economical. Nevertheless, this does not mean that the information obtained from this method is totally irrelevant. Because, determination of infestation levels on crop residue (stubble in this case) may still provide sufficient information which help to decide on the use of some control measures (such as management of crop residue) that help reduce the potential build-up of inoculum in the soil and thus future disease epidemics. Furthermore, knowledge on the extent of inoculum on crop residues provide valuable information on the amount of pathogen resting propagules (microsclerotia) joining the soil bank. Estimation of inoculum levels in the soil is one of the basic and most important inputs required for forecasting disease pressure and this information will help to plan future production schemes such as decision making in crop rotation programs.

On the other hand, the use of qPCR for the evaluation of disease severity or VL resistance in OSR under field conditions seems to have several merits. First of all, with this method it is possible to detect even a very slight level of infection (up to few pictograms of fungal DNA per gram of stem material) and at a relatively early growth stage (at least at BBCH65). Later at crop maturity (BBCH75-80), the method allows distinct differentiation of resistant and susceptible plants. More importantly, qPCR results are not only significantly correlated across locations and seasons but also are more consistent with the results of the experiments in controlled environments (greenhouse and outdoor) and occasionally with field disease index evaluations as well. In conclusion, quantification of *V. longisporum* by qPCR seems to be the most consistent, fast and possibly cheaper alternative to survey VL disease severity or evaluate resistance of OSR genotypes in the field.

In general, it can be concluded that with the currently available sampling techniques, primers and PCR conditions, reliable and consistent assessment of VL disease severity in the field is mainly possible by qPCR and particularly when the crop is approaching the ripening stage (BBCH 75-80). Development of a method that enables early (in autumn or spring) assessment of infection or disease severity by qPCR from root or hypocotyl tissue may provide a better and timely information about disease epidemics or level of plant resistance. Therefore, in order to reduce quantitative or qualitative yield losses that might be caused due to the late recognition of *V. longisporum* epidemics in OSR, development of a more elegant method (for example monitoring disease severity in roots during early stages of crop growth or development of a model that enables early detection or forecast of disease severity), should be the focus of future research.

5.2 Searching for VL resistance factors in OSR xylem sap

Understanding mechanisms of VL-resistance was one of the major objectives of the present study. To this end, the investigations on xylem sap residing cultivar-related *V. longisporum* resistance factors in oilseed rape were undertaken in different experiments involving greenhouse studies, *in vitro* bioassays and biochemical analyses. Three *B. napus* genotypes with differential resistance towards VL were used. Greenhouse studies were conducted for the purpose of verifying resistance responses of the genotypes and collection of xylem sap. *In vitro* bioassays and biochemical analyses were performed to study the effects of OSR xylem sap constituents on VL growth.

Analysis of the disease evaluation data obtained from assessment of disease index and quantification of VL DNA (Fig. 3.2) showed the development of very slight symptoms or disease level in genotype Aviso and SEM, confirming their resistance to VL. On the other hand, the very fast and high level of disease

development in cultivar Falcon demonstrated its high susceptibility to VL. Further evidences for the consistent response of the three genotypes to VL-infection was shown in agronomic traits where unlike SEM, significantly higher reduction of plant height, stem thickness and shoot dry mater accumulation due to infection occurred in cultivar Falcon (Figure 3.3). Correlation analysis also showed very strong, significant, positive correlation within disease or agronomic parameters and negative correlations between disease and agronomic parameters (Table 3.6). VL-resistance of genotype SEM (SEM 05-500256) and susceptibility of Falcon under different experimental conditions (greenhouse, outdoor and field) was also shown in another independent disease screening experiments (Chapter 2). Furthermore, similar results from previously field and greenhouse disease screening studies have been reported (Knüfer, 2013; Eynck *et al.,* 2009b; Rygulla *et al,* 2007b). Greenhouse and field studied by Keunecke (2009) also demonstrated the resistance of cultivar AVISO to VL.

From the phenotypic and molecular greenhouse disease evaluation results described in Chapter 2 and Chapter 4 and from previous studies on mechanisms of VL resistance in OSR (Eynck *et al.,* 2009b; Obermeier *et al.,* 2013), it is well known that 21-28 DPI is the critical time point for resistance and susceptible genotypes to show marked differences in disease symptoms. That means, the level of accumulation of resistant factors in resistant genotypes at this time point is sufficient to reduce or restrict further growth and development of the pathogen. Hence, in order to compare the nature of VL growth in xylem sap of resistant (SEM) and susceptible (Falcon) plants, xylem sap derived from different aged plants was used in *in vitro* bioassay. Assessment of xylem sap fungal growth by spectrophotometry revealed that regardless of plant genotype, xylem sap served as a suitable medium for the growth of VL. Initiation of spore germination and further fungal growth was detectable 2-3 days after incubation. Later 5 days after incubation, robust fungal growth that covered the whole area of the microtitre plate wells was observed. Compared to its typical slow growth on artificial medium (such as PDA) which usually takes several weeks to cover a full radius of a 90mm Petri dish (Fig. 1.2), VL growth on xylem sap seems a bit faster. Further inspection of fungal growth even one week after incubation shows a similar story, no significant growth difference in xylem sap derived from plants with contrasting response to VL infection. Similarly, comparisons of fungal growth in xylem sap of mock and VL-inoculated plants also revealed the absence of infection induced VL-resistance factor contributing to significant reduction of *in vitro* VL growth. Nevertheless, whether xylem sap constitutes of resistant and susceptible plants differentially affect fungal sporulation, was not investigated here.

Interestingly, fungal growth on filtered xylem sap was slightly reduced, but did not significantly affect fungal growth. The minor protein binding property of the syringe filter used to prepare filtered xylem sap treatments might adsorb some substances that are required by the fungus and this might cause the slight reduction of fungal growth. Results of protein assay also showed slightly reduced amounts of total soluble proteins in filtered samples. Again, the difference in concentration of total soluble proteins in xylem sap of resistant and susceptible genotypes was not significant (Table 3.3). Even the infection-induced slight increase in protein concentration occurred similarly in both susceptible and resistant genotypes. Similar results have been reported in tomato where infection with *V. albo-atrum* caused a general increase of xylem sap proteins irrespective of plant resistance to the disease (Dixon and Pegg, 1972). The above mentioned facts are in strong agreement with the present bioassay results where no significant effect of plant genotype, VL-infection and filtration of xylem sap on *in vitro* growth of VL is shown. Unfortunately, compared to other plant fluids or tissue extracts, very little is known about the composition and role of xylem sap constituents in plants in general and in *B. napus* in particular. In agreement with the findings of the present study, the work by Floerl *el al.* (2008) showed no effect of VL-infection on *B. napus* total xylem sap protein concentration. This study further demonstrated *B. napus* xylem sap as a suitable medium for the growth of VL. However, in contrast to this work, the study used a single susceptible genotype and found significant reduction of *in vitro* growth of VL on xylem sap of infected plants. As it is shown in another pathosystem, several factors such as symbiotic or pathogenic interactions can determine the level and composition of xylem sap proteins and other constitutes (Subramanian *et al,* 2009).

The other xylem sap constitutes related to plant defence are plant hormones. The role of the well-known defence hormone SA in *B. napus*-VL interaction was investigated in previous studies. Results provided a strong evidence for the lack of correlation between the enhanced accumulation of SA in xylem sap or tissue extracts and cultivar-related resistance of OSR against VL. These studies rather demonstrated VL-infection induced increased accumulation of SA in susceptible cultivars than in resistant plants (Ratzinger *et al.,* 2009; Siebold, 2012; Kamble et al., 2013). Similarly, in *V. dahliae*-Arabidopsis interaction, impairment of SA does not cause either high sensitivity to disease or any change in symptom development (Veronese *et al.*, 2003). Other plant hormones such as JA and ABA seems to have no or insignificant role in Arabidopsis- and *B. napus*- VL interaction (Ratzinger et al., 2009; Veronese *et al.*, 2003).

The other interesting observation of the present study that exactly correlates with natural (field) lifecycle of the pathogen is its enhanced *in vitro* growth on xylem sap of older aged plants (Fig. 3.8). As thoroughly discussed in previous chapters (Chapter 1 and 2) and section 5.1 of this chapter as well, VL shows a long biothrophic latency period during early stages of plant development. Late in the growing season, the pathogen undergoes fast development and rapidly disseminate to the above-hypocotyl shoot part, leading to the development of the typical phenotypic symptoms (Knüfer, 2013). It is possible that VL can sense signals of plant developmental stages present in OSR xylem sap. This may possibly explain why the fungus displayed fast *in vitro* growth in xylem sap of older plants. The increased accumulation xylem sap sugars in older plants which is also known for other crop species such as tomato (Wang and Bergeson, 1974)and Arabidopsis (Yu *et al.,* 2013) could also be a possible explanation for enhanced growth of VL in xylem sap of older aged plants.

This is a first study on functional analysis of cultivar related VL-resistance factors in winter OSR xylem sap. It provided concrete evidence that OSR xylem sap, irrespective of plant genotype, provide a suitable nutritional and chemical environment for the growth of VL. The slightly increased total soluble protein and sugar content in xylem sap of infected plants also demonstrates possible VL-induced changes in the composition or level of OSR xylem sap constitutes. However, since these quantitative changes were not significantly different between resistant and susceptible genotypes, it can be concluded that OSR soluble xylem sap constituents are not playing a role as major resistance factors for cultivar-related winter oilseed rape resistance against VL. This is in strong agreement with the findings of previous studies on mechanisms of VL-resistance in OSR that demonstrated the significant role of cell wall bound metabolites and physical barriers in resistance of OSR to *V. longisporum* (Eynck *et al.,* 2009b; Obermeier *et al.,* 2013). Nevertheless, further studies that encompass a large number of genotypes and assessment of other parameters such as fungal sporulation are suggested.

5.3 *B. napus - V. longisporum* interaction under drought stress conditions

The main abiotic stress factor affecting plant production particularly under the changing global climate is drought stress. Understanding the extent of disease development and the nature of plant resistance under abiotic stress factors is therefore indispensable. An extensive study that looked at the impact of VL-infection on drought stress resistance of OSR and also the influence of drought stress on *B. napus - VL* interaction was conducted. Here a brief discussion of the results is provided. Interested reader on the detailed results and discussions of this topic is advised to see section 4.4 of Chapter 4.

It is well known that, among other factors, vascular occlusions are one of the mechanisms contributing to VL resistance in OSR. Occlusions induced by wilt causing vascular pathogens have similar effect as different abiotic stress factors such as drought. In the present study, analysis of physiological processes, gene expression, and agronomic and biomass yield accumulation in VL-infected OSR plants revealed that infection induced changes in the vascular system (such as accumulation of occlusions) have no significant effect on plants response to drought stress. Except for slight changes observed in a few cases (see section 4.3), neither VL alone nor its interaction with drought or the genotype had a significant effect on the response of OSR to drought stress. Similar results from previous studies also showed no effects of VL infection in gas exchange of *B. napus* (Floerl *et al.*, 2008) and Arabidopsis (Floerl *et al.,* 2010). The presence of occlusion free vessels in VL-infected *B. napus* plants (Eynck *et al.,* 2007) might provide sufficient room for the transport of adequate amount of water and mineral nutrients. This may possibly explain why most physiological processes are not significantly affected by infection and the resulting blockage of vessels by occlusions. More importantly, this could also be one of the reasons why VL infection in *B. napus* is not causing wilting symptom (Eynck *et al.,* 2007).

On the other hand, as expected, drought stress was the major factor that caused substantial changes in all physiological parameters and expression of drought induced genes considered in this study. These effects were also significant irrespective of genotype and pathogen infection. Drought-induced significant reduction in gas exchange (Naderikharaji *et al.,* 2008; Sangtarash *et al.,* 2009) and RWC (Ullah *et al.,* 2012; Khalili *et al.,* 2012) is well documented in previous studies in *B. napus* and other crop species. Furthermore, drought-induced increased accumulation of proline and expression of drought inducible genes is reported in several previous studies (Ghaffari *et al.,* 2011; Omidi, 2010; Kagale *et al.,* 2007; Saadia *et al.,* 2012; Xue *et al.,* 2009).

Other factors responsible for the variation in drought-induced changes in plant physiology were genotype and plant part. In general, the VL-susceptible cultivar Falcon was less sensitive to drought than the resistant genotype SEM. Comparisons of drought induced genes expression also suggest that the accumulation of P5SC1, DREB2 and CIPK1 gene transcripts were relatively higher in Falcon than in SEM. This is in agreement with physiological and yield assessment results that showed better performance of Falcon under drought conditions. Nevertheless, since only two genotypes are analyzed in the present study (which does not represent the genetic diversity in *B. napus*), it should not be generalized that OSR genotypes with resistance to VL are always sensitive to drought stress.

The other interesting observation regarding physiological parameters was the difference in physiological changes between mock- and VL-inoculated plants. Comparison of drought induced changes in gas exchange, hypocotyl proline content and WUE under mock- and VL-inoculation conditions indicated a slightly reduced impact of drought stress during infection with VL. The reduced impact of drought stress during infection regardless of plant resistance to the pathogen might be associated with the infection induced anatomical changes in the xylem tissue. As described in Eynck *et al.* (2007), colonization of xylem vessels with VL and accumulation of VL-induced vascular occlusions in *B. napus* are restricted to individual vessels and other adjacent vessels remain completely uninfected and free of obstructions. In general, it is known that plants with smaller number of pit pores or smaller vessel diameter are well adapted to environments prone to frequent soil water deficit. This is because such plants extract water at a lower rate and as a result they will not run out of water quickly. In *B. napus*-OSR pathosystem, unlike control plants with large number of occlusion free vessels, the reduced number of occlusion free vessels in VL-infected plants might possibly helped plants to easily transport the very small amount of water present in soil and also not to suffer from cavitation that usually occur at high water potentials. The slightly reduced expression of the drought inducible genes (HB6, P5SC1, DREB2, and P5SC2) in VL-infected treatments of at least for one of the genotypes might also suggest a reduced impact of drought stress during infection with VL. Yadeta and Thomma *et al.* (2013) also suggested that if less numbers of vessels are closed by occlusions, the host plant will not suffer from drought stress. Furthermore, a recent study in Arabidopsis has shown enhanced drought tolerance of VL-infected plants due to infection induced *de novo* xylem formation (Reusche *et al.,* 2014). In another pathosystem, Xu *et al.* (2008) showed viral infection induced increase in osmoprotectant and antioxidant substances which provided better drought tolerance to several crop species. Enhanced mycorrhizal induced drought tolerance due to increased accumulation of osmolites and sugars, nutrient uptake and root growth etc. has been reported in different crop families including vegetables, cereals and legumes.

Regarding the impact of drought stress on disease development, the consistent responses of resistant and susceptible genotypes as measured by AUDPC, relative stunting and fungal DNA quantification measurements; and the very strong correlation of these parameters at all watering regimes (see Chapter 2 and 3) verified the stable response of plants to VL-infection regardless of the amount of water supply. This is a strong evidence for stable functioning of the quantitative VL-resistance mechanisms known in OSR even under conditions of severe drought conditions. Likewise, the high disease severity observed in the susceptible cultivar which was even significantly higher at severe drought stress suggests more

vulnerability of susceptible plants to the disease under conditions of additional stresses, drought in this case.

Concerning agronomic and yield traits, the effect of both stress factors was obvious. While VL-infection caused significant reduction of plant height and stem thickness, it induced increased production of excessive branches. The effect of VL on these parameters was evident regardless of water supply and plant genotype; but, compared to the resistant genotype, the magnitude of the effect was significantly greater on the susceptible cultivar. Furthermore, in agreement to the physiological data, slightly lower effect of infection on plant height, stem thickness and branching was observed under severe drought condition. Unfortunately and partly because of their short life span, the abnormal excessive branches induced by VL did not result in increased biomass yield. In contrast to field conditions (Dunker et al., 2008; Gladders, 2009), symptoms of stunting, excessive branching and reduced stem thickness were only observed under greenhouse (Chapter 3) and outdoor (Chapter 2) conditions. Apart from climatic factors, the source, amount and method of inoculations (Chapter 2) might also contribute for the exceptional development of these symptoms in controlled environments. Similar to VL, drought stress also significantly reduced plant height and stem thickness but its effect on branching particularly on resistant genotype was not significant. In general, the results from the above three agronomic parameters provided additional evidence for the absence of significant additive adverse effect of drought stress on VL-resistance of OSR.

The other agronomic trait considered in this study was plant growth rate. Interestingly, results were in line with the differential reactions of the two genotypes towards VL-infection and drought stress. The VL-resistant genotype SEM showed relatively faster growth and development. As it is known from Verticillium resistance mechanisms in other crop species (Bell and Stipanovic, 1978; Hutson and Smith, 1980), early and rapid accumulation of resistance factors might contributed to VL-resistance of genotype SEM.

Regarding biomass yield, as expected, both VL-infection and drought stress had significant effect. The severity of biomass yield reduction was also in agreement with the differential reactions of the two genotypes to drought stress and VL-infection. Furthermore, regardless of plant genotype, the extent of DM yield loss was directly proportional to the magnitude of drought stress. The reasonable SDM obtained from VL-infected fully and moderately irrigated resistant plants, no effect of VL-infection on SDM yield of severely stressed resistant plants, and again no impact of infection on RDM yield of the resistant genotype SEM are in agreement with disease evaluation results. This is a clear evidence for

stable operation of VL-resistance mechanisms regardless of drought stress. Besides, the relatively low rate of pathogen induced yield reduction under severely-stressed tolerant and susceptible plants (Table 4.13), which also correlated with reduced impact of drought on most physiological parameters in infected plants and the disease development (AUDPC) as well imply that VL-drought interaction may favor the host plant.

In general, when the impact of the two stress factors on OSR is compared, the effect of VL was more important than drought stress. The deep and numerous root system and other typical drought tolerance associated traits of OSR such as leaf hair and waxes might provide *B. napus* the ability to tolerate drought stress and perform very well under water deficit situations. This is strongly supported by a recent study that showed increased root growth of *B. napus* cultivars with an increase in duration and intensity of drought stress (Ashraf *et al.,* 2013). In conclusion, results of the present comprehensive study suggested that severe drought may not favor VL while at the same time VL-infection is possibly triggering changes in plant metabolism or architecture that provide plants with some level of tolerance against drought stress. However, this does not mean that simultaneous exposure of *B. napus* to drought stress and VL-infection has less impact than the independent effects of each stress factor. Because, despite the consistent resistance to VL under drought conditions and the better drought tolerance of VL-infected plants, compared to the effects of either VL or drought stress, simultaneous exposure of OSR to both stresses factors cause signifiacntly higher yield loss; indicating the very significance of VL epidemics during periods of drought stress.

5.4 References

Agrios G. N. 2005. Plant pathology (5th edition). *Elsevier-Academic Press*. San Diego, CA.

Ashraf M., Shahbaz M. and Ali Q. 2013. Drought-induced modulation in growth and mineral nutrients in canola (*Brassica napus* L.). *Pakistan Journal of Botany*, 45(1): 93-98.

Bell A. A. and Stipanovic R. D. 1978. Biochemistry of disease and pest resistance in cotton. *Mycopathologia*, 65:91-106.

CABI and EPPO, 2011. *Verticillium longisporum*. Distribution maps of plant diseases. Map no. 1117 (Edition 1). ISSN 0012-396X. CAB International, Wallingford, UK.

Carré P. and Pouzet A. 2014. Rapeseed market, worldwide and in Europe. Oilseeds and fats crops lipids, 21 (1) D102.

Dixon G. R. and Pegg G. F. 1972. Changes in amino-acid content of tomato xylem sap following infection with strains of *Verticillium albo-atrum*. *Annals of Botany*, 36:147-54.

Dunker S., Keunecke H., Steinbach P. and von Tiedemann A. 2008. Impact of *Verticillium longisporum* on yield and morphology of winter oilseed rape (*Brassica napus*) in relation to systemic spread in the plant. *Journal of Phytopathology* 156: 698-707.

Eynck C. 2008. Identification of resistance sources and characterization of resistance factors in *Brassica* species to *Verticillium longisporum*. PhD thesis. Georg-August-Universität Göttingen, Göttingen, Germany.

Eynck C., Koopmann B. and von Tiedemann A. 2009a. Identification of *Brassica* accessions with enhanced resistance to *Verticillium longisporum* under controlled and field conditions. *Journal of Plant Diseases and Protection*, 116(2), 63-72.

Eynck C., Koopmann B., Grunewaldt-Stoecker G., Karlovsky P. and von Tiedemann A. 2007. Differential interactions of *Verticillium longisporum* and *V. dahliae* with *Brassica napus* detected with molecular and histological techniques. *European Journal* of *Plant Pathology*, 118:259-274.

Eynck C., Koopmann B., Karlovsky P. and von Tiedemann A. 2009b. Internal resistance in winter oilseed rape inhibits systemic spread of the vascular pathogen *Verticillium longisporum*. *Phytopathology*, 99:802-811.

Floerl S, Druebert C, Aroud HI, Karlovsky P, Polle A. 2010. Disease symptoms and mineral nutrition in *Arabidopsis thaliana* in response to *Verticillium longisporum* Vl43 infection. *Journal of Plant Pathology*, 92 (3), 693-700.

Floerl S**,** Druebert C.**,** Majcherczyk A.**,** Karlovsky P.**,** Kües U. and Polle A. 2008. Defence reactions in the apoplastic proteome of oilseed rape (*Brassica napus* var. *napus*) attenuate *Verticillium longisporum* growth but not disease symptoms. *BMC Plant Biology,* 8:129.

Ghaffari G., Toorchi M., Aharizad S. and Shakiba M. R. 2011. Evaluation of traits related to water deficit stress in winter rapeseed cultivars. *Universal Journal of Environmental Research and Technology*, 1(3):338-350.

Gladders P. 2009. Relevance of Verticillium wilt (*Verticillium longisporum*) in winter oilseed rape in the UK. HCGA Research Review, 72:1-39.

Happstadius I., Ljungberg A., Kristiansson B. and Dixelius C. 2003. Identification of *Brassica oleracea* germplasm with improved resistance to *Verticillium wilt*. *Plant Breeding*, 122:30-34.

Hutson R. A. and Smith I. M. 1980. Phytoalexins and tyloses in tomato cultivars infected with *Fusarium oxysporum* f.sp. *lycopersici* or *Verlicillium albo-atrum*. *Physiological Plant Pathology*, 17:245-257.

Inderbitzin P., Davis R. M., Bostock R. M. and Subbarao K. V. 2011a. The Ascomycete *Verticillium longisporum* is a hybrid and a plant pathogen with an expanded host range. *PLoS ONE*, 6(3): e18260.

Johansson A., Goud Jan-K. C. and Dixelius C. 2006. Plant host range of *Verticillium longisporum* and microsclerotia density in Swedish soils. *European Journal of Plant Pathology*, 114:139-149.

Kagale S., Divi U. K., Krochko J. E., Keller W. A. and P. Krishna. 2007. Brassinosteroid confers tolerance in *Arabidopsis thaliana* and *Brassica napus* to a range of abiotic stresses. *Planta*, 225:353-364.

Kamble A., Koopmann B. and von Tiedemann A. 2013. Induced resistance to *Verticillium longisporum* in *Brassica napus* by b-aminobutyric acid. *Plant Pathology*, 62:552-561.

Keunecke H.. 2009. Einfluss von Kohlfliegenbefall auf die Infektion und Schadwirkung von *Verticillium longisporum* und *Phoma lingam* an Raps. PhD thesis. Georg-August-Universität Göttingen, Göttingen, Germany.

Khalili M., Aboughadareh A. P., Naghavi M. R. and Talebzadeh S. J. 2012. Response of spring canola (*Brassica napus* L.) genotypes to water deficit stress. *International Journal of Agriculture and Crop Sciences,* 4 (21):1579-1586.

Knüfer J. 2013. Improvement of winter oilseed rape resistance to *Verticillium longisporum* – Assessment of field resistance and characterization of ultrastructural plant responses. PhD thesis. Georg-August-Universität Göttingen, Göttingen, Germany.

Naderikharaji R., Pakniyat H. and Biabani A. R. 2008. Effect of drought stress on photosynthetic rate of four rapeseed (*Brassica napus*) cultivars. *Journal of Applied Sciences*, 8(23): 4460-4463.

Obermeier C., Hossain M. A., Snowdon R., Knüfer J., von Tiedemann A. and Friedt W. 2013. Genetic analysis of phenylpropanoid metabolites associated with resistance against *Verticillium longisporum* in *Brassica napus*. *Molecular Breeding*, 31:347-361.

Omidi H., 2010. Changes of proline content and activity of antioxidative enzymes in two canola genotype under drought stress. *American Journal of Plant Physiology*, 5: 338-349.

Ratzinger A., Riediger N., von Tiedemann A. and Karlovsky P. 2009. Salicylic acid and salicylic acid glucoside in xylem sap of *Brassica napus* infected with *Verticillium longisporum*. *Journal* of *Plant Research*, 122:571-579.

Reusche M., Truskina J., Thole K., Nagel L., Rindfleisch S., Tran Van T. and Braus-Stromeyer S. A. 2014. Infections with the vascular pathogens *Verticillium longisporum* and *Verticillium dahliae* induce distinct disease symptoms and differentially affect drought stress tolerance of *Arabidopsis thaliana*. *Environmental and Experimental Botany,* in press, Accepted manuscript.

Rygulla W., Friedt W., Seyis F., Lühs W., Eynck C., von Tiedemann A. and R. J. Snowdon. 2007a. Combination of resistance to *Verticillium longisporum* from zero erucic acid *Brassica oleracea* and oilseed *Brassica rapa* genotypes in resynthesized rapeseed (*Brassica napus*) lines. *Plant Breeding*, 126:596-602.

Rygulla W., Snowdon R. J., Eynck C., Koopmann B., von Tiedemann A., Lühs W. and Friedt W. 2007b. Broadening the genetic basis of *Verticillium longisporum* resistance in *Brassica napus* by interspecific hybridization. *Phytopathology*, 97:1391-1396.

Saadia M., Jamil A., Akram N. A. and Ashraf M.. 2012. A Study of proline metabolism in canola (*Brassica napus* L.) seedlings under salt stress. *Molecules*, 17: 5803-5815.

Sangtarash M. H., Qaderi M. M., Chinnappa C. C. and Reid D. M. 2009. Differential sensitivity of canola (*Brassica napus*) seedlings to ultraviolet-B radiation, water stress and abscisic acid. *Environmental and Experimental Botany*, 66:212-219.

Siebold M. 2012. Effects of rising air and soil temperatures on the life cycle of important pathogens in oilseed rape (*Brassica napus* L.) in Lower Saxony. PhD thesis. Georg-August-Universität Göttingen, Göttingen, Germany.

Subramanian S., Cho U. H., Keyes C. and Yu O. 2009. Distinct changes in soybean xylem sap proteome in response to pathogenic and symbiotic microbe interactions. *BMC Plant Biology*, 9(119):1-11.

Ullah, F., Bano A. and Nosheen A.. 2012. Effects of plant growth regulators on growth and oil quality of canola (*Brassica napus* L.) under drought stress. *Pakistan Journal of Botany*, 44(6): 1873-1880.

Veronese P., Narasimhan M. L., Stevenson R. A., Zhu J. K., Weller S. C., Subbarao K. V. and Bressan R. A. 2003. Identification of a locus controlling *Verticillium* disease symptom response in *Arabidopsis thaliana*. *The Plant Journal*, 35(5):574-87.

Wang E. L. H. and Bergeson G. B. 1974. Biochemical changes in root exudate and xylem sap of tomato plants infected with *Meloidogyne incognita*. *Journal of Nematology,* 6(4): 194-202.

Xu P., Chen F., Mannas J. P., Feldman T., Sumner L. W. and Roossinck M. J. 2008. Virus infection improves drought tolerance. *New Phytologist*, 180: 911-921.

Xue X., Liu A. and Hua X. 2009. Proline accumulation and transcriptional regulation of proline biothesynthesis and degradation in *Brassica napus*. *Biochemistry and Molecular Biology Reports*, 42(1):28-34.

Yadeta K. A. and Thomma B. P. H. J. 2013. The xylem as battleground for plant hosts and vascular wilt pathogens. *Frontiers in Plant* Science, 4(97):1-12.

Yu S., Cao L., Zhou C. M., Zhang T. Q., Lian H., Sun Y., Wu J., Huang J., Wang G. and Wang J. W. 2013. Sugar is an endogenous cue for juvenile-to-adult phase transition in plants. *eLife*, 2:e00269.

Summary

Oilseed rape (*Brassica napus*) is one of the most important sources of vegetable oil in the world. Due to the growing demands in the biodiesel industry in particular, the global OSR production is gradually increasing since the last decade. Among other factors, the intensive production of OSR may have largely contributed to the emergence and increased economic importance of pest and diseases. One of the most important pathogens, *Verticillium longisporum* (VL), is a recently evolved vascular pathogen of crucifers. In recent periods, it has become a potential threat to OSR production in major OSR growing countries of the temperate region. This soil borne host-specific fungus causes foliar chlorosis, reduced growth, and premature senescence and ripening which ultimately leads to substantial yield losses. Unavailability of VL-effective fungicides and production of abundant and highly durable microsclerotia contributing to the soil inoculum are among the major factors that greatly hampers the management of VL. The only possible alternative management option available at present is the use of genotypes with enhanced resistance. The present study therefore focused on the identification of *B. napus* lines resistant to VL. Besides, the applicability of qPCR as an alternative method for the assessment of VL disease severity in the field was validated. Furthermore, mechanisms of cultivar related disease resistance and the significance of plant resistance mechanisms to VL under drought stress conditions were investigated.

Chapter two describes the resistance screening experiments conducted under greenhouse, outdoor and field conditions. Initially, a large number (>230) of *B. napus* DH lines and accessions were screened for VL resistance in multiple greenhouse experiments. Results of disease severity (AUDPC) and stunting effect assessments not only demonstrated the availability of VL-resistance in *B. napus* but also showed the presence of a wide range of variation in the level of resistance. Accordingly, *B. napus* lines that showed high degree of resistance in the greenhouse conditions were identified and recommended for further evaluation in the field. It was also found that the greenhouse resistance screening method used in this study, that involved the use of non-vernalized few-weeks old plants, provided consistent and reliable information. The method further enabled the screening of quite a large number of plants within a relatively small space and short period of time.

The outdoor screening experiment was conducted with the specific objective of identifying the sources/causes of variations in VL symptoms (mainly stunting and excessive branching) between greenhouse and field conditions. Results showed that stunting is significantly correlated with disease severity (genotype's susceptibility). This is in agreement with greenhouse observations and suggests the possible effect of type/source of inoculum and method of inoculation on the development of this

symptom. In contrast, increased branching as a result of VL infection was genotype dependent and is apparently not related to susceptibility to VL.

In the field experiments, more than 80 best performing lines selected based on previous greenhouse screening results were evaluated in three different locations that vary in climatic factors, soil conditions and level of natural disease infestation. From the field trials conducted for three consecutive seasons (from 2010/11 to 2012/13), it was understood that plant genotype, seasonal variations and locational differences have a significant influence on the extent of disease development and the resulting response of plants to VL infection. Like in greenhouse conditions, significant differences among genotypes were observed in the field. Accordingly, best performing *B. napus* DH lines were identified and recommended for use as parent materials in future breeding programs that aim at integrating VL resistance in commercial OSR varieties. The other interesting observation from the field experiments was the substantial variation of disease severity across locations. The highest disease severity was observed in Svaöv followed by Fehmarn. In contrast, the least disease level was recorded in Göttingen, where unlike the other locations trials were conducted with additional artificial application of inoculum. Regarding seasonal variations, comparisons were not conclusive as no complete data sets were available. Nevertheless, compared to the later seasons, disease severity in 2010/10 was relatively higher. More importantly, correlation analysis showed that disease index assessment results are poorly and only occasionally correlated across locations, years as well as with results of greenhouse experiments. In contrast, qPCR was the only field disease evaluation parameter that showed very strong correlations across locations. Furthermore, unlike disease index, this parameter was more significantly correlated to field disease index recordings in different locations and years, and with AUDPC and stunting effect results of greenhouse and outdoor experiments. With this method, detection of OSR infection in the field was possible as early as BBCH65 (50% flowering stage). However, distinct separation of resistance and susceptible varieties and differentiation of infestation levels across locations was achieved at BBCH80. Hence, for reliable quantification of VL disease severity and identification of differential plant resistance by qPCR in the field, sampling between growth stages BBCH70 and BBCH70 are recommended. In conclusion, the use of qPCR method for detection and quantification of VL infection in OSR field samples has several merits over the use of post-harvest stubble disease index screening. The consistency and rapidity of this method and possibly the cost effectiveness in comparing plant resistance or assessing disease epidemics in the field is much more reliable than post- harvest stubble disease index assessment. Nevertheless, further improvement of this method to achieve early (in late autumn or early spring) detection of infection or disease severity will provide a more applicable and timely information.

Chapter three mainly focused on the investigation of cultivar related VL-resistance factors in winter OSR xylem sap, an environment where the pathogen spends most part of its life cycle. Three *B. napus* genotypes with known differential resistance towards VL were used. Greenhouse studies were conducted to obtain xylem sap and verify resistance responses of the genotypes. *In vitro* bioassays and biochemical analyses were performed to study the effects of OSR xylem sap constituents on the growth of VL. From *in vivo* phenotypic (AUDPC and stunting effect) and molecular (qPCR) disease severity evaluations, the resistance of genotype SEM and susceptibility of Falcon was confirmed. Further evidences for the consistent response of these genotypes to VL-infection were obtained from the assessment of stem thickness and biomass yield. *In vitro* fungal growth analysis showed uniform growth of VL on xylem sap of 28 DPI old susceptible and resistant genotypes. Similarly, comparisons of fungal growth in xylem sap of mock and VL-inoculated plants revealed the absence of infection induced VL-resistance, which would contribute to significant reduction in VL growth. Quantification of total soluble protein content also showed no significant difference between xylem sap of resistant and susceptible genotypes. Even the infection-induced slight increase in protein concentration was similar in both susceptible and resistant genotypes. Interestingly, a time course independent study using the resistant genotype AVISO and susceptible cultivar Falcon indicated that regardless of plant resistance to VL, xylem sap collected from older plants provide enhanced fungal growth. This phenomenon correlates with natural lifecycle of the pathogen on field grown OSR plants. The increased accumulation of xylem sap sugars in older plants could also be one of the possible explanations for enhanced growth of VL in xylem sap of older plants. This is a first study on functional analysis of cultivar related VL-resistance factors in winter OSR. It provided concrete evidence that OSR xylem sap, regardless of disease resistance, provides a favourable nutritional and chemical environment for the growth of VL. The slightly increased total soluble protein and sugar content in xylem sap of infected plants also demonstrates possible VL-induced changes in the composition or level of OSR xylem sap constituents. However, since these quantitative changes were not significantly different between resistant and susceptible genotypes, it can be concluded that soluble xylem sap constituents do not play a role as major resistance factors for cultivar-related resistance of winter OSR against VL. This is in strong agreement with previous studies on the mechanisms of VL-resistance in OSR that demonstrated the significant role of cell wall bound metabolites and physical barriers. Further studies with large number of genotypes and assessment of other parameters such as effects of xylem sap constituents on fungal sporulation are suggested.

In the last chapter, the study on the response of *B. napus* to the combined effects of VL infection and drought stress was conducted. Previously, substantial amounts of vascular occlusions that obstruct

xylem vessels have been detected in resistant VL-infected OSR genotypes. This mechanism of resistance to the pathogen may however alter the rate of water and nutrient transport and consequently plant response to drought stress. To investigate whether genotypic VL resistance is associated with a reduced drought tolerance, drought resistance of VL-resistant and susceptible winter OSR genotypes were studied in combination with infection with VL. Furthermore, the influence of drought stress on the response of plants to VL infection was investigated. This study was conducted in a controlled pot experiment where seedlings of the VL susceptible cultivar Falcon and the tolerant line SEM were inoculated with VL and exposed to three watering levels (optimum, moderate deficiency and severe deficiency i.e. watering at 100, 60 and 30% field capacity). Analysis of disease parameters (AUDPC, stunting and qPCR) showed a significantly lower rate and level of disease development in the resistant genotype across all watering regimes. Likewise, regardless of the water supply at different field capacity levels, high disease severity and stunting effects were observed in the susceptible cultivar. Furthermore, the amount of fungal DNA was up to 31fold in Falcon as compared to SEM. qPCR results showed that levels of fungal DNA were positively correlated with the intensity of drought stress. At 49 DPI, the respective average fungal DNA in dry hypocotyl tissue at 100, 60, and 30% field capacity was 27.1, 29.0 and 36.0 ng/g in SEM and 839.1, 1,032.4 and 1,096.4 ng/g in Falcon; indicating more pronounced effect of VL during drought stress, particularly on susceptible *B. napus* varieties. Significant changes in physiological parameters (gas exchange, relative water content, proline accumulation and water use efficiency) and up-regulation of drought stress marker genes confirmed the reaction of both genotypes to drought stress. On the other hand, neither VL alone nor its interaction with drought or the genotype had any significant effect on physiological parameters. Further comparisons of the drought induced physiological changes under mock- and VL-inoculation conditions showed a cultivar-independent trend of a slightly reduced impact of drought stress during VL infection. The main and interactive effects of VL and drought on biomass yield and other agronomic traits (stem diameter, branching and phenological growth stage) were significant but the magnitude of their impact was dependent on differential disease and physiological responses of the genotypes. In general, the consistent and interrelated results from ANOVA, correlation, regression and principal component analyses of the present comprehensive study not only proved that VL-resistance mechanisms have no additive negative consequence on plant performance under drought stress but also demonstrate effective functioning of the quantitative VL-resistance mechanisms even under conditions of severe drought stress. Nevertheless, despite the stable VL-resistance under water deficit conditions and the slightly smaller effects of drought on infected plants, simultaneous exposure of OSR to both stresses can cause considerable yield loss.

Appendices

Appendix 2.1 List and descriptions of *Brassica napus* lines screened for *Verticillium longisporum* resistance under greenhouse conditions.

No.	Genotype code	Screening no. /year	Seed company	No.	Genotype code	Screening no. /year	Seed company
1	DSV 10-10	GHS42/2011	DSV	41	SRG 204	GHS42/2011	SRG
2	DSV 10-11	GHS42/2011	DSV	42	SRG 205	GHS42/2011	SRG
3	DSV 10-12	GHS42/2011	DSV	43	SRG 206	GHS42/2011	SRG
4	DSV 10-13	GHS42/2011	DSV	44	SRG 207	GHS42/2011	SRG
5	DSV 10-14	GHS42/2011	DSV	45	SRG 208	GHS42/2011	SRG
6	DSV 10-15	GHS42/2011	DSV	46	SRG 209	GHS42/2011	SRG
7	DSV 10-16	GHS42/2011	DSV	47	SRG 210	GHS42/2011	SRG
8	DSV 10-5	GHS42/2011	DSV	48	SRG 211	GHS42/2011	SRG
9	DSV 10-6	GHS42/2011	DSV	49	SRG 212	GHS42/2011	SRG
10	DSV 10-7	GHS42/2011	DSV	50	SRG 213	GHS42/2011	SRG
11	DSV 10-8	GHS42/2011	DSV	51	SW 309009	GHS42/2011	SW
12	DSV 10-9	GHS42/2011	DSV	52	SW 309010	GHS42/2011	SW
13	KWS 188	GHS42/2011	KWS	53	SW 309011	GHS42/2011	SW
14	KWS 189	GHS42/2011	KWS	54	SW 309012	GHS42/2011	SW
15	KWS 190	GHS42/2011	KWS	55	SW 309013	GHS42/2011	SW
16	KWS 191	GHS42/2011	KWS	56	SW 309014	GHS42/2011	SW
17	KWS 192	GHS42/2011	KWS	57	SW 309015	GHS42/2011	SW
18	KWS 193	GHS42/2011	KWS	58	SW 309016	GHS42/2011	SW
19	KWS 194	GHS42/2011	KWS	59	SW 309017	GHS42/2011	SW
20	KWS 195	GHS42/2011	KWS	60	SW 309018	GHS42/2011	SW
21	KWS 196	GHS42/2011	KWS	61	SW 309019	GHS42/2011	SW
22	KWS 197	GHS42/2011	KWS	62	SW 309020	GHS42/2011	SW
23	KWS 198	GHS42/2011	KWS	63	SYN VER 228	GHS42/2011	SYN
24	KWS 199	GHS42/2011	KWS	64	SYN VER 229	GHS42/2011	SYN
25	NICK 183	GHS42/2011	NICK	65	SYN VER 230	GHS42/2011	SYN
26	NICK 184	GHS42/2011	NICK	66	SYN VER 231	GHS42/2011	SYN
27	NPZ 198/08	GHS42/2011	NPZ	67	SYN VER 232	GHS42/2011	SYN
28	NPZ 199/08	GHS42/2011	NPZ	68	SYN VER 233	GHS42/2011	SYN
29	NPZ 200/08	GHS42/2011	NPZ	69	SYN VER 235	GHS42/2011	SYN
30	NPZ 201/08	GHS42/2011	NPZ	70	SYN VER 236	GHS42/2011	SYN
31	NPZ 202/08	GHS42/2011	NPZ	71	SYN VER 237	GHS42/2011	SYN
32	NPZ 203/08	GHS42/2011	NPZ	72	SYN VER 239	GHS42/2011	SYN
33	NPZ 204/08	GHS42/2011	NPZ	73	SYN VER 240	GHS42/2011	SYN
34	NPZ 205/08	GHS42/2011	NPZ	74	WVB 203	GHS42/2011	WVB
35	NPZ 206/08	GHS42/2011	NPZ	75	WVB 204	GHS42/2011	WVB
36	NPZ 207/08	GHS42/2011	NPZ	76	WVB 205	GHS42/2011	WVB
37	NPZ 208/08	GHS42/2011	NPZ	77	WVB 206	GHS42/2011	WVB
38	NPZ 209/08	GHS42/2011	NPZ	78	WVB 207	GHS42/2011	WVB
39	SRG 202	GHS42/2011	SRG	79	WVB 208	GHS42/2011	WVB
40	SRG 203	GHS42/2011	SRG	80	WVB 209	GHS42/2011	WVB

Appendix 2.1 continued

No.	Genotype code	Screening no. /year	Seed company	No.	Genotype code	Screening no. /year	Seed company
81	WVB 210	GHS42/2011	WVB	121	SRG 221	GHS43/2011	SRG
82	WVB 211	GHS42/2011	WVB	122	SRG 222	GHS43/2011	SRG
83	WVB 212	GHS42/2011	WVB	123	SRG 223	GHS43/2011	SRG
84	WVB 213	GHS42/2011	WVB	124	SW 08-309021	GHS43/2011	SW
85	WVB 214	GHS42/2011	WVB	125	SW 08-309022	GHS43/2011	SW
86	DSV 10-17	GHS43/2011	DSV	126	SW 08-309023	GHS43/2011	SW
87	DSV 10-18	GHS43/2011	DSV	127	SW 08-309024	GHS43/2011	SW
88	DSV 10-19	GHS43/2011	DSV	128	SW 08-309025	GHS43/2011	SW
89	DSV 10-20	GHS43/2011	DSV	129	SW 08-309026	GHS43/2011	SW
90	DSV 10-21	GHS43/2011	DSV	130	SW 08-309027	GHS43/2011	SW
91	DSV 10-22	GHS43/2011	DSV	131	SW 08-309028	GHS43/2011	SW
92	DSV 10-23	GHS43/2011	DSV	132	SW 08-309029	GHS43/2011	SW
93	DSV 10-24	GHS43/2011	DSV	133	SW 08-309030	GHS43/2011	SW
94	DSV 10-25	GHS43/2011	DSV	134	SYN VER 234	GHS43/2011	SYN
95	DSV 10-26	GHS43/2011	DSV	135	SYN VER 241	GHS43/2011	SYN
96	KWS 200	GHS43/2011	KWS	136	SYN VER 242	GHS43/2011	SYN
97	KWS 201	GHS43/2011	KWS	137	SYN VER 243	GHS43/2011	SYN
98	KWS 202	GHS43/2011	KWS	138	SYN VER 244	GHS43/2011	SYN
99	KWS 203	GHS43/2011	KWS	139	SYN VER 245	GHS43/2011	SYN
100	KWS 204	GHS43/2011	KWS	140	SYN VER 246	GHS43/2011	SYN
101	KWS 205	GHS43/2011	KWS	141	SYN VER 247	GHS43/2011	SYN
102	KWS 206	GHS43/2011	KWS	142	SYN VER 248	GHS43/2011	SYN
103	NPZ 210/08	GHS43/2011	NPZ	143	SYN VER 249	GHS43/2011	SYN
104	NPZ 211/08	GHS43/2011	NPZ	144	SYN VER 250	GHS43/2011	SYN
105	NPZ 212/08	GHS43/2011	NPZ	145	SYN VER 251	GHS43/2011	SYN
106	NPZ 213/08	GHS43/2011	NPZ	146	WVB 215	GHS43/2011	WVB
107	NPZ 214/08	GHS43/2011	NPZ	147	WVB 216	GHS43/2011	WVB
108	NPZ 215/08	GHS43/2011	NPZ	148	WVB 217	GHS43/2011	WVB
109	NPZ 216/08	GHS43/2011	NPZ	149	WVB 218	GHS43/2011	WVB
110	NPZ 217/08	GHS43/2011	NPZ	150	WVB 219	GHS43/2011	WVB
111	NPZ 218/08	GHS43/2011	NPZ	151	WVB 220	GHS43/2011	WVB
112	NPZ 219/08	GHS43/2011	NPZ	152	WVB 221	GHS43/2011	WVB
113	NPZ 220/08	GHS43/2011	NPZ	153	WVB 222	GHS43/2011	WVB
114	SRG 214	GHS43/2011	SRG	154	WVB 223	GHS43/2011	WVB
115	SRG 215	GHS43/2011	SRG	155	WVB 224	GHS43/2011	WVB
116	SRG 216	GHS43/2011	SRG	156	DSV 10-27	GHS44/2012	DSV
117	SRG 217	GHS43/2011	SRG	157	DSV 10-28	GHS44/2012	DSV
118	SRG 218	GHS43/2011	SRG	158	DSV 10-29	GHS44/2012	DSV
119	SRG 219	GHS43/2011	SRG	159	DSV 10-30	GHS44/2012	DSV
120	SRG 220	GHS43/2011	SRG	160	DSV 10-31	GHS44/2012	DSV

Appendix 2.1 continued

No.	Genotype code	Screening no. /year	Seed company	No.	Genotype code	Screening no. /year	Seed company
161	DSV 10-32	GHS44/2012	DSV	200	DSV 10-41	GHS46/2012	DSV
162	DSV 10-33	GHS44/2012	DSV	201	DSV 10-42	GHS46/2012	DSV
163	DSV 10-34	GHS44/2012	DSV	202	DSV 10-43	GHS46/2012	DSV
164	NICK 185	GHS44/2012	NICK	203	DSV 10-44	GHS46/2012	DSV
165	NICK 186	GHS44/2012	NICK	204	DSV 10-45	GHS46/2012	DSV
166	NICK 187	GHS44/2012	NICK	205	DSV 10-46	GHS46/2012	DSV
167	NICK 188	GHS44/2012	NICK	206	KWS 217	GHS46/2012	KWS
168	NICK 189	GHS44/2012	NICK	207	KWS 218	GHS46/2012	KWS
169	NICK 190	GHS44/2012	NICK	208	KWS 219	GHS46/2012	KWS
170	NICK 191	GHS44/2012	NICK	209	KWS 220	GHS46/2012	KWS
171	NICK 192	GHS44/2012	NICK	210	KWS 221	GHS46/2012	KWS
172	SRG 224	GHS44/2012	SRG	211	KWS 222	GHS46/2012	KWS
173	SRG 225	GHS44/2012	SRG	212	NICK 194	GHS46/2012	NICK
174	SRG 226	GHS44/2012	SRG	213	NICK 195	GHS46/2012	NICK
175	SRG 227	GHS44/2012	SRG	214	NICK 196	GHS46/2012	NICK
176	SRG 228	GHS44/2012	SRG	215	NICK 197	GHS46/2012	NICK
177	SRG 229	GHS44/2012	SRG	216	NICK 198	GHS46/2012	NICK
178	SW 08-309026	GHS44/2012	SW	217	NICK 199	GHS46/2012	NICK
179	SW 08-309031	GHS44/2012	SW	218	SW 12-1	GHS46/2012	SW
180	SW 08-309032	GHS44/2012	SW	219	SW 12-2	GHS46/2012	SW
181	SW 08-309033	GHS44/2012	SW	220	SW 12-3	GHS46/2012	SW
182	SW 08-309034	GHS44/2012	SW	221	SW 12-4	GHS46/2012	SW
183	SW 08-309035	GHS44/2012	SW	222	SW 12-5	GHS46/2012	SW
184	SYN VER 252	GHS44/2012	SYN	223	SW 12-6	GHS46/2012	SW
185	SYN VER 253	GHS44/2012	SYN	224	SYN VER 260	GHS46/2012	SYN
186	SYN VER 254	GHS44/2012	SYN	225	SYN VER 261	GHS46/2012	SYN
187	SYN VER 255	GHS44/2012	SYN	226	SYN VER 262	GHS46/2012	SYN
188	SYN VER 256	GHS44/2012	SYN	227	SYN VER 263	GHS46/2012	SYN
189	SYN VER 257	GHS44/2012	SYN	228	SYN VER 264	GHS46/2012	SYN
190	SYN VER 258	GHS44/2012	SYN	229	SYN VER 265	GHS46/2012	SYN
191	SYN VER 259	GHS44/2012	SYN	230	WVB 225	GHS46/2012	WVB
192	WVB 225	GHS44/2012	WVB	231	WVB 226	GHS46/2012	WVB
193	WVB 226	GHS44/2012	WVB	232	WVB 227	GHS46/2012	WVB
194	WVB 227	GHS44/2012	WVB	233	WVB 228	GHS46/2012	WVB
195	WVB 228	GHS44/2012	WVB	234	WVB 229	GHS46/2012	WVB
196	WVB 229	GHS44/2012	WVB	235	WVB 230	GHS46/2012	WVB
197	WVB 230	GHS44/2012	WVB	236	Express (RC)	All trials	NPZ
198	WVB 231	GHS44/2012	WVB	237	Falcon (SC)	All trials	NPZ
199	WVB 232	GHS44/2012	WVB				

DSV, Deutsche Saatveredelung AG. **KWS**, KWS SAAT AG. **NICK**, Limagrain GmbH. **NPZ**, NorddeutschePflanzenzucht Hans-Georg Lembke KG. **SRG**, Raps GbR Saatzucht Lundsgaard. **SW**, Lantmännen SW Seed. **SYN**, Syngenta Seeds GmbH. **WVB**, W. von Borries-Eckendorf GmbH & Co. **VL**, *Verticillium longisporum*. (**RC**), resistant reference variety. (**SC**), susceptible reference variety.

Appendix 2.2 List and descriptions of *Brassica napus* lines evaluated for *Verticillium longisporum* resistance under field conditions.

No.	Genotype code	Season	Seed company
1	SEM 05-500256	All	SW
2	SW 08-190001-1*	All	SW
3	SW 08-190001-6*	All	SW
4	SW 08-190001-7*	All	SW
5	SW 08-190001-11*	All	SW
6	SW 08-190001-12*	All	SW
7	SW 08-190001-25*	All	SW
8	SW 08-190002-1*	All	SW
9	SW 08-190002-5*	All	SW
10	SW 08-190002-8*	All	SW
11	SW 08-190002-9*	All	SW
12	SW 08-190002-11*	All	SW
13	SW 08-190002-16*	All	SW
14	SW 08-190002-18*	All	SW
15	DH 7*	2010/11	JLU
16	DH 16*	2010/11	JLU
17	DH 24*	2010/11	JLU
18	DH 69*	2010/11	JLU
19	DH 88*	2010/11	JLU
20	DH 101*	2010/11	JLU
21	DH 108*	2010/11	JLU
22	DH 118*	2010/11	JLU
23	DH 123*	2010/11	JLU
24	DH 126*	2010/11	JLU
25	DH 138*	2010/11	JLU
26	DH 140*	2010/11	JLU
27	DH 141*	2010/11	JLU
28	DH 152*	2010/11	JLU
29	DH 165*	2010/11	JLU
30	DH 179*	2010/11	JLU
31	DH 196*	2010/11	JLU
32	DH 272*	2010/11	JLU
33	DH 282*	2010/11	JLU
34	DH 290*	2010/11	JLU
35	DH 294*	2010/11	JLU
36	DH 359*	2010/11	JLU
37	DSV-DH-Ver-1(1575-1)*	2011/12	DSV
38	DSV-DH-Ver-2(1575-2)*	2011/12	DSV
39	DSV-DH-Ver-3(1575-2)*	2011/12	DSV
40	DSV-DH-Ver-4(1575-3)*	2011/12	DSV
41	DSV-DH-Ver-5(1575-1)*	2011/12	DSV
42	DSV-DH-Ver-6(1575-1)*	2011/12	DSV
43	DSV-DH-Ver-7(1575-2)*	2011/12	DSV
44	DSV-DH-Ver-8(1605-2)*	2011/12	DSV
45	DSV-DH-Ver-9(1605-2)*	2011/12	DSV
46	DSV-DH-Ver-10(1605-2)*	2011/12	DSV
47	DSV-DH-Ver-11(1605-2)*	2011/12	DSV
48	DSV-DH-Ver-12(1605-2)*	2011/12	DSV
49	DSV-DH-Ver-13(1605-1)*	2011/12	DSV
50	DSV-DH-Ver-14(1605-2)*	2011/12	DSV
51	DSV-DH-Ver-15(1605-2)*	2011/12	DSV
52	DSV-DH-Ver-16(1605-2)*	2011/12	DSV
53	DSV-DH-Ver-17(1605-2)*	2011/12	DSV
54	DSV-DH-Ver-18(1605-2)*	2011/12	DSV
55	DSV-DH-Ver-19(1605-2)*	2011/12	DSV
56	DSV-DH-Ver-20(1605-3)*	2011/12	DSV
57	DSV-DH-Ver-21(1605-1)*	2011/12	DSV
58	DSV-DH-Ver-22(1605-2)*	2011/12	DSV
59	DM 12-01	2012/13	DM
60	DM 12-02	2012/13	DM
61	DSV 1	2012/13	DSV
62	DSV 2	2012/13	DSV
63	DSV 3	2012/13	DSV
64	KWS 187	2012/13	KWS
65	KWS 195	2012/13	KWS
66	KWS 200	2012/13	KWS
67	NICK 219	2012/13	NICK
68	NICK 220	2012/13	NICK
69	NICK 221	2012/13	NICK
70	SW 08-190001-10*	2012/13	SW
71	SW 08-190002-2*	2012/13	SW
72	SW 08-190002-17*	2012/13	SW
73	SW 08-190002-25*	2012/13	SW
74	SW 12-1	2012/13	SW
75	SW 12-2	2012/13	SW
76	SW 12-3	2012/13	SW
77	SW 12-4	2012/13	SW
78	SYN 09033483	2012/13	SYN
79	SYN 10091232	2012/13	SYN
80	SYN 11091465	2012/13	SYN
81	Express(RC)	All	NPZ
82	Falcon(SC)	All	NPZ
83	Laser(SC)	All	SYN
84	Oase(RC)	All	DSV

Key: **DM**, Dieckmann GmbH & Co. KG. **DSV**, Deutsche Saatveredelung AG. **JLU**, Justus-Liebig-University of Gießen, department of Plant breeding. **KWS**, KWS SAAT AG. **NICK**, Limagrain GmbH. **NPZ**, NorddeutschePflanzenzucht Hans-Georg Lembke KG. **SW**, Lantmännen SW Seed. **SYN**, Syngenta Seeds GmbH. **VL**, *Verticillium longisporum*. **(RC)**, resistant control. **(S)**, susceptible control**. ***, Double haploid line.

Appendix 2.3 Response of *Brassica napus* lines to *Verticillium longisporum* under greenhouse conditions as measured by AUDPC and relative stunting.

No.	Genotype code	Normalized net AUDPC (28 DPI)	Normalized relative stunting (28DPI)	Experiment No.
1	NICK 191*	-0.20±0.53	0.03±0.74	GHS44
2	NICK 189*	-0.09±0.52	0.23±0.65	GHS44
3	NICK 188	-0.04±0.24	0.34±0.40	GHS44
4	DSV 10-33*	0.02±0.16	0.12±0.41	GHS44
5	NICK 186*	0.02±0.26	0.11±0.63	GHS44
6	DSV 10-30*	0.04±0.68	0.32±0.55	GHS44
7	NICK 190*	0.04±0.42	0.10±0.30	GHS44
8	DSV 10-32*	0.07±0.72	0.26±0.50	GHS44
9	SW 08-309026*	0.07±0.51	-0.13±0.67	GHS44
10	DSV 10-27	0.16±0.61	0.43±0.59	GHS44
11	SRG 227	0.16±0.85	0.71±0.44	GHS44
12	SW 08-309029*	0.18±0.23	-0.18±0.16	GHS43
13	NICK 187*	0.18±0.32	0.19±0.36	GHS44
14	SYN VER 250	0.18±0.22	0.58±0.20	GHS43
15	SRG 225	0.20±0.39	0.63±0.43	GHS44
16	SRG 228*	0.20±0.62	0.09±0.55	GHS44
17	WVB 232	0.20±2.07	1.31±1.78	GHS44
18	NICK 184*	0.24±0.11	0.14±0.25	GHS42
19	WVB 231*	0.25±0.35	0.22±0.55	GHS44
20	SW 08-309033*	0.27±0.28	0.15±0.38	GHS44
21	SW 08-309035	0.29±0.35	1.02±0.52	GHS44
22	NICK 192*	0.29±0.42	0.30±0.44	GHS44
23	SW 309016*	0.29±0.07	0.30±0.09	GHS42
24	SW 08-309024*	0.31±0.18	-0.08±0.16	GHS43
25	SW 08-309030*	0.31±0.29	0.03±0.14	GHS43
26	DSV 10-29*	0.31±0.43	-0.44±0.61	GHS44
27	DSV 10-28*	0.31±0.54	0.22±0.54	GHS44
28	WVB 225	0.34±0.63	1.23±0.72	GHS44
29	WVB 226	0.34±1.04	1.03±0.77	GHS44
30	SW 309019*	0.34±0.07	0.09±0.13	GHS42
31	NPZ 219/08*	0.35±0.17	0.33±0.21	GHS43
32	SYN VER 248	0.35±0.18	0.57±0.10	GHS43
33	KWS 195	0.36±0.12	0.56±0.22	GHS42
34	KWS 200*	0.36±0.14	0.20±0.17	GHS43
35	WVB 228	0.36±0.40	0.45±0.52	GHS44
36	WVB 230	0.36±0.65	0.79±0.48	GHS44
37	NPZ 199/08	0.36±0.20	0.58±0.30	GHS42
38	SW 12-3	0.37±0.26	0.54±0.33	GHS46
39	WVB 227	0.38±0.47	1.29±0.94	GHS44
40	SW 309018	0.40±0.08	0.25±0.07	GHS42

Appendix 2.3 continued

No.	Genotype code	Normalized net AUDPC	Normalized relative stunting	Experiment No.
41	NPZ 217/08	0.40±0.13	0.06±0.14	GHS43
42	DSV 10-26	0.40±0.17	0.52±0.30	GHS43
43	DSV 10-23	0.41±0.14	0.21±0.19	GHS43
44	DSV 10-9	0.41±0.19	0.50±0.24	GHS42
45	WVB 207	0.41±0.15	0.51±0.27	GHS42
46	NICK 195	0.41±0.19	0.43±0.29	GHS46
47	NPZ 216/08	0.42±0.18	0.43±0.16	GHS43
48	SYN VER 264	0.42±0.24	0.63±0.20	GHS46
49	NPZ 203/08	0.42±0.26	0.47±0.13	GHS42
50	SRG 211	0.43±0.13	0.64±0.09	GHS42
51	SRG 221	0.44±0.15	0.32±0.17	GHS43
52	SW 309010	0.45±0.07	0.44±0.12	GHS42
53	SW 309017	0.45±0.06	0.38±0.08	GHS42
54	SW 12-6	0.45±0.36	0.49±0.25	GHS46
55	KWS 188	0.45±0.15	0.48±0.21	GHS42
56	SW 309012	0.46±0.08	0.65±0.10	GHS42
57	SW 08-309028	0.46±0.26	0.03±0.15	GHS43
58	SW 08-309027	0.46±0.16	0.15±0.14	GHS43
59	SYN VER 251	0.46±0.28	0.81±0.13	GHS43
60	WVB 210	0.46±0.10	0.51±0.19	GHS42
61	SW 12-4	0.47±0.23	0.35±0.27	GHS46
62	DSV 10-34	0.47±0.41	0.69±0.74	GHS44
63	KWS 201	0.47±0.27	0.32±0.33	GHS43
64	SW 08-309022	0.48±0.32	0.05±0.08	GHS43
65	NPZ 198/08	0.48±0.14	0.53±0.10	GHS42
66	SW 309014	0.49±0.07	0.38±0.14	GHS42
67	NPZ 211/08	0.49±0.20	0.43±0.21	GHS43
68	SW 12-1	0.49±0.24	0.53±0.43	GHS46
69	SW 309015	0.50±0.09	0.32±0.16	GHS42
70	WVB 208	0.50±0.10	0.62±0.19	GHS42
71	KWS 202	0.51±0.30	0.37±0.21	GHS43
72	DSV 10-17	0.51±0.29	0.64±0.33	GHS43
73	DSV 10-6	0.51±0.26	0.60±0.31	GHS42
74	SYN VER 262	0.52±0.33	0.62±0.36	GHS46
75	NICK 194	0.52±0.18	0.62±0.29	GHS46
76	SYN VER 256	0.52±0.50	1.68±0.96	GHS44
77	SW 08-309026*	0.52±0.14	0.11±0.09	GHS43
78	SW 309009	0.52±0.18	0.68±0.14	GHS42
79	NICK 197	0.52±0.29	0.81±0.24	GHS46
80	WVB 205	0.52±0.20	0.64±0.27	GHS42

Appendix 2.3 continued

No.	Genotype code	Normalized net AUDPC	Normalized relative stunting	Experiment No.
81	SYN VER 261	0.53±0.18	0.86±0.27	GHS46
82	KWS 189	0.53±0.10	0.79±0.09	GHS42
83	WVB 230	0.54±0.14	0.36±0.20	GHS46
84	SW 309013	0.54±0.19	0.93±0.28	GHS42
85	WVB 203	0.54±0.20	0.68±0.30	GHS42
86	NPZ 204/08	0.54±0.33	0.68±0.26	GHS42
87	DSV 10-12	0.54±0.12	0.79±0.17	GHS42
88	SRG 213	0.56±0.19	0.92±0.25	GHS42
89	SRG 226	0.56±0.92	0.17±0.39	GHS44
90	SYN VER 231	0.57±0.23	0.89±0.30	GHS42
91	DSV 10-11	0.58±0.21	0.42±0.17	GHS42
92	**Express**	**0.58±0.31**	**0.53±0.21**	**Resistant standard**
93	SRG 204	0.59±0.10	0.57±0.17	GHS42
94	SW 08-309025	0.59±0.21	0.33±0.14	GHS43
95	KWS 198	0.60±0.25	0.72±0.37	GHS42
96	SRG 219	0.61±0.20	0.38±0.14	GHS43
97	SYN VER 260	0.62±0.19	0.74±0.19	GHS46
98	SW 309020	0.62±0.17	0.65±0.21	GHS42
99	SYN VER 237	0.62±0.22	0.98±0.22	GHS42
100	SRG 222	0.62±0.23	0.34±0.20	GHS43
101	SRG 217	0.62±0.29	0.69±0.40	GHS43
102	SRG 209	0.62±0.22	0.59±0.15	GHS42
103	DSV 10-13	0.63±0.22	1.03±0.28	GHS42
104	WVB 227	0.63±0.15	0.36±0.12	GHS46
105	SRG 210	0.63±0.18	0.94±0.17	GHS42
106	SYN VER 233	0.63±0.15	0.92±0.28	GHS42
107	SW 08-309021	0.64±0.22	-0.16±0.11	GHS43
108	DSV 10-46	0.64±0.09	0.81±0.17	GHS46
109	WVB 225	0.64±0.18	0.61±0.29	GHS46
110	NPZ 220/08	0.64±0.19	0.99±0.18	GHS43
111	SRG 205	0.64±0.13	0.88±0.18	GHS42
112	WVB 209	0.64±0.19	0.84±0.25	GHS42
113	SW 309011	0.65±0.19	0.69±0.28	GHS42
114	SW 08-309034	0.65±0.56	0.73±0.57	GHS44
115	WVB 211	0.65±0.12	0.59±0.20	GHS42
116	NPZ 212/08	0.66±0.25	0.47±0.24	GHS43
117	SYN VER 246	0.66±0.36	0.92±0.28	GHS43
118	KWS 194	0.66±0.15	0.77±0.23	GHS42
119	DSV 10-7	0.66±0.18	0.96±0.30	GHS42
120	SRG 212	0.67±0.18	1.16±0.14	GHS42

Appendix 2.3 continued

No.	Genotype code	Normalized net AUDPC	Normalized relative stunting	Experiment No.
121	KWS 197	0.67+0.24	0.94+0.22	GHS42
122	SW 08-309023	0.67+0.25	0.09+0.16	GHS43
123	DSV 10-10	0.67+0.36	0.90+0.35	GHS42
124	SYN VER 232	0.67+0.29	0.91+0.33	GHS42
125	NPZ 200/08	0.68+0.22	0.52+0.21	GHS42
126	WVB 214	0.69+0.29	0.68+0.26	GHS42
127	NPZ 215/08	0.70+0.22	0.31+0.16	GHS43
128	SRG 216	0.70+0.09	0.47+0.06	GHS43
129	WVB 228	0.70+0.11	0.21+0.28	GHS46
130	SRG 202	0.71+0.21	0.70+0.18	GHS42
131	WVB 204	0.71+0.16	0.75+0.19	GHS42
132	NPZ 213/08	0.71+0.30	0.82+0.19	GHS43
133	SYN VER 239	0.72+0.28	1.05+0.28	GHS42
134	NICK 199	0.72+0.27	1.03+0.22	GHS46
135	SYN VER 265	0.72+0.31	0.85+0.40	GHS46
136	SYN VER 247	0.73+0.33	0.94+0.30	GHS43
137	NPZ 201/08	0.73+0.34	0.76+0.31	GHS42
138	NPZ 209/08	0.73+0.15	0.76+0.15	GHS42
139	SRG 214	0.73+0.15	0.79+0.27	GHS43
140	WVB 224	0.74+0.25	0.85+0.41	GHS43
141	SRG 208	0.74+0.27	0.84+0.21	GHS42
142	WVB 215	0.75+0.30	0.67+0.39	GHS43
143	SYN VER 230	0.75+0.29	0.93+0.33	GHS42
144	SYN VER 228	0.75+0.21	0.87+0.33	GHS42
145	KWS 196	0.75+0.13	0.84+0.27	GHS42
146	NPZ 202/08	0.76+0.32	0.82+0.35	GHS42
147	SYN VER 229	0.76+0.24	0.97+0.14	GHS42
148	SRG 223	0.76+0.25	0.63+0.28	GHS43
149	NPZ 214/08	0.76+0.23	0.74+0.22	GHS43
150	SYN VER 252	0.76+0.70	0.76+0.33	GHS44
151	SYN VER 236	0.77+0.16	0.93+0.16	GHS42
152	SYN VER 242	0.77+0.17	1.11+0.16	GHS43
153	SYN VER 254	0.79+0.28	0.46+0.54	GHS44
154	SRG 224	0.79+0.88	-0.21+0.86	GHS44
155	DSV 10-20	0.79+0.32	0.56+0.42	GHS43
156	SRG 215	0.79+0.29	0.87+0.34	GHS43
157	WVB 216	0.79+0.24	0.44+0.24	GHS43
158	WVB 218	0.81+0.24	0.53+0.19	GHS43
159	SYN VER 258	0.81+0.74	1.57+1.35	GHS44
160	SRG 203	0.81+0.39	1.06+0.38	GHS42

Appendix 2.3 continued

No.	Genotype code	Normalized net AUDPC	Normalized relative stunting	Experiment No.
161	DSV 10-22	0.81±0.23	0.93±0.25	GHS43
162	KWS 206	0.82±0.29	0.89±0.27	GHS43
163	WVB 212	0.83±0.23	0.89±0.30	GHS42
164	DSV 10-31	0.83±0.76	0.79±0.61	GHS44
165	NPZ 206/08	0.83±0.38	0.88±0.23	GHS42
166	SYN VER 263	0.84±0.27	0.72±0.21	GHS46
167	KWS 217	0.85±0.26	1.08±0.24	GHS46
168	WVB 219	0.85±0.43	0.89±0.44	GHS43
169	SW 08-309031	0.85±0.59	0.96±0.79	GHS44
170	SRG 229	0.85±0.94	0.36±0.84	GHS44
171	WVB 206	0.86±0.34	0.91±0.36	GHS42
172	WVB 217	0.87±0.30	0.53±0.21	GHS43
173	DSV 10-18	0.89±0.44	0.84±0.58	GHS43
174	NICK 198	0.89±0.36	1.11±0.32	GHS46
175	DSV 10-5	0.89±0.22	1.23±0.17	GHS42
176	SYN VER 245	0.89±0.43	1.02±0.40	GHS43
177	NPZ 210/08	0.90±0.24	0.54±0.20	GHS43
178	NPZ 218/08	0.90±0.39	0.85±0.35	GHS43
179	SYN VER 240	0.91±0.16	1.18±0.23	GHS42
180	WVB 220	0.92±0.26	0.09±0.14	GHS43
181	SRG 207	0.92±0.27	1.16±0.37	GHS42
182	SW 12-5	0.94±0.42	0.94±0.34	GHS46
183	SRG 206	0.94±0.31	1.13±0.30	GHS42
184	NPZ 205/08	0.95±0.26	1.00±0.31	GHS42
185	SW 12-2	0.96±0.52	0.89±0.33	GHS46
186	KWS 190	0.97±0.28	1.25±0.22	GHS42
187	NICK 185	0.97±0.67	0.91±1.06	GHS44
188	WVB 229	0.97±1.00	0.71±0.60	GHS44
189	NPZ 208/08	0.98±0.35	1.22±0.24	GHS42
190	KWS 203	0.98±0.46	0.96±0.42	GHS43
191	SRG 220	1.01±0.31	0.96±0.36	GHS43
192	DSV 10-44	1.02±0.42	1.26±0.32	GHS46
193	KWS 193	1.02±0.23	1.35±0.21	GHS42
194	KWS 220	1.03±0.49	1.22±0.46	GHS46
195	SW 08-309032	1.03±0.72	0.29±0.41	GHS44
196	NPZ 207/08	1.04±0.37	1.16±0.32	GHS42
197	DSV 10-24	1.04±0.33	1.14±0.28	GHS43
198	DSV 10-25	1.06±0.16	1.10±0.25	GHS43
199	DSV 10-45	1.06±0.27	0.99±0.18	GHS46
200	WVB 221	1.07±0.31	1.05±0.29	GHS43

Appendix 2.3 continued

No.	Genotype code	Normalized net AUDPC	Normalized relative stunting	Experiment No.
201	DSV 10-43	1.07+0.28	1.18+0.34	GHS46
202	WVB 213	1.09+0.39	1.10+0.31	GHS42
203	SRG 218	1.10+0.50	1.11+0.36	GHS43
204	KWS 219	1.10+0.27	0.94+0.22	GHS46
205	KWS 191	1.11+0.19	1.27+0.16	GHS42
206	KWS 205	1.11+0.97	0.99+0.13	GHS43
207	DSV 10-16	1.12+0.40	1.22+0.22	GHS42
208	SYN VER 259	1.12+1.24	0.81+0.54	GHS44
209	SYN VER 249	1.13+0.31	1.36+0.17	GHS43
210	DSV 10-19	1.17+0.21	1.00+0.26	GHS43
211	NICK 196	1.19+0.56	1.17+0.43	GHS46
212	KWS 221	1.21+0.30	1.37+0.22	GHS46
213	DSV 10-8	1.21+0.29	1.28+0.24	GHS42
214	NICK 183	1.22+0.30	1.37+0.19	GHS42
215	SYN VER 235	1.23+0.29	1.20+0.21	GHS42
216	WVB 222	1.23+0.30	0.88+0.27	GHS43
217	SYN VER 257	1.24+1.03	0.47+0.43	GHS44
218	KWS 199	1.25+0.29	1.31+0.18	GHS42
219	DSV 10-21	1.27+0.27	0.88+0.35	GHS43
220	DSV 10-15	1.28+0.26	1.37+0.13	GHS42
221	SYN VER 244	1.30+0.54	1.24+0.54	GHS43
222	SYN VER 243	1.31+0.45	1.34+0.37	GHS43
223	DSV 10-41	1.32+0.43	1.59+0.34	GHS46
224	WVB 229	1.32+0.25	0.99+0.18	GHS46
225	KWS 222	1.34+0.25	1.21+0.31	GHS46
226	WVB 226	1.34+0.29	1.07+0.36	GHS46
227	KWS 192	1.37+0.25	1.33+0.16	GHS42
228	DSV 10-42	1.37+0.43	1.58+0.32	GHS46
229	**Falcon**	**1.42+0.31**	**1.47+0.21**	**Susceptible standard**
230	DSV 10-14	1.43+0.41	1.38+0.24	GHS42
231	SYN VER 234	1.46+0.22	1.43+0.09	GHS43
232	SYN VER 241	1.61+0.65	1.39+0.29	GHS43
233	KWS 218	1.70+0.43	1.43+0.58	GHS46
234	SYN VER 255	1.71+1.66	0.70+0.58	GHS44
235	SYN VER 253	2.11+1.01	-0.03+0.63	GHS44
236	WVB 223	2.22+0.30	1.66+0.05	GHS43
237	KWS 204	2.35+0.47	1.59+0.21	GHS43

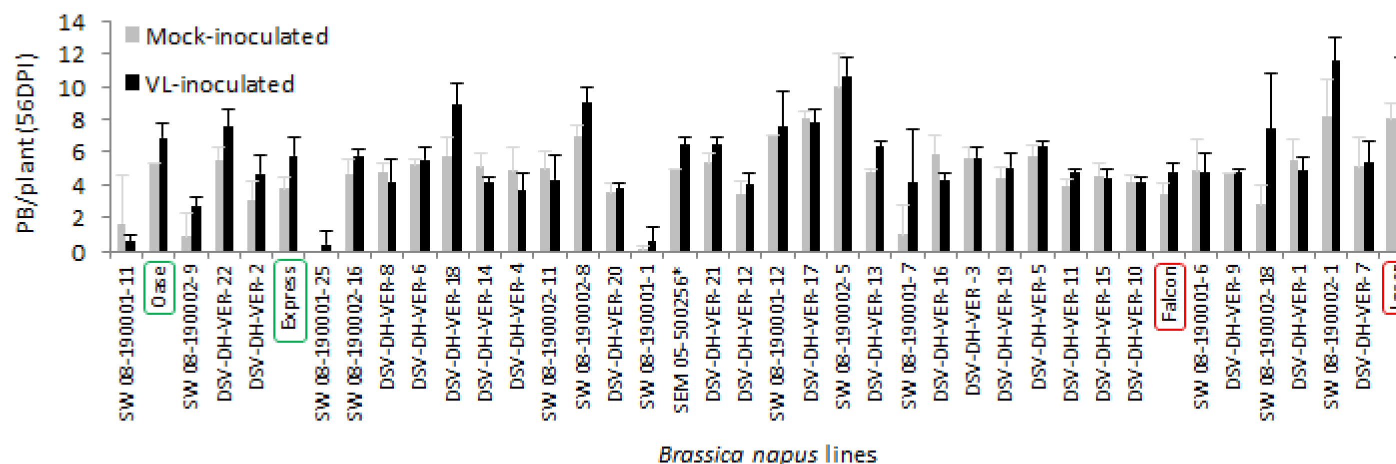
Mean + standard deviation data obtained from 20 plants of 235 *Brassica napus* lines evaluated in four different experiments are shown. Genotypes are sorted according to increasing values of normalized net AUDPC results. The values shown for resistant (Express; No. 92) and susceptible (Falcon; No.229) controls are indicated in **bold**. For the reference varieties, mean values obtained from four independent experiments (GHS42, GHS43, GHS44 and GHS46) are shown. Genotypes marked with * are best performing lines recommended for further verification under field conditions.

Appendix 2.4 Monthly air temperature, relative humidity and rainfall at the outdoor experimental area.

Month	Total RF(mm)	T (^oC)	RH (%)
April 2012	21.2	8.6	70.1
May 2012	31.4	15.2	69.0
June 2012	97.6	15.3	78.6
July 2012	90.9	18.0	80.7

RF, rain fall. **T**, temperature. **RH**, relative humidity.

Appendix 2.5 Comparison of branching in control and infected *Brassica napus* double haploid lines screened for *Verticillium longisporum* resistance in the outdoor experiment.

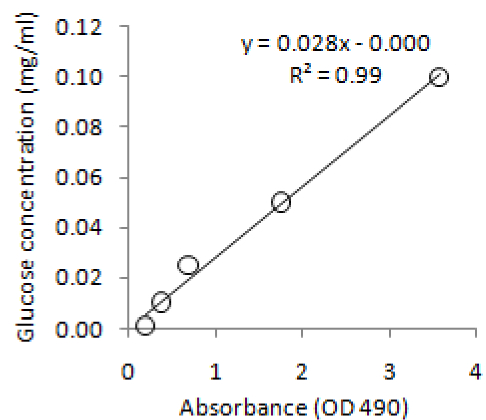


PB, primary branches. **VL**, *Verticillium longisporum*. *****, not a double haploid line. Mean ± standard deviation data obtained from three biological replicates is shown. Values of resistant and susceptible controls are indicated in green and red marks, respectively. Genotypes are sorted according to increasing net AUDPC values.

Appendix 2.6 Assessment key for evaluation of *Verticillium longisporum* disease severity on stubble and root of *Brassica napus*.

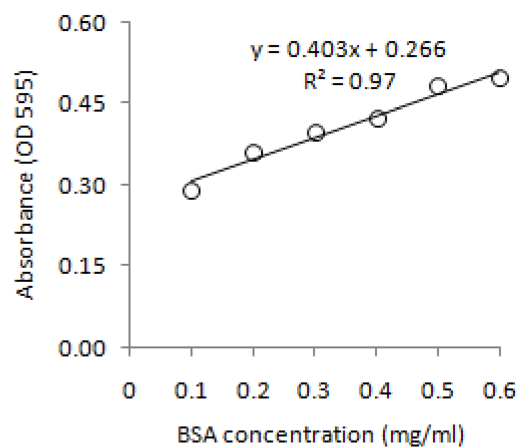
Index	Disease category	Symptom descriptions
1	Healthy tissue	Surface free of any symptoms and fungal structures; pith white and with cottony appearance; no microsclerotia visible
2	Slight infestation	Narrow and short longitudinal lesions on the surface; pith still whitish but a few patches of microsclerotia visible
3	Advanced infestation	Very long lesions and abundant microsclerotia visible under the epidermis; epidermis peeling off; long patches of black microsclerotia visible in the pith
4	Severe infestation	Most part of the stem epidermis peeling off and disintegrated; the surface black and covered with microsclerotia; pith becomes dark, disintegrated and filled with microsclerotia

Appendix 3.1 Standard curve for quantification of total carbohydrate content in xylem sap.



Points represent means of triplicates. Bovine serum albumin (BSA) was used as protein standard.

Appendix 3.2 Standard curve for quantification of total protein concentration in xylem sap.



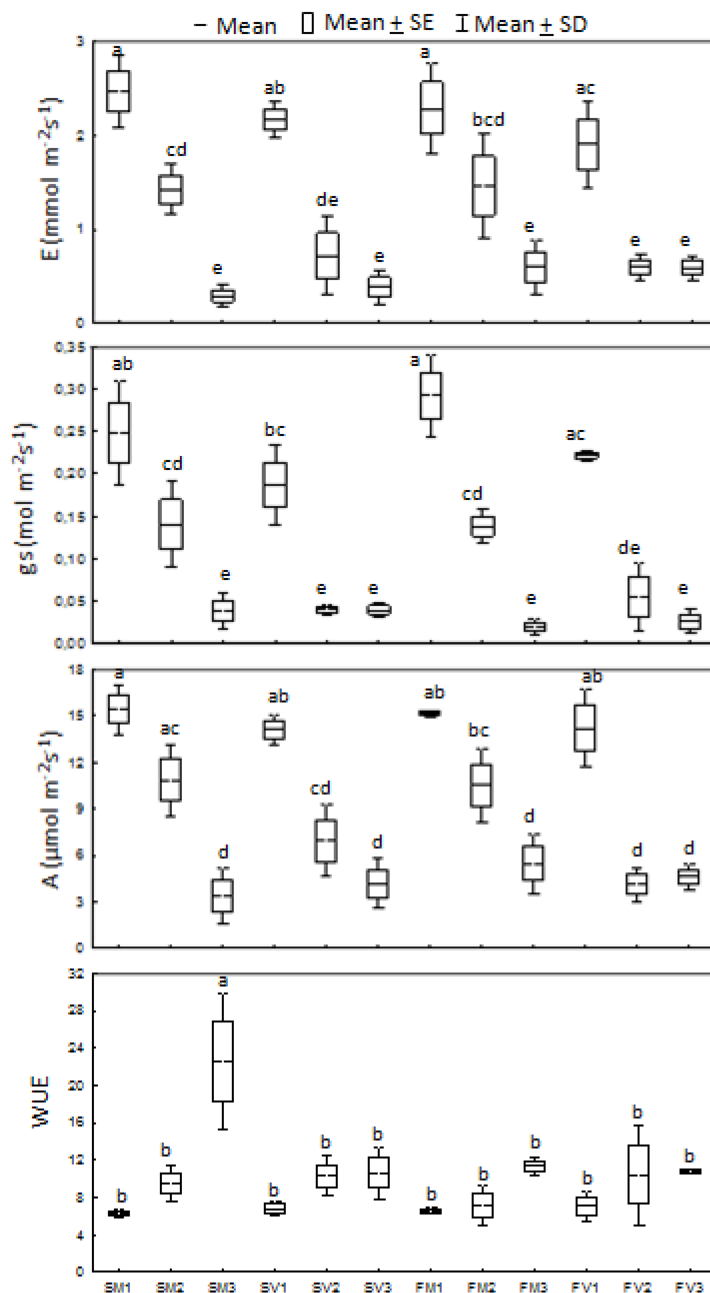
Points represent means of triplicates. Bovine serum albumin (BSA) was used as protein standard.

Appendix 3.3 Measurement of *Verticillium longisporum* growth in xylem sap of resistant (SEM) and susceptible (Falcon) *Brassica napus* plants. Xylem sap was collected 28 days after inoculation *with Verticillium longisporum*.

	Fungal growth on xylem sap (OD 580)					
Treatments	2 DAI	3 DAI	4 DAI	5 DAI	6 DAI	7 DAI
FM	0.044±0.02 [ab]	0.195±0.07 [a]	0.223±0.06 [a]	0.317±0.09 [ab]	0.358±0.09 [ab]	0.407±0.12 [a]
FMf	0.029±0.02 [b]	0.213±0.06 [a]	0.254±0.04 [a]	0.363±0.07 [ab]	0.395±0.06 [ab]	0.448±0.05 [a]
FVL	0.086±0.06 [ab]	0.291±0.22 [a]	0.320±0.26 [a]	0.441±0.34 [ab]	0.465±0.35 [ab]	0.489±0.34 [a]
FVLf	0.028±0.00 [b]	0.181±0.03 [a]	0.192±0.02 [a]	0.273±0.04 [b]	0.295±0.06 [b]	0.350±0.10 [a]
SM	0.045±0.03 [b]	0.182±0.04 [a]	0.243±0.10 [a]	0.359±0.12 [ab]	0.435±0.14 [ab]	0.548±0.21 [a]
SMf	0.038±0.02 [b]	0.208±0.02 [a]	0.270±0.01 [a]	0.386±0.04 [ab]	0.443±0.03 [ab]	0.508±0.03 [a]
SVL	0.114±0.05 [a]	0.383±0.16 [a]	0.418±0.17 [a]	0.515±0.19 [a]	0.585±0.22 [a]	0.611±0.23 [a]
SVLf	0.039±0.02 [b]	0.228±0.03 [a]	0.291±0.04 [a]	0.430±0.08 [ab]	0.493±0.13 [ab]	0.573±0.17 [a]
BDWVL	0.009±0.00 [c]	0.020±0.02 [a]	0.017±0.00 [a]	0.038±0.03 [c]	0.036±0.00 [c]	0.018±0.00 [a]
BDW	0.007±0.00 [c]	0.010±0.00 [a]	0.009±0.00 [a]	0.037±0.01 [c]	0.019±0.00 [c]	0.009±0.00 [a]

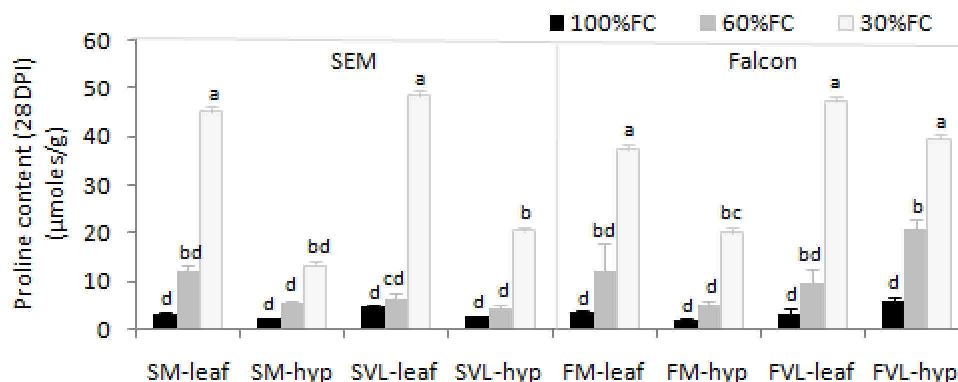
SM and **SMf**, respective fungal growth in unfiltered and filtered xylem sap collected from mock-inoculated plants. **SVL** and **SVLf**, respective fungal growth in unfiltered and filtered xylem sap extracted from VL-inoculated plants. **FM** and **FMf**, respective fungal growth in unfiltered and filtered xylem sap of mock-inoculated plants. **SVL** and **SVLf**, respective fungal growth in unfiltered and filtered xylem sap of VL-inoculated plants. **BDWVL**, Fungal growth in sterile bi-distilled water. **BDW**, sterile bi-distilled water without VL inoculum. Means ± standard deviation were obtained from three biological replicates. Mean values assigned with the same superscript are not significantly different at P=0.05.

Appendix 4.1 Transpiration rate (E), Stomatal conductance of CO_2 (gs), photosynthesis rate (A), and water use efficiency (WUE) of two *Brassica napus* genotypes subjected to *Verticillium longisporum* infection and/or drought stress under greenhouse conditions at 28 DPI.



Mock and/or VL-inoculated plants supplied with water at 100% FC were used as control. Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for one week starting from 21 days after inoculation with *Verticillium longisporum*. Mean data obtained from 30 plants of two independent experiments are presented. For each parameter, different letters on the bars indicate significant differences between treatments at P≤0.05. **SM**, SEM-05-500526 mock-inoculated. **FM**, Falcon mock-inoculated. **SV**, SEM-05-500526 VL-inoculated. **FV**, Falcon VL-inoculated. Treatment names followed by the numbers **1**, **2** and **3** refers to watering at 100, 60 and 30% field capacity, respectively.

Appendix 4.2 Changes in proline content in leaf and hypocotyl tissue of two *Brassica napus* genotypes exposed to different levels of drought stress and *Verticillium longisporum* infection at 28 DPI.



Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for one week starting from 21 days after inoculation with *Verticillium longisporum*. Mock and/or VL-inoculated plants supplied with water at 100% FC were used as control. Mean data obtained from 30 plants of two independent experiments are presented. Bars indicate standard deviations. Different letters on the bars indicate significant differences between treatments at $P \leq 0.05$. **FC**, field capacity. **hyp**, hypocotyl. **SM**, SEM-05-500526 mock-inoculated. **FM**, Falcon mock-inoculated. **SVL**, SEM-05-500526 VL- inoculated. **FVL**, Falcon VL-inoculated.

Appendix 4.3 Number of primary branches per plant measured from two *Brassica napus* genotypes exposed to drought stress and/or *Verticillium longisporum* infection at 28 DPI.

Treatments	100%FC	60%FC	30%FC
SM	1.8 ± 0.4 [bc]	1.9 ± 0.7 [bc]	1.9 ± 0.7 [bc]
SVL	4.2 ± 1.0 [ab]	3.9 ± 1.4 [ab]	4.2 ± 1.3 [ab]
FM	0.6 ± 0.2 [c]	0.5 ± 0.2 [c]	0.8 ± 0.4 [c]
FVL	3.8 ± 0.6 [ab]	5.1 ± 1.8 [a]	4.9 ± 1.2 [a]

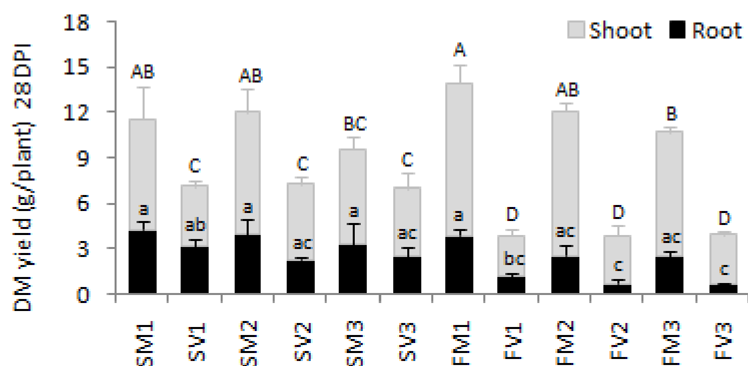
Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for one week starting from 21 days after inoculation with *Verticillium longisporum*. Mock and/or VL-inoculated plants supplied with water at 100% FC were used as control. Mean ± Standard deviation data obtained from 30 plants of two independent experiments are presented. Mean values containing the same superscript are not significantly different at P=0.05. **FC**, field capacity. **SM**, SEM-05-500526 mock-inoculated. **FM**, Falcon mock-inoculated. **SVL**, SEM-05-500526 VL-inoculated. **FVL**, Falcon VL-inoculated.

Appendix 4.4 Average phenological growth stages of two *Brassica napus* genotypes exposed to drought stress and/or *Verticillium longisporum* infection.

Treatments	21 DPI	28 DPI	35 DPI	42 DPI	49 DPI
SM1	50	59	62	65	67
SM2	50	58	63	65	68
SM3	48	55	60	60	63
SV1	49	56	60	63	65
SV2	52	59	62	64	66
SV3	47	54	57	59	60
FM1	47	51	56	59	62
FM2	45	51	56	58	60
FM3	46	51	56	58	59
FV1	43	49	53	57	61
FV2	44	50	54	57	59
FV3	45	50	56	58	62

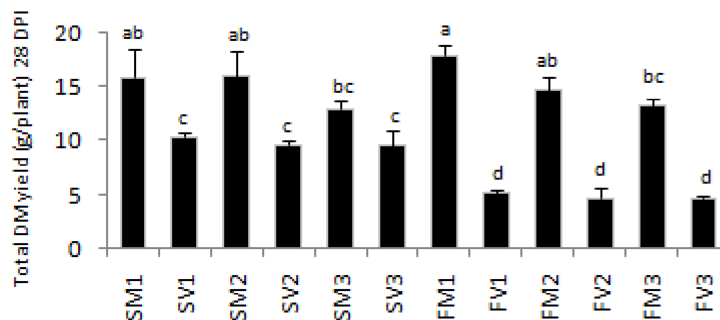
Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for four weeks starting from 21 days after inoculation with *Verticillium longisporum*. Mock and/or VL-inoculated plants supplied with water at 100% FC were used as control. Mean data obtained from 30 plants of two independent experiments are presented. **FC**, field capacity. **S**, genotype SEM-05-500526. **F**, cultivar Falcon. **M**, mock-inoculated. **V**, infected with *Verticillium longisporum*. **1**, **2** and **3** refers to watering at 100, 60 and 30% field capacity, respectively. Assessment was done using BBCH scale.

Appendix 4.5 Shoot and root dry biomass yields of two *Brassica napus* genotypes subjected to *Verticillium longisporum* infection and/or drought stress conditions in a greenhouse experiment at 28 DPI.



Mock and/or VL-inoculated plants supplied with water at 100% FC were used as control. Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for one week starting from 21 days after inoculation with *Verticillium longisporum*. Mean data obtained from 30 plants of two independent experiments are presented. Bars indicate standard deviation. For each parameter, different letters on the bars with the same letter-case indicate significant differences between treatments at P≤0.05. **DM**, dry matter. **S**, genotype SEM-05-500526. **F**, cultivar Falcon. **M**, mock-inoculated. **V**, infected with *Verticillium longisporum*. Treatments names followed by numbers **1**, **2** and **3** refer to watering at 100, 60 and 30% field capacity, respectively.

Appendix 4.6 Total dry biomass yield of two *Brassica napus* genotypes subjected to *Verticillium longisporum* infection and/or drought stress in a greenhouse experiment at 28 DPI.



Mock- and/or VL-inoculated plants supplied with water at 100% FC were used as control. Drought treatments were made by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for one week starting from 21 days after inoculation with *Verticillium longisporum*. Mean data obtained from 30 plants of two independent experiments are presented. Bars indicate standard deviation. For each parameter, different letters on the bars indicate significant difference between treatments at P≤0.05. **S**, genotype SEM-05-500526. **F**, cultivar Falcon. **M**, mock inoculated. **V**, infected with *Verticillium longisporum*. Treatments names followed by numbers **1**, **2** and **3** refer to watering at 100, 60 and 30% field capacity, respectively.

Appendix 4.7 F-values of analysis of variance for the effects of genotype, *Verticillium longisporum* infection, drought stress and interaction factors on disease development, plant physiolgy and agronomic trtaits measured at 28 DPI.

	Parameters	G	VL	D	G*VL	G*D	VL*D	G*VL*D
	DF	1	1	2	1	2	2	2
Disease	AUDPC	103.6***	650.3***	5.2*	103.6***	4.0*	5.2*	4.0*
	VL DNA	183.3***	224.7***	2.3^{ns}	183.3***	2.4^{ns}	2.3^{ns}	2.4^{ns}
	RS	31.0*	491.1***	1.9^{ns}	41.7**	3.9*	1.9^{ns}	3.9*
	PB	1.6^{ns}	98.8***	0.8^{ns}	7.4*	0.9^{ns}	0.5^{ns}	1.0^{ns}
Physiological	E	0.0^{ns}	19.0***	151.6***	0.5^{ns}	2.8^{ns}	8.3**	0.0^{ns}
	gs	0.9^{ns}	24.6***	139.3***	0.0^{ns}	2.3^{ns}	7.6**	0.1^{ns}
	A	0.1^{ns}	16.1**	133.2***	1.4^{ns}	2.4^{ns}	8.9**	0.7^{ns}
	WUE	5.2*	1.7^{ns}	19.8***	6.1*	3.5*	7.4**	3.4^{ns}
	RWC	0.2^{ns}	0.9^{ns}	23.1***	2.2^{ns}	0.4^{ns}	0.9^{ns}	0.9^{ns}
Agronomic	PH	4.7^{ns}	87.9***	1.6^{ns}	24.6***	0.0^{ns}	5.2*	2.5^{ns}
	SDM	9.4**	339.8***	5.8**	44.5***	1.2^{ns}	5.2*	0.9^{ns}
	RDM	34.6***	53.9***	6.2**	4.2^{ns}	0.2^{ns}	0.7^{ns}	0.8^{ns}
	TDM	28.7***	319.0***	8.7**	37.9***	1.1^{ns}	4.7*	1.4^{ns}

Drought treatments were applied by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for one week starting from 21 days after inoculation with *Verticillium longisporum* (28 days post inoculation). ANOVA was performed using the PROC MIXED procedure (SAS 9.3, SAS Inst. 2002). P-values are indicated in superscripts and the values less than 0.05 are considered a significant effect. ***,** significant at P=0.05. ****,** significant at P=0.01. *****,** significant at P=0.001. **ns**, not significant. **DF**, degree of freedom. **G**, genotype. **VL**, *Verticillium longisporum*. **D**, drought. **AUDPC**, net area under disease progress curve, **VL DNA**, *Verticillium longisporum* DNA in hypocotyl. **RS**, relative stunting. **PB**, number of primary branches per plant. **E,** transpiration rate. **gs,** stomatal conductance of CO_2. **A,** photosynthesis rate. **WUE,** water use efficiency. **LPC,** leeaf proline content. **HPC,** hypocotyl proline content. **RWC,** leaf relative water content. **PH,** plant height. **SDM,** shoot dry matter. **RDM,** root dry matter. **TDM,** total dry matter.

Appendix 4.8 Analysis of variance for the effects of genotype, *Verticillium longisporum* infection, drought stress, and interaction factors on accumulation of free proline in leaf and hypocotyl tissue of *Brassica napus* at 28 DPI.

Factors	F-value	DF
G	8.5^{*}	1
VL	15.2^{**}	1
D	306.0^{***}	2
PP	78.4^{***}	1
G*VL	9.2^{*}	1
G*D	1.6^{ns}	2
G*PP	25.4^{***}	1
VL*D	7.0^{**}	2
VL*PP	14.1^{**}	1
D*PP	65.0^{***}	2
G*VL*D	2.0^{ns}	2
G*VL*PP	6.1^{*}	1
G*D*PP	7.0^{**}	2
VL*D*PP	2.9^{ns}	2
G*VL*D*PP	0.7^{ns}	2

Drought treatments were applied by exposing plants to moderate (60% FC) or severe (30% FC) drought stress for one week starting from 21 days after inoculation with *Verticillium longisporum* (28 days post inoculation). ANOVA was performed using the PROC MIXED procedure (SAS 9.3, SAS Inst. 2002). P-values are indicated in superscripts and the values less than 0.05 are considered a significant effect. ***,** significant at P=0.05. ****,** significant at P=0.01. *****,** significant at P=0.001. **ns**, not significant. **DF**, degree of freedom. **G**, genotype. **VL**, *Verticillium longisporum*. **D**, drought. **PP**, plant part.

Appendix 4.9 Coefficients of Pearson's correlation (r) describing relationships within and between disease, physiological and agronomic parameters measured from two *Brassica napus* genotypes exposed to drought stress and/or *Verticillium longisporum* infection 7 days after initiation of drought treatments (28 DPI).

	Parameters	Disease				Physiological							Agronomic		
		AUDPC	VL DNA	RS	PB	E	gs	A	WUE	RWC	LPC	HPC	PH	SDM	RDM
Disease	VL DNA	0.87***	0.82***												
	RS	0.97***	0.63***												
	PB	0.76***	-0.21^{ns}	0.84***											
Physiological	E	-0.20^{ns}	-0.18^{ns}	-0.17^{ns}	-0.26^{ns}										
	gs	-0.19^{ns}	-0.24^{ns}	-0.18^{ns}	-0.27^{ns}	0.92***									
	A	-0.21^{ns}	-0.02^{ns}	0.18^{ns}	-0.27^{ns}	0.96***	0.91***								
	WUE	-0.11^{ns}	-0.18^{ns}	-0.12^{ns}	0.05^{ns}	-0.54***	-0.50**	-0.55***							
	RWC	-0.15^{ns}	-0.11^{ns}	0.14^{ns}	-0.17^{ns}	0.58***	0.63***	0.63***	0.35*						
	LPC	-0.02^{ns}	0.59***	0.01^{ns}	0.10^{ns}	-0.72***	-0.70***	-0.74***	0.58***	-0.53**					
	HPC	-0.43**	-0.77***	0.43**	0.40*	-0.63***	-0.62***	-0.68***	0.32*	-0.52**	0.73***				
Agronomic	PH	-0.85***	-0.77***	-0.87***	-0.71***	0.30*	0.29*	0.31*	-0.11^{ns}	0.24^{ns}	-0.14^{ns}	0.48**			
	SDM	-0.91***	-0.76***	-0.92***	-0.83***	0.35*	0.36*	0.33*	-0.06^{ns}	0.21^{ns}	-0.18^{ns}	-0.49**	0.83***		
	RDM	-0.79***	-0.80***	-0.74***	-0.56***	0.43**	0.43**	0.44**	-0.01^{ns}	0.35*	-0.22^{ns}	-0.54***	0.69***	0.77***	
	TDM	-0.92***	0.82***	-0.91***	-0.79***	0.39*	0.39*	0.38*	-0.05^{ns}	0.26^{ns}	-0.20^{ns}	-0.53**	0.83***	0.98***	0.87***

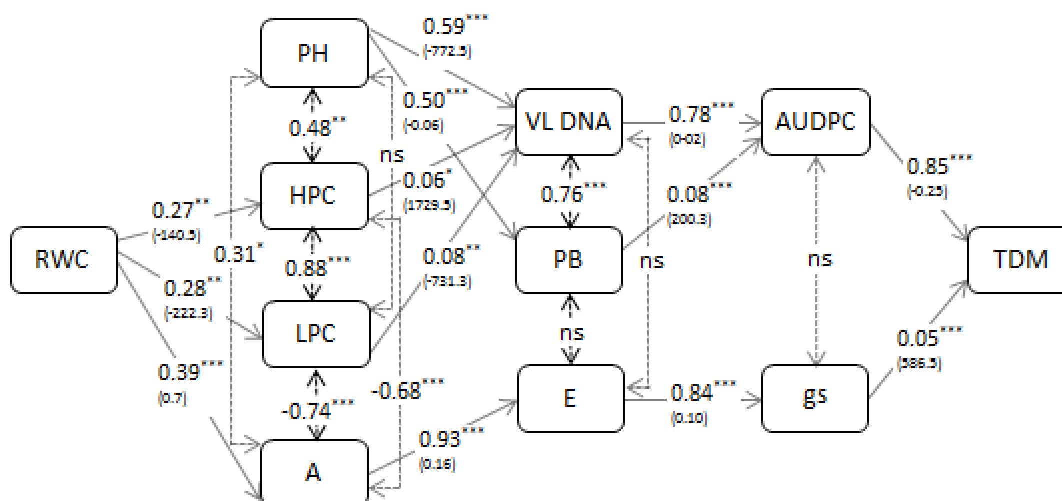
Correlation analysis was performed using PROC CORR procedure (SAS 9.3, SAS Inst. 2002). Minus signs indicate negative correlations. **ns**, not significant. ***,** significant at P=0.05. ****,** significant at P=0.01. *****,** significant at P=0.001. **DPI**, Days post inoculation with *Verticillium longisporum*. **AUDPC**, net area under disease progress curve, **VL DNA**, *Verticillium longisporum* DNA in hypocotyl. **RS**, relative stunting. **PB**, number of primary branches per plant. **E,** Transpiration rate. **gs,** stomatal conductance of CO_2. **A,** photosynthesis rate. **WUE,** water use efficiency. **LPC,** leaf proline content. **HPC,** hHypocotyl proline content. **RWC,** leaf relative water content. **PH,** pPlant height. **SDM,** shoot dry matter. **RDM,** root dry matter. **TDM,** total dry matter.

Appendix 4.10 Coefficients of determination (b), partial regression coefficients (r^2), and p-values of stepwise regression analysis indicating the effects of genotype, *Verticillium longisporum* infection and drought stress on disease, physiological and agronomic traits of two *B. napus* genotypes at 28 DPI.

	Factors	Genotype		*V. longisporum*		Drought	
	Parameters	b	Partial R^2	b	Partial R^2	b	Partial R^2
Disease	AUDPC	9.3	0.11^{***}	24.6	0.76^{***}	ns	ns
	VL DNA	331.7	0.29^{***}	368.9	0.35^{**}	ns	ns
	RS	13.7	0.05^{**}	57.5	0.87^{***}	ns	ns
	PB	ns	ns	3.0	0.77^{***}	ns	ns
Physiological	E	ns	ns	ns	ns	-1.7	0.89^{***}
	gs	ns	ns	ns	ns	-0.2	0.88^{***}
	A	ns	ns	ns	ns	-10.3	0.93^{***}
	WUE	ns	ns	ns	ns	7.1	0.41^{***}
	RWC	ns	ns	ns	ns	-7.2	0.66^{***}
	LPC	3.8	0.01^{*}	ns	ns	41.0	0.97^{***}
	HPC	7.3	0.08^{*}	7.7	0.09^{*}	20.1	0.58^{***}
Agronomic	PH	ns	ns	-32.7	0.61^{***}	ns	ns
	SDM	ns	ns	-6.0	0.73^{***}	ns	ns
	RDM	-1.2	0.23^{**}	-1.6	0.39^{**}	-0.9	0.12^{**}
	TDM	-1.9	0.05^{*}	-7.5	0.70^{***}	-2.2	0.06^{*}

Mean data obtained from fully irrigated and severely stressed treatments of two independent experiments were used. Drought treatments (30% FC) were applied for four weeks starting from 7 days after inoculation with *Verticillium longisporum*. **AUDPC,** net area under disease progress curve. **VL DNA,** *Verticillium longisporum* DNA in hypocotyl. **RS,** relative stunting. **PB, n**umber of primary branches per plant. **E,** transpiration rate. **gs,** stomatal conductance of CO_2. **A,** photosynthesis rate. **WUE,** water use efficiency. **RWC,** leaf relative water content. **LPC,** leaf proline content. **HPC,** hypocotyl proline content. **PH,** plant height. **SDM,** shoot dry matter. **RDM,** root dry matter. **TDM,** total dry matter. **ns,** not significant. ***,** significant at P≤0.05. ****.** significant at P≤0.01. *****,** significant at P≤0.001.

Appendix 4.11 Sequential path model indicating stepwise regression coefficients of determination (b), partial regression coefficients (r^2), Pearson’s correlation coefficients (r) and probability values of variables predicting total dry matter yield in *Brassica napus* genotypes subjected to drought stress and *Verticillium longisporum* infection at 28 DPI.



Mean data obtained from 30 plants of two independent experiments were used for analysis. Drought treatments were applied for four weeks starting from 7 days after inoculation with *Verticillium longisporum*. Solid arrows show stepwise regression analysis with Partial regression coefficients. Coefficients of determination are indicated in brackets. Dotted arrows show coefficients of Pearson’s correlation indicating relationships within the predicting variables. **AUDPC,** net area under disease progress curve. **VL DNA,** *Verticillium longisporum* DNA in hypocotyl. **PB,** **n**umber of primary branches per plant. **E,** transpiration rate. **gs,** stomatal conductance of CO_2. **A,** photosynthesis rate. **RWC,** leaf relative water content. **LPC,** leaf proline content. **HPC,** hypocotyl proline content. **PH,** plant height. **TDM,** total dry matter. **ns**, not significant. ***,** significant at P=0.05. ****,** significant at P=0.01. *****,** significant at P=0.001.

Appendix 4.12 Principal component analysis of 16 diseases, physiological and agronomic variables measured from two *Brassica napus* genotypes subjected to drought stress and infection with *Verticillium longisporum* at 49 DPI.

Variables	**PC1**	**PC2**
AUDPC	0,251	-0,297
VL DNA	0,256	-0,251
PB	0,215	-0,252
RS	0,248	-0,295
E	-0,221	-0,287
gs	-0,225	-0,250
A	-0,209	-0,296
WUE	0,169	0,295
RWC	-0,159	-0,304
LPC	0,176	0,336
HPC	0,144	0,345
PH	-0,310	0,178
HD	-0,310	-0,019
SDM	-0,332	0,037
RDM	-0,317	0,129
TDM	-0,335	0,061
Variance explained	51%	32%

Only the first two principal components that explained much of the total variation are shown. Parameters included in the PCA: **AUDPC,** net area under disease progress curve. **VL DNA,** *Verticillium longisporum* DNA in hypocotyl. **PB, n**umber of primary branches per plant. **RS,** relative stunting. **E,** transpiration rate. **gs,** stomatal conductance of CO_2. **A,** photosynthesis rate. **RWC,** leaf relative water content. **LPC,** leaf proline content. **HPC,** hypocotyl proline content. **PH,** plant height. **HD,** hypocotyl diameter. **SDM,** shoot dry matter. **RDM,** root dry matter. **TDM,** total dry matter.

Appendix 4.13 Phenological growth stages and BBCH-identification keys of oilseed rape

Oilseed rape

Weber and Bleiholder, 1990; Lancashire et al., 1991

Phenological growth stages and BBCH-identification keys of oilseed rape
(*Brassica napus* L. ssp. *napus*)

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

[1] Stem elongation may occur earlier than stage stage 19; in this case continue with stage 20
[2] Visibly extended internode n develops between leaf n and leaf n+1

Code	Description
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")
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

Acknowledgements

First and foremost, I would like to thank God for giving me the wisdom, strength and endurance to complete my study.

I would like to extend my sincere gratitude to my supervisor Prof. Dr. Andreas von Tiedemann first of all for accepting me as his student then for his supervision, guidance and advice throughout my study period. He was always keen to give valuable and brilliant ideas which enriched my study. I learned a lot from his extraordinary experiences and expertise in phytopathology and skills in leadership. Thank you for your availability without any preconditions, for your precious time to go throughout my thesis and the critical comments and suggestions. My great gratitude goes to Prof. Dr. Petr Karlovsky for supervising my study and for his advice and support particularly with regard to the molecular studies. I am highly indebted to Prof. Dr. Heiko C. Becker for his absolute willingness to be my examiner.

My special thanks goes to Dr. Birger Koopmann for his unreserved guidance and advice from the first date of my study until the end. I really appreciate your multipurpose role in the group as a whole. Thank you for your guidance during my work on molecular topics and for your valuable comments and suggestions on my thesis in particular. Dr. Jessica Knüfer, Dr. Mark Winter and Dr. Magdalena Siebold are gratefully acknowledged for sharing me their skills and expertise and for those interesting scientific and social discussions. I would like to thank Dr. Anke Sirrenberg for her interest to read part of my thesis and her comments.

Jutta shapper is the first person to be sincerely acknowledged for all her assistance in the field trials and greenhouse studies. I also acknowledge the significant contribution of Hubertus Reintke, Evelin Vorbeck and Dagmar Tacke in the field and lab experiments. Thank you all for your help in the last years. Without your support, my work would not have been successful.

Many thanks go to the project partners at the University of Giessen, Department of Plant Breeding. Particularly Dr. Christian Obermeier, Prof. Dr. Wolfgang Friedt and Prof. Dr. Rod Snowdon are sincerely acknowledged for their unreserved collaboration which led to the successful completion of the project. I also thank all the breeding companies in the GFP for providing plant materials and for the fruitful cooperation. Special thanks go to Dr. Detlef Hauska, Dr. Maria Kaliff, Dr. Tobias Jesske, Dr. Werner Horn, Dr. Reinhard Hemker, Dr. Andreas Girke, Dr. Annika Spies, Dr. Peter Duchscherer, Ms Simone Sendke, Dr. Andreas Gertz, Dr. Hanna Meier zu Beerentrup. My sincere gratitude also goes to Mr. Stefan Lütke Entrup for the productive cooperation and organization of the fruitful annual GFP meetings. I am deeply

grateful to Christine Kettner and Prof. Dr. Andrea Polle (Institute for Forest Botany, Forest Botany and Tree Physiology Section, Göttingen University) for their cooperation and technical help during my drought experiments.

To all staff members of the Division of General Plant Pathology and Crop Protection, Göttingen University, I would like to say thank you for the enjoyable social events and for providing me with a nice research facilities and condusive working environment. In particular, my special thanks go to Ms Martina Bode for the efficient facilitation of the academic and project related issues which substantially helped me to smoothly accomplish my study. Collective and individual acknowledgments are also owed to all my PhD colleagues; Christian Comberg, Geoffrey Onaga, Hendrik Hanekamp, Ines Eikenberg, Kerstin Höch, Lucia Ramos-Romero, Sandra Gerth, Tingting Wei and Xia Ha. Thank you guys for the nice time we had during the social events and our Tuesday seminar which greatly helped us to improve our work. It was really great to know you all and work with you.

I want to take this opportunity to thank my Ethiopian friends and their families here in Göttingen. Dr. Zerihum Teklemariam, Wondimeneh Taye, Denabo Billo and Dr. Yabibal Mulualem your presence was helpful and memorable. My special thanks goes to Cathrin Horstmann for her help in several administrative issues, for her constant encouragement and for the nice social events.

Last but not least, I extend my gratefulness to my brothers and sisters who contributed a lot for my today's position and for their everlasting love and faith. My Special thanks go to Gash Girma Gebray and his family who were my motivation since the beginning of my earliest studies. Emebet, Techane, Belayneh, Alganeh, Yimer, Yoseph, Zemede, Wondwesen and Shewangizaw, you have a special place in my life in particular and in Lopisso's family as a whole. Without your support, I would have not been reached to today's situation. Thank you Kokobe Gizaw for your continues support, advise, encouragement, and pray that helped me a lot for the successful completion of my study. May God bless you all!

This study was financed by GFP (an association for the promotion of private plant breeding in Germany), BMELV (German Federal Ministry for Food, Agriculture and Consumer Protection) and FNR (German Federal Agency of renewable resources) to which I am highly indebted.

Selected contributions in non-reviewed journal paper or conference proceedings

Pfordt A., Zheng X., Eseola A. B., Wilch A., Koopmann B., **Lopisso D. T**., von Tiedemann A. 2016. Time course of infection, seed transmission and damage of *Verticillium longisporum* in oilseed rape. In: Berne S. and Javornik B. (Eds).12th International Verticillium Symposium. Ljubljana, Slovenia. Book of Abstracts, P.28.

Lopisso D. T., Eseola A. B., Koopmann B., and von Tiedemann A. 2016. The role of seed transmission of *Verticillium longisporum* in oilseed rape. The 60th German Plant Protection Conference. Haale, Germany.

Alnajar D., **Lopisso D. T**., Koopmann B., and von Tiedemann A. 2016. Potential of root associated fungal and bacterial biocontrol agents (BCAs) against *Verticillium longisporum* infection of oilseed rape. The 60th German Plant Protection Conference. Haale, Germany.

von Tiedemann A, Eynck C., Riediger N., **Lopisso D. T.**, Kamble A., Knüfer J., Novakazi F., Zheng X., Siebold M., Koopmann B., 2015. A hidden pathogen with uncommon properties – reviewing the state-of-the-art of *Verticillium longisporum* on oilseed rape. 14th International Rapeseed Congress, Book of Abstracts, p.115.

Hossain M. A., **Lopisso D. T**., Snowdon R., von Tiedemann A., Friedt W., Obermeier C. 2015. Mining QTl for candidate genes involved in resistance of oilseed rape against *Verticillium longisporum* by an integrative omics approach. 14th International Rapeseed Congress, Book of Abstracts, p.365.

Lopisso D. T., Knüfer J., Koopmann B., von Tiedemann A. 2015. Effects of host-resistance to *Verticillium longisporum* on the performance of winter oilseed rape (Brassica napus) under drought stress. XVIII. International Plant Protection Congress, Berlin 2015. Book of Abstracts, p.540.

Knüfer J. **Lopisso D. T**., Koopmann B., von Tiedemann A. 2014. Classification of winter oilseed rape resistance towards the soil-borne pathogen *Verticillium longisporum* by quantitative PCR. 59. Deutsche Pflanzenschutztagung, Freiburg, Germany, Kurzfassungen der Beiträge, Julius-Kühn-Archiv 447, S.354.

Lopisso D. T., Knüfer J., Koopmann B. and von Tiedemann A. 2014. Impact of cultivar resistance to *Verticillium longisporum* on drought stress tolerance of winter oilseed rape (*Brassica napus*). 59. Deutsche Pflanzenschutztagung, Freiburg, Germany, Kurzfassungen der Beiträge, Julius-Kühn-Archiv 447, S.355.

Lopisso D. T., Knüfer K., Koopmann B, and von Tiedemann A. 2015. Phenotypinig of a ‘hidden’ pathogen - quantifying resistance in winter oilseed rape lines (*Brassica napus*) to *Verticillium longisporum*, PhenoDays 2014. Beaune, France.

Lopisso D. T., Knüfer J., Koopmann B: and von Tiedemann A. 2013. Winter oilseed rape physiology, gene

expression and agronomic performance during drought stress and infection with *Verticillium longisporum*. In: Koopmann B., von Tiedemann A. (Hg.) 11th International Verticillium Symposium. Braunschweig, Germany, Book of Abstracts, P.47.

Hossain M. A., Obermeier C., Snowdon R., **Lopisso D. T**., von Tiedemann A. and Friedt W. 2013. Association of phenylpropanoid metabolites with resistance of rapeseed to *Verticillium longisporum*. 11th International Verticillium Symposium. Braunschweig, Germany, Book of Abstracts, P.100.

Lopisso D. T., Knüfer J., Koopmann B., von Tiedemann A. 2013. Response of oilseed rape (*Brassic napus* L.) to combined effects of drought stress and *Verticillium longisporum* infestation. In: Koopmann B, Cook S, Evans N, Ulber B (Hg.) Integrated Control in Oilseed Crops - IOBC-WPRS Bulletin VOL.96. IOBC-Verlag, Gent, Belgium, 213-214.

Lopisso D. T., Koopmann B., von Tiedemann A. 2012. Physiological and morphological responses in oilseed rape (*B. napus*) during drought stress and infection with *Verticillium longisporum*. 58. Deutsche Pflanzenschutztagung, 10.-14.September 2012, Braunschweig. Kurzfassungen der Beiträge, Julius-Kühn-Archiv 438, P.403.

Master's project supervision

Eseola A. B. The role of seed transmission of *Verticillium longisporum* in oilseed rape. Division of General Plant Pathology and Crop Protection, Department of Crop Sciences, Faculty of Agricultural Sciences, Georg-August-Universität Göttingen, Germany. November, 2016.

Alnajar D. Potential of root associated fungal and bacterial biocontrol agents against *Verticillium longisporum* in oilseed rape. Division of General Plant Pathology and Crop Protection, Department of Crop Sciences, Faculty of Agricultural Sciences, Georg-August-Universität Göttingen, Germany. Germany. July, 2016.

Kühlmann V. Plant growth promotion and disease control potential of soilborne fungal biocontrol agents on sugar beets infected with Verticillium wilt. Division of General Plant Pathology and Crop Protection, Department of Crop Sciences, Faculty of Agricultural Sciences, Georg-August-Universität Göttingen, Germany. Germany. December, 2015.

Farooq M. Differential responses of oilseed rape (*Brassica napus* L.) to infection with fungal and bacterial vascular pathogens. Division of General Plant Pathology and Crop Protection, Department of Crop Sciences, Faculty of Agricultural Sciences, Georg-August-Universität Göttingen, Germany. September, 2013.

CURRICULUM VITAE

PERSONAL DETAILS

Full name: Daniel Teshome Lopisso
Date of Birth: August 27, 1980
Place of birth: Addis Ababa, Ethiopia
Nationality: Ethiopian

EDUCATIONAL BACKGROUND

1. **PhD in Plant Pathology** (2011-2014), graduated with highest honor (Summa Cum Laude) from Georg-August-Universität Göttingen, Germany
2. **MSc in Plant Pathology and Entomology** (2008 – 2010), graduated with distinction from Wageningen University, The Netherlands
3. **BSc degree in Plant Production and Dry Land Farming** (2003-2005), graduated with distinction from Debub University, Ethiopia
4. **Diploma in Plant Science and Technology** (1997-1999), graduated with distinction from Debub University, Ethiopia

WORK EXPERIENCE

1. Post Doctorate at Georg-August-Universität Göttingen, Germany since September 2014: Coordination and supervision (master's students and project staff) of a bilateral (German-Greece) project on biological crop protection.
2. PhD student and Researcher at Georg-August-Universität Göttingen, Germany from April 2011 to August 2014: Conducted and supervised a trilateral project (plant pathology and crop protection) funded by the German private plant breeding association and several European seed companies.
3. Lecturer and Researcher at Jimma University, Ethiopia from 2005 to 2008: Teaching, research, student and staff supervision, community based training, etc.
4. Academic and Research Technical Assistant at Harramaya University, Ethiopia from 2002 to 2003: Assisted field and laboratory researches in the crop protection section of the department of plant sciences.
5. Research Technical Assistant at Awassa Agricultural Research Organization, Ethiopia from 2000 to 2001: Assisted plant breeding and crop protection field researches.

HONORS AND AWARDS

1. **Outstanding international student**: Netherlands Organization for International Cooperation in Higher Education (Nuffic), August,2008
2. **Excellence in teaching**: Jimma University College of Agriculture and Veterinary Medicine, Ethiopia, August 2007
3. **Innovative and outstanding research and scientific presentation**: Scientific Committee of the IOBC working group "Integrated Control in Oilseed Crops" October 2013, Luxemburg.

Declaration

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

Göttingen, September 2014