Elucidating the interaction between *Brassica napus* and *Rhizoctonia solani* AG 2-1

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

September 2017

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This thesis is dedicated to my parents
For always reminding me what is important in this life
Without them I wouldn't be who I am

'*Ithaka gave you the marvelous journey.*
Without her you would not have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka won't have fooled you.
Wise as you will have become, so full of experience,
you will have understood by then what these Ithakas mean.'

(Ithaka, C.P. Cavafy, Collected Poems)

Acknowledgements

Firstly I would like to sincerely thank my three supervisors, Toby Bruce, Rumiana Ray and Neil Graham, for their advice, instructions and help during my PhD. I feel privileged for working with you. I would especially like to thank Toby whose work has always been an inspiration and the reason that I chose this PhD. Also Neil, with his calm and patience helped me incredibly even during the most stressful periods. Additionally, I would like to thank the BBSRC-DTP for offering me this PhD fellowship and especially Jerry Roberts; I admired his great sense of humour and positive attitude that always brought a solution.

Special thanks also to Sam Grange for his enthusiasm, willingness to learn and hard work during the screening of the mapping population, to the SB glasshouse team and particularly to Mark Meacham for his help throughout the PhD with the glasshouses, the aphids and every other little thing that allowed me to accomplish my experiments. Also to Julietta Marquez not only for her help in the lab but also because she has been a true friend. I feel I should particularly offer my thanks to Matt Brown and Jassy Drakulic; we met by sharing the same office but you have always been two great friends, thank you for every memory! Also, to Guille Mendiondo, I'm grateful not only for her advices as a friend but also as a scientist. My thanks also to Moe Abbas for his useful advice during the gene expression analysis, to Tom Alcock for his help during my first weeks in the A38 lab and to Diriba Kumssa for helping me during the formatting of the thesis. Also many thanks go to everyone in the two groups (Crop Pathology and Plant Nutrition) for their support.

I offer my thanks to all the friends I've made during these years in SB: Dasuni, Brian, Dimitra and Mark, the 'beet group' and Cristina. But also to three people that I met during my arrival in Nottingham 4 years ago and since then they have been probably some of the closest friends in my life; Suzanne, my dear Partridge, thank you for all the great memories down in RRes, for your incredible birthday cards, for being there for me whenever I needed you. Ruth, we started our friendship with a gig; Back then I though we just had the same taste in music, now I know we had much more in common; thanks for all the

memories and for your honest friendship. My dear friend John, thank you for all the interesting conversations about classical music, cinema and books, for being my friend.

I honestly believe that my life during these 4 years would be very different without the support of certain people. Maybe with some of them we got lost but I will be always thankful for the good memories I have with them: Many thanks to my dearest friends Fyllio, Voula, Maria and Giorgo, Evi and Yiota that our friendship remains strong for so many years. To Aliki and Lyda for all the funny/crazy moments back at home, to my cousin Dimitra for bringing optimism in my life. Also to Galini, James, Matt and Jess, Mim, Ben, my housemate Tim who patiently carried on with my stress about the PhD and the house-cleaning, to Ben and Christos. Also my thanks go to my comrades from the communist party of Greece (KKE).

I owe thanks to everyone in my family back home for their love and support all these years. Especially, I would like to express my gratitude to my uncle George and my late godmother Fryni, who unconditionally supported me as second parents. Also, my siblings Iason and Christina. Last but definitely not least, my parents; Mum and Dad I'm grateful for your love, support, freedom, criticism, for everything I have.

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List of Abbreviations

OSR	oilseed rape
AG	anastomosis group
GSL	glucosinolates
ITC	Isothiocyanates
SA	salicylic acid
JA	jasmonic acid
ET	ethylene
ABA	abscisic acid
SDHI	succinate dehydrogenase inhibitors
DMI	demethylation inhibitors
LECA	light expanded clay aggregate
PGA	potato glucose agar
dpi	days post inoculation
DI	disease index
ANOVA	analysis of variance
GLM	generalised linear model
s.e.d	standard errors of differences
d.f	degrees of freedom
LSD	least significant difference of means
RL	root length
QTL	quantitative trait loci
AMF	arbuscular mycorrhizal fungi
TCDH	Temple x Canard Doubled Haploid

P+	plants produced from inoculated plants
P-	plants produced from control plants
HAMPs	herbivore associated molecular patterns
PAMPs	pathogen associated molecular patterns
ETI	effectors triggered immunity
cv	cultivars
PA	pathogen infected plants followed by aphid infestation
A	aphid infested plants
P	pathogen inoculated plants
AP	aphid infested plants followed by pathogen inoculation
MRGR	Mean Relative Growth Rate
r_m	population increase
h	hours
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
ITS	Internal Transcribed Spacer region
FW	Fresh Weight

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Published

Drizou F., Graham NS, Bruce TJA and Ray RV. Development of high-throughput methods to screen disease caused by *Rhizoctonia solani* AG 2-1 in oilseed rape. **Plant Methods, 2017**, 13(1): p. 45

In preparation

Drizou F., Bruce TJA, Ray RV and Graham NS. Infestation by *Myzus persicae* increases susceptibility of *Brassica napus* cv. 'Canard' to *Rhizoctonia solani* AG 2-1.

Drizou F., Bruce TJA and Graham NS. *Rhizoctonia solani* AG 2-1 and *Brassica napus*: a review of current knowledge.

Publication from PIP

Anastasaki E., **Drizou F** and Milonas PG. Elecrophysiological and oviposition responses of *Tuta absoluta* females on herbivore induced volatiles in tomato plants **(**under review in the **Journal of Chemical Ecology**).

Attendance in international conferences

Drizou F., Bruce TJA and Graham NS.Aphid infestation increases susceptibility of *B. napus* to *Rhizoctonia solani* AG 2-1. BSPP/BMS Presidential Conference**,** 11-13 of September 2017, Nottingham**,** UK **(Scientific poster)**.

Drizou F., Bruce TJA and Graham NS. Herbivory by aphid increases susceptibility of *Brassica napus* to *Rhizoctonia solani* AG 2-1. SCI Young Researchers in Crop Sciences 2017, 11th of July, Harpenden, UK **(flash presentation and scientific poster)**.

Drizou F., Bruce TJA and Graham NS. Insect-pathogen interaction in oilseed rape (OSR): *Myzus persicae* increases susceptibility of *Brassica napus* to *Rhizoctonia solani* AG 2-1. **16th** International Symposium of Plant-insect Interactions, 3-6 of July 2017, Tour, France (**Scientific poster**).

Abstract

Brassica napus, oilseed rape (OSR), is a worldwide cultivated crop belonging to the family Brassicaceae, broadly used in crop rotations with cereals. Production is focused on oil for human consumption, biodiesel and feedstock. OSR has undergone intensive breeding for optimization of oil content, disease resistance and augmentation of yields, and today is considered one of the most profitable crops. Nonetheless, oilseed rape is the primary host for the necrotrophic soil-borne pathogen *Rhizoctonia solani* anastomosis group (AG) 2-1. Infection of seedlings causes damping off disease and decreases crop establishment and yields. AG 2-1 is the most prevalent AG of *R. solani* in wheat fields in the UK. Currently there is no OSR germplasm resistant to *R. solani* AG 2-1. Available control methods include cultural practices and chemical seed treatments, which aim to postpone the infection and hence improve crop establishment. Changes in agronomic practices and crop management, including choice of cultivars, tillage, application of fertilisers and pesticides, mean that there is a danger of future outbreaks of diseases that in the past were not considered as major problem. This includes *R. solani* AG 2-1 which can infect other rotational crops as well and due to its saprophytic nature remains in the fields for years. The aim of the PhD was to elucidate interactions between *R. solani* AG 2-1 and *B. napus*, by identifying potential resistant traits and understanding how the pathogen counteracts OSR plant defences.

The first objective was to develop and compare different high-throughput screening methods that could be used for the phenotyping of OSR germplasm interactions with *R. solani* AG 2-1. Four methods were developed and compared: (1) nutrient media plates, (2) compost trays, (3) light expanded clay aggregate (LECA) trays and (4) a hydroponic pouch and wick system. Inoculation of LECA was the most suitable method for screening disease caused by AG 2-1 to OSR germplasm, because it allowed the detection of differences in disease severity between the tested OSR genotypes 5 days post infection (dpi) and also to conduct measurements in whole plants.

The second objective was to identify any sources of disease resistance by screening a diversity of OSR germplasm. To start the screening, I selected randomly germplasm from commercial cultivars and parental lines of mapping populations that was available in our seed bank. Overall, the germplasm tested consisted of commercial cultivars, genotypes from diversity sets and a mapping population. All genotypes tested appeared to be susceptible to AG 2-1 infection as shown by high disease levels, reduced emergence and survival. Additionally, I tested if any induced defence responses from exposure to disease could be inherited in the next generation through an epigenetic stress response. However, all progeny plants were also highly susceptible indicating that there was no evidence for transgenerational induction of resistance in this system.

The third objective was to gain insight into OSR plant defences when exposed to a combination of attacking organisms, as this often occurs in real field situations. I investigated the role of *M. persicae* infestation on OSR susceptibility to *R. solani* AG 2-1. There was no effect of AG 2-1 infection on aphid performance. However, *M. persicae* infestation resulted in significantly more disease symptoms in *B. napus* cv. 'Canard' plants although there were no significant differences in the amount of fungal DNA. Marker genes *LOX3* and *MYC2* had an augmented expression under AG 2-1 treatment but were downregulated in plants exposed to both aphids and pathogen. Hence, it appears that aphid infestation induced changes in the jasmonic acid (JA) signalling pathway, which resulted in the increased susceptibility to AG 2-1.

In conclusion, the present work provided a new high-throughput screening method suitable to phenotype disease by AG 2-1 in the early seedling stage within a short time period. Unfortunately, the current results confirm previous studies indicating that AG 2-1 is an extremely aggressive isolate to OSR germplasm that lacks genetic resistance. Nonetheless, the observed differences between the germplasm tested in the present work suggest that there are potential tolerant traits. For the first time, the current work provided evidence that *M. persicae* infestation can negatively affect plant defences against *R. solani* AG 2-1, through suppression of genes involved in JA signalling. Additionally, it was demonstrated that *R. solani* AG 2-1 induces the activation

of defence mechanism related to both JA and salicylic acid (SA) pathways. Future studies aiming to identify resistant/tolerant traits should screen wider *Brassica* germplasm, including wild species. Additionally, it will be particularly interesting to explore how *R. solani* overcomes OSR defences by examining the expression of a broader array of genes involved in plant defence mechanisms.

Chapter 1. *Rhizoctonia solani* AG 2-1 and *Brassica napus* – a review of current knowledge

This chapter presents a review of literature of *Rhizoctonia solani* AG 2-1 and oilseed rape, focusing on pathogen's global distribution, the available control methods the absence of resistance and AG 2-1 ability to manipulate plant defences. This chapter is a manuscript in preparation for publication.

1.1 Author contribution

This manuscript is composed and researched by F. Drizou. Editing and supervision guidance was provided by N. Graham and T. Bruce.

1.2 Abstract

Rhizoctonia solani is a globally distributed necrotrophic fungus with a wide range of crop-hosts including cereals, legumes and brassicas. *Brassica napus*, oilseed rape (OSR), is the primary host of *R. solani* anastomosis group (AG) 2-1. Infection by AG 2-1 causes seed decay and damping off disease in young seedlings which reduce crop establishment and yields. In our view, *R. solani* AG 2-1 due to its global distribution and prevalence, its saprophytic nature and its high virulence to OSR could be a potential threat for future epidemics. As there is no recent review of the *R. solani* AG 2-1 – OSR system, we provide here an update on knowledge of the global occurrence of the pathogen, the available control methods, the lack of genetic resistance in OSR and the mechanisms used by AG 2-1 to trigger plant defences.

Key words: *Rhizoctonia solani*, *Brassica napus*, control, resistance

1.3 Introduction

The *Brassicaceae* plant family includes some of the most important cultivated crops worldwide including *Brassica oleracea*, *Brassica rapa* and *Brassica napus*. The latter is a tetraploid species (AACC) and the result of crossing between *B. oleracea* (CC) and *B. rapa* (AA) (Mason and Snowdon, 2016). It is considered as a recent crop; its cultivation started in Europe and extended worldwide (Chalhoub et al., 2014). Today it is one of the most profitable and cultivated crops in Canada, China, India and EU (Carré and Pouzet, 2014, USDA, 2017). Oilseed rape has undergone intensive breeding for the elimination of eluric acid and glucosinolates (GSL), improvement of oil content and quality and disease resistance (Chalhoub et al., 2014). Advances in breeding and agronomy are expected to improve production and increase yields in the future (Hu et al., 2017). However, so far breeding strategies for disease management target specific plant pathogens without taking into account the potential risk of others that also occur in the field. In addition, the increasing global demand for food, the need to minimise the negative impact of intensive agriculture on the environment and climate changes have resulted in changes to crop management practices (Kremen and Miles, 2012, Stavi et al., 2016); these include alterations to crop rotations, choice of cultivars, tillage, fertilisation and pesticides application (Hannukkala et al., 2016, Kremen and Miles, 2012, Stavi et al., 2016). These changes can directly and indirectly affect the environment for many pathogens, leading to outbreaks of diseases that previously were not considered significant problems and can decrease productivity (Hannukkala et al., 2016).

1.4 *Rhizoctonia* spp.

The fungal complex of the *Rhizoctonia* genus comprises many important soil-borne necrotrophic plant pathogens worldwide. *Rhizoctonia* species are primarily allocated in three groups according to the number of nuclei per cell; the multinucleate *Rhizoctonia solani* Kühn with sexual morph *Thanatephorus cucumeris* Donk, the binucleate *Rhizoctonia cerealis* with sexual morph *Ceratobasidium cereale* and *Rhizoctonia oryzae* and *Rhizoctonia zeae*, both multinucleate with sexual morph in the genus Waitea (Ogoshi, 1987, Vilgalys and Cubeta, 1994). Moreover their pathogenicity, morphology and genetic

similarities lead to further classification into anastomosis groups (AG) based on their ability for hyphal fusion (Cubeta and Vilgalys, 1997, Vilgalys and Cubeta, 1994). *Rhizocotnia solani* consists of 13 AGs (AG 1 to AG 13) (Carling et al., 2002) and molecular methods have identified more subsets within AG 1, -2, -3 and -4 (Guillemaut et al., 2003, Stodart et al., 2007) (Figure 1.1).

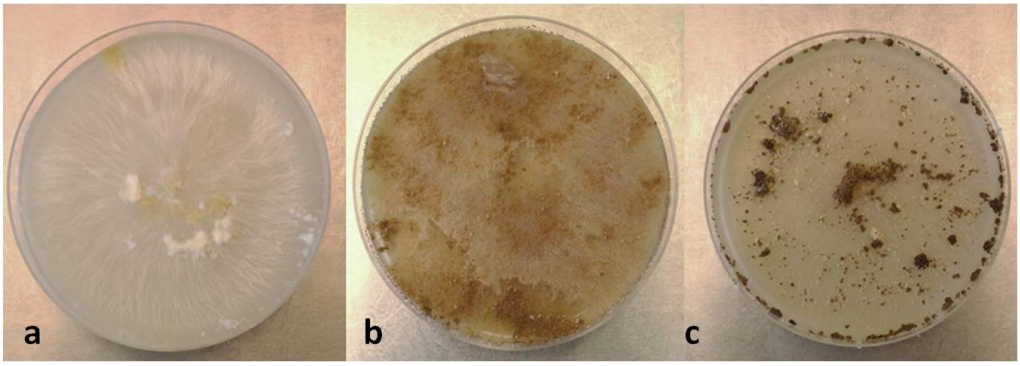


Figure 1.1 *Rhizoctonia* spp. colonies in petri dishes: (a) *R. cerealis*, (b) *R. solani* AG 4 and (c) *R. solani* AG 2-1.

Rhizoctonia solani host plants include many important agricultural crops such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), brassica species, sugar beet (*Beta vulgaris*), peas (*Pisum sativum* L. and other members in the Fabaceae family), cotton (*Gossypium* spp.), potato (*Solanum tuberosum*), corn (*Zea mays*) and soybean (*Glycine max*). However, there is variability in the pathogenicity of each AG to these crops, with some isolates more specialised to certain plant species while others have a wider range of hosts (Anderson, 1982). For example AG 8 is known to be mostly pathogenic to wheat and barley (Ogoshi et al., 1990) and AG 2-1 to brassicaceous species (Babiker et al., 2013), whereas AG 4 is more generalised with a wider range of hosts (Tomaso-Peterson and Trevathan, 2007). Despite the high virulence and specialisation of some AGs towards certain hosts, they can still infect other crops causing less severe symptoms if the plant is able to effectively defend against them. Pannecoucque et al., showed a difference between pathogenic and non-pathogenic AGs in their interaction with *B. oleracea*, driven by the effectiveness of basal plant defences (Pannecoucque and Hofte, 2009). The non-pathogenic interaction with AG 3 and AG 5 resulted in less severe disease compared to the pathogenic interactions with AG 2-1

(Pannecoucque and Hofte, 2009). The diversity of *R. solani* and its wide plant-host range in combination with its saprophytic nature, which enables its survival in crop residues, makes it an important crop pathogen. However, its presence in fields may not be immediately apparent due to its cryptic nature that does not result in visual symptoms in non host plants. In precise, the build-up of the pathogen may occur slowly over time due to changes in crop management and/or environmental factors but in the absence of the primary host plant infection could be asymptomatic (Hannukkala et al., 2016, Melzer et al., 2016). Crop losses can be high, for example, yield losses of marketable onion bulbs could be reduced by 25% to 60% due to stunting caused by *Rhizoctonia* complex including *R. solani* AG 3, AG 4, AG 2-1, AG 8, within diseased patches (Sharma-Poudyal et al., 2015). Infection of sugar beet with AG 2-2, negatively affects 5-10% of European and U.S. fields (Buttner et al., 2004) and *R. solani* infections in oilseed rape can result in significant losses as root rot can lead to 17% yield loss (in a single plant) which can further increase up to 65% if the roots are completely damaged compared to healthy plants or plants with low disease incidence (Klein-Gebbinck and Woods, 2002). In the present review we are interested in the interaction between AG 2-1 and *Brassica napus* (oilseed rape, OSR), its primary host.

1.5 *Rhizoctonia solani* AG 2-1

Rhizoctonia solani AG 2-1 is an asexual Basidiomycetes that does not form spores compared to its sexual morph *T. cucumeris*. It survives in soil, crop debris or/and on seeds, in the form of hyphae or sclerotia (survival structures consisted of a dense mass of harden hyphae). Infection process occurs as hyphae growing on plant tissues, adhere on the surface of plant stem and forms T-shaped branches, followed by the formation of infection cushions (Pannecoucque and Hofte, 2009). Penetration occurs soon after either by infection cushions or through stomata and within 3 days AG 2-1 colonises cortex and vascular tissues. During this process, AG 2-1 is able to strongly degrade pectin and alter plant cell walls (Pannecoucque and Hofte, 2009). AG 2-1 infects germinating seeds and young oilseed rape seedlings causing seed decay, pre- and post-emergence damping off (Agrios, 2005) (Figure 1.2).

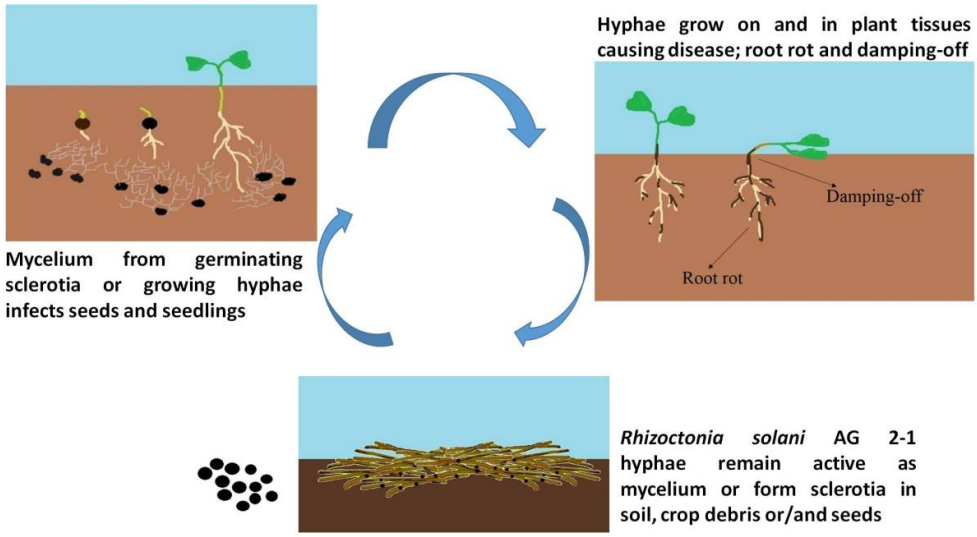


Figure 1.2. Life cycle of *Rhizoctonia solani* AG 2-1 on oilseed rape; pathogen remains as active hyphae or forms sclerotia in soil, crop debris and/or seeds. Under the presence of the host-plant and favourable environmental conditions, sclerotia germinate and hyphae penetrate plant tissues causing disease.

Characteristic symptoms of the disease are root rot and hypocotyl rot (damping-off), where dark brown lesions are formed on the hypocotyl of the young seedlings near the soil surface (Figure 1.3). Hypocotyls eventually become thinner, dry out and unable to support the seedling (Figure 1.3) (Agrios, 2005, Khangura et al., 1999, Lamichhane et al., 2017). *Rhizoctonia solani* AG 2-1 is favoured by cool temperatures during the establishment phase of the crop but during later growth stages and warmer temperatures AG 4 is also pathogenic to OSR causing stem rot (Yitbarek et al., 1988).

Figure 1.3 Damping-off disease on oilseed rape seedlings with the characteristic brown lesions on the hypocotyl near the soil surface, caused by *R. solani* AG 2-1 infection.

1.6 Geographical distribution of AG 2-1

Rhizoctonia solani AG 2-1 is known to be a cosmopolitan species, it has been isolated from fields in Canada, USA, Australia, Europe and Asia (Brown et al., 2014, Goll et al., 2014, Hannukkala et al., 2016, Jaaffar et al., 2016, Khangura

et al., 1999, Melzer et al., 2016, Tewoldemedhin et al., 2006, Zhang et al., 2016). Surveys of cereal fields, in Washington State (USA), between 2000 and 2011 showed that AG 2-1 was consistently the most frequent AG (32%) isolated. Although AG 8 was the second most isolated group (14%), it had considerable variation between the years surveyed compared to the consistency observed with AG 2-1 (Jaaffar et al., 2016). Extensive research has been carried out in Canada and studies from the 1980's identified AG 2-1 as the main AG in oilseed rape fields. Gugel et al., found that AG 2-1 represented the 92.8% of the *Rhizoctonia* spp. isolated from fields in Peach River Region during the summer of 1987 Gugel et al., 1987). Also, another study identified AG 2-1 (36%) to be the second most isolated group after AG 4 (53%) in oilseed rape fields in Saskatchewan during the summer of 1984 (Yitbarek et al., 1987). Additionally, surveys conducted in 2009-2011 in different crop fields in Canada identified AG 2-1 as the most frequent AG isolated in Western Canada (Melzer et al., 2016). Taken together, these studies indicate the stable occurrence of AG 2-1 in Canada where canola is a major crop grown. Moreover, Tewoldemedhin et al., also identified AG 2-1 as the second most isolated AG from crops in a rotational program between 2000 and 2003, in South Africa (Tewoldemedhin et al., 2006). Surveys in Europe have also revealed the presence of AG 2-1; Goll et al., found that AG 2-1 represented 7% of the *R. solani* isolates from soil samples collected in Germany, Poland and UK (Goll et al., 2014). Additionally, Brown et al., found AG 2-1 to be the most prevalent AG isolated from 69% of soil samples collected from wheat fields across the UK (Brown et al., 2014) and Hannukkala et al., also found that *R. solani* AG 2-1 is the most frequent isolated pathogen in OSR during the late 2000s compared to surveys during 1980s in Finland (Hannukkala et al., 2016). Recently Zhang et al., identified AG 2-1 as the main agent causing damping-off in oat (*Avena sativa*) seedlings in China (Zhang et al., 2016). These studies show that despite the differences of its occurrence between countries, AG 2-1 it is present in cultivated areas throughout the world.

1.7 Pathogenicity and epidemiology of AG 2-1

Pathogenicity studies have consistently confirmed that, although AG 2-1 is extremely aggressive to *B. napus* and other brassica species, it is also able to

infect and cause disease in other plant species. Meltzer et al., revealed that AG 2-1 isolates (isolated from OSR, pea and wheat seedlings) were pathogenic to lentil (*Lens culinaris*), pea, soybean and wheat (56%, 36%, 28% and 29% of AG 2-1 isolates respectively) (Melzer et al., 2016). Additionally, Tewoldemedhin et al., demonstrated that AG 2-1 infection caused damping-off not only in oilseed rape but also in medic (annual *Medicago* spp.), lupin (*Lupinus* spp.) and lucerne (*Medicago sativa* L.) (Tewoldemedhin et al., 2006). However, the virulence of AG 2-1 was different for other crops and only very low levels of damping-off were observed for clover (*Trifolium* spp.) , barley and wheat (Tewoldemedhin et al., 2006). Similarly, AG 2-1 has been shown to be pathogenic but is less virulent to wheat (Jaaffar et al., 2016, Sturrock et al., 2015). A factor that affects pathogenicity of AG 2-1 is the source of the isolate, with isolates from young seedlings known to be more aggressive to OSR than isolates from adult plants (Verma, 1996). In addition there is variability in the pathogenicity between isolates even within AG 2-1 and isolates from non-symptomatic plants in the field could be pathogenic and cause disease under control conditions to other crop hosts (Melzer et al., 2016). Considering the different epidemiological factors that influence disease severity, it is expected that there will be variation in pathogenicity between isolates of AG 2-1 and in their aggressiveness towards different hosts and different regions. Furthermore, due to its saprophytic capabilities and the formation of sclerotia in adverse environmental conditions AG 2-1 is capable of maintaining its presence in crop fields without being a significant problem to other non-host crops grown in the rotation, but when the primary plant host, OSR, is present it can cause severe disease, reducing crop establishment and yields.

1.8 Control methods

Due to the great variability in the *R. solani* complex, available control methods are not designed specifically for AG 2-1 and therefore here we discuss general control methods that are used. Control methods against *R. solani* aim to eliminate pathogen occurrence in the field or postpone infection of plants in the young seedling stage.

1.8.1 Chemical control

Chemical control is probably the most efficient available control method against *R. solani*. Seed treatments with fungicides are a preventative method that protect young seedlings against pre-emergence damping off leading to increased establishment (Kataria and Verma, 1992). Several broad spectrum fungicides are available to control *R. solani*, including sedaxane, metalaxyl, iprodione and carboxin (Cook, 2001, Lamichhane et al., 2017, Yang and Verma, 1992, Zeun et al., 2013). Sedaxane belongs to the class of fungicides known as succinate dehydrogenase inhibitors (SDHI). This class of fungicides, bind to the SDH complex and inhibits the action of enzymes interfering with the TCA cycle and respiration chain (Zeun et al., 2013). Sedaxane, have been shown to be effective against a range of *R. solani* AGs: isolates of different AGs collected from different soils across Europe were all found to be sensitive (*in vitro*) to sedaxane with EC_{50} values ranging between 0.001 and 0.093 p.p.m. indicating that sedaxane could be used for the control of multiple AGs (Goll et al., 2014). Additionally, *in vitro* experiments have demonstrated that different AGs of *R. solani*, isolated from soybean fields (from soybean and sugar beet plants), were extremely sensitive (EC_{50} values less than 1 p.p.m.) to penflufen and sedaxane, while they were extremely or moderately sensitive (EC_{50} values between 1 p.p.m. and 10 p.p.m.) to ipconazole and prothioconazole (DMI class of fungicides that interfere with the C14-demethylase, a vital ezyme for the biosynthesis of sterols in plasma membrane), indicating that SDHI fungicides are better for the control of *R. solani* (Ajayi-Oyetunde et al., 2017). Following on from this, positive results from the use of those four fungicides were also obtained from glasshouse experiments, with seed-treated soybean plants showing less disease compared to non-treated controls (Ajayi-Oyetunde et al., 2017). Although none of the seed-treatments completely controlled the disease in soybean, seed treatments can aid the establishment of the crop in the field despite the presence of the pathogen. Research has shown that a combination of fungicides with different modes of action gives better results in terms of seedling survival, emergence and reduced damping-off disease. Seed treatments with a combination of difenconazole, fludioxonil, metalaxyl-M and thiamethoxam or metalaxyl,

thiram and iprodione one day prior to sowing in inoculated medium (mix of soil, perlite and sand) have been shown to result in reduced disease incidence and improved oilseed rape survival and growth against AG 2-1 and AG 4 (Lamprecht et al., 2011). Xu et al., demonstrated that seed treated with metalaxyl alone was not sufficient to control AG 4 infection in soybean, but when combined with other fungicide chemicals (e.g. carbathin, fludioxonil, trifloxystrobin, HEC5725) both emergence and yield were improved compared to the untreated control (Xue et al., 2007). Nonetheless, the authors stated that none of the seed treatments provided the same emergence and yields as non-inoculated control treatment and that a more holistic approach that takes into account environmental conditions and agricultural practices is needed for the control of *R. solani* (Xue et al., 2007).

In addition to seed treatments, although rarely used, application of fungicide in the field can also control *R. solani* infection but its efficacy depends on epidemiological factors such as pathogen's spread which should define the site and method of application (Le Cointe et al., 2016). Considering that *R. solani* exists in the soil either as mycelia or sclerotia and targets host-plant's roots or seeds, general surface application of the fungicide will not be effective. Le Cointe et al., studied the chemical control of AG 4 in *Raphanus sativus* with pencycuron (a fungicide primary developed to have a selective mode of action to *R. solani* on rice and potato) and they found that the fungicide was not effectively controlling the pathogen if the latter was in close proximity to or had reached the plant (Le Cointe et al., 2016). However, localised application of pencycuron eliminated pathogen growth and the best approach to prevent infection was to apply the fungicide in thin strips between plants/seeds in order to protect the rhizosphere of the plant (Le Cointe et al., 2016).

Overall, chemical control of *R. solani* to prevent early infection is promising but factors such as the specificity of the AGs towards the crop host and the environmental conditions that exist in the field should be considered prior to application of any fungicide. However, chemical control should not be the sole method of controlling *R. solani* but part of an integrated control strategy that also includes cultural and biological practices.

1.8.2 Cultural control

Cultural practices such as soil tillage, fertilisation and sowing date are important factors that can alter the population dynamics of the pathogen in the field. Considering the saprophytic nature of *R. solani*, the presence of crop residues could be deleterious as they will enable pathogen survival and spread in the soil in the absence of a host (Papavizas et al., 1975). Tillage is another practice that influences the incidence and severity of *R. solani*, as the plant residues and the undisturbed soil enable the survival of the pathogen (Cook, 2001). Contrary to this, a recent interesting study found that the change from conventional or reduced tillage to no tillage in combination with fertilisation seemed to reduce the risk of *R. solani* disease in oilseed fields in Finland (Hannukkala et al., 2016). The authors however, stated that the higher risk of disease incidence was due to a combination of risk factors (Hannukkala et al., 2016). Fertilisation is considered as an essential cultural practice to manage damping-off disease (Lamichhane et al., 2017). Providing young seedlings with nutrients improves their emergence and growth and helps them to escape the pathogen infection (Lamichhane et al., 2017). It has also been found that direct application of nitrogen, phosphorus and sulphur beneath the seed is beneficial because it promotes the availability of nutrients to young seedlings lacking a developed root system (Cook, 2001, Lamichhane et al., 2017). Removal of the 'green bridge' (living plants between cultivations) is argued to be an efficient way to control *R. solani*, as it minimises the available living plant material that can serve as host for the pathogen (Cook, 2001). Nonetheless, considering the saprophytic nature of this pathogen, even with the removal of the 'green bridge' *R. solani* would still be present in the field.

Another factor that significantly influences disease incidence and is linked with disease escape, is seeding depth. Shallow seeding enables faster emergence and reduces pre-emergence damping-off compared to seeding in deeper soil layers that increases the exposure time of the seed/seedling to *R. solani* (Kharbanda and Tewari, 1996). Sowing OSR seeds into AG 2-1 inoculated soil mix at 3 cm depth reduced the emergence of seedlings by 58% compared to 15% at 2 cm, 12 % at 1 cm and the non-inoculated controls (79% at 3 and 2 cm and 85% at 1 cm), indicating that increased sowing depth

favours infection by AG 2-1 (Khangura et al., 1999). Moreover, shallow seeding in combination with fast growing cultivars could be very beneficial. It is known that AG 2-1 attacks OSR roots and significantly reduces their volume and surface area within 6 days which impairs the establishment of a strong root system and the acquisition of nutrients (Sturrock et al., 2015). The establishment of a long primary root in OSR is crucial as it is positively correlated with higher seed yields (Thomas et al., 2016b). Therefore, genotypes with faster growth of the primary root and more lateral roots will probably be able to escape AG 2-1 infection in the crucial seedling stage, without put at risk yields. A key factor is also the sowing date as it is linked with soil conditions such as temperature and moisture (Hannukkala et al., 2016). It is known that AG 2-1 is favoured by lower temperatures and during cool weather disease incidence can increase (Yitbarek et al., 1988) and therefore earlier sowing is expected to benefit crop establishment. However, as AG 4 prefers warmer temperatures for infection of OSR (Yitbarek et al., 1988), planting time decisions should be made carefully. Although there is no direct effect of soil moisture on AG 2-1 and seedling emergence and infection, increased soil moisture benefits disease occurrence in adult plants (Teo et al., 1988). Although it has been stated that crop rotations have a benefit and reduce soil-borne pathogens (Kharbanda and Tewari, 1996), in the case of *R. solani* they do not seem to be helpful due to their wide host range. As stated previously, despite AG 2-1 being very aggressive towards OSR, it is also pathogenic to other crops such as wheat, potatoes and peas that are used in crop rotations. Consequently, rotations are unable to control the pathogen population and they can actually benefit its build up in the field and even a long break with cereals are not sufficient; Hannukkala et al., demonstrated that a break with cereals for 4-6 years actually resulted in increased disease caused by AG 2-1 (Hannukkala et al., 2016).

1.8.3 Biological control

Over the last few decades interest in developing alternative control measures against pathogens has increased. This is not only due to the harmful effects of fungicides on the environment but also due to the ability of pathogens to evolve fungicide resistance. The genus *Trichoderma* consists of different

fungal species known to antagonise plant pathogens, directly attack them and also promote plant growth (Vinale et al., 2008). They produce a range of cell-wall degrading enzymes, lytic enzymes and secondary metabolites during mycoparasitim which negatively affect pathogens growth (Benitez et al., 2004, Karlsson et al., 2017, Vinale et al., 2008). Additionally, these properties are known to vary according to the *Trichoderma* species. Atanasova et al., showed that *Trichoderma* species differ in their strategies when counteracting *R. solani*; for example when *Trichoderma virens* and *Trichoderma atroviride* sense the presence of *R. solani*, genes related with attack are regulated, while *Trichoderma reesei* modifies gene expression for competition of nutrients (Atanasova et al., 2013). Additionally, differences are also observed between *T. virens* and *T. atroviride* with the first preparing for direct attack and poisoning the pathogen with the production of gliotoxin and the second exploiting a less aggressive tactic towards parasitism with elements of antibiosis and the use of hydrolytic enzymes (Atanasova et al., 2013). Moreover, the presence of *Trichoderma* in the field and the colonisation of the plants is known to enhance plant growth and performance through bio-fertilisation (Benitez et al., 2004). Currently several commercial products with *Trichoderma* are available across the world, however, as with other biocontrol agents, their effectiveness is variable and closely related to the interactions that are taking place within the soil environment (O'Brien, 2017, Vinale et al., 2008).

Another potential biocontrol agent is the non-pathogenic binucleate *Rhizoctonia*, which has been shown to reduce damping off and root rot, caused by AG 2-1 and AG 4, on oilseed rape (Verma, 1996). In addition, hypovirulent binucleate *Rhizoctonia* has been shown to induce systemic acquired resistance (SAR) and induce systemic resistance (ISR) in *Arabidopsis thaliana* plants against AG 4 infection, which resulted in a minor increase in plant protection (Sharon et al., 2011).

Plant members of the *Brassicaceae* family contain chemical compounds known as glucosinolates (GSL) that have a major role in plant defences (van Dam et al., 2009). The hydrolytic products of GSL such as isothiocyanates (ITC) have antimicrobial activity and inhibit pathogen growth. Incorporation

of plant material from brassica plants as well as seed meals in the soil enables the release of these compounds and can be used as an alternative control method termed biofumigation (Kirkegaard et al., 2000). In the case of *R. solani*, an in vitro study showed that hyphal growth was eliminated when exposed to allyl ITC from *Brassica juncea* seeds at concentrations 100-400 p.p.m. and repressed at higher concentrations (Chung et al., 2002). However, the authors did not observe a suppressive activity from the volatile compounds from *B. oleracea* and *Brassica campestris* seeds, indicating that there is probably a species specificity in their effectiveness against the pathogen (Chung et al., 2002). Moreover, variability exists within *R. solani*; Smith and Kirkegaard (Smith and Kirkegaard, 2002) found that *R. solani* exhibits an intraspecific variability, between different AGs, regarding its responses to 2-phenylethyl ITC in vitro and they suggest that we need to be careful when we target specific pathogens. Although, there is a clear indication from in vitro studies that there is a suppressive effect of GSL and their hydrolytic products, field studies indicate that the reality is more complicated. For example, oilseed rape seed meal increased *Streptomyces spp* communities and reduced apple root infection by *R. solani* AG 5 in treated soils compared to untreated, but had no effect on hyphal growth and the authors concluded that these modifications were not due to GSL content in the seed meal but from another unknown mechanism (Cohen et al., 2005). In addition, soils treated with seed meal from *B. juncea, B. napus and Sinapis alba* reduced root rot incidence on wheat caused by *R. solani* AG 8 compared to untreated soils (Handiseni et al., 2013). Although, the three plants varied in the amount of GSL they produced, all reduced AG 8 incidence in wheat, hence probably other mechanisms contributed to the suppression of AG 8 (Handiseni et al., 2013). Another approach of biofumigation and seed meal application is the use of ITC compounds in seed coating, aiming to eliminate the pathogen infection during seeding; Chung et al., showed that ground seed meal from *B. juncea* with the appropriate carrier (biolan peat B3 mix), improved the control of damping-off in *B. oleracea* caused by AG 4 (Chung et al., 2002). So far, it is clear that biofumigation approaches have potential in the biocontrol of *R. solani*, nonetheless further research focusing on the variation between GSL from

different brassica plants and the specificity of each AG is vital for the efficient control of specific AGs.

1.9 Resistance

All current control methods and practices only provide a partial solution to control damping-off disease in OSR caused by AG 2-1. They aim to promote the faster emergence and growth of seedlings by suppressing the fungal growth, however, even the most efficient chemical control does not provide complete control of the pathogen. Thus, identifying resistant OSR germplasm to AG 2-1 would probably be the most sustainable and effective way to control damping-off disease. Identifying resistance can be divided in two components: identifying genetic resistance (true resistance) and identifying 'escaping' traits (tolerance) to overcome the infection. More than 30 years have passed since the first published research aimed at identifying resistance in *B. napus* to AG 2-1 and yet no resistant germplasm has yet been identified. Acharya et al. (1984), were the first to screen *B. napus* lines for resistance to damping-off using AG 2-1. Among the 300 lines from *B. napus* and *B. campestris* that they screened during chamber experiments none were resistant, nevertheless they identified differences in their emergence and survival under control and field conditions. Overall they showed that there was no genetic resistance and variation existed even within the same plant genotype regarding response to AG 2-1 (Acharya et al., 1984).

For the identification of resistance traits and their integration through breeding, screening of other *Brassica* species is also important. Another study attempted to identify resistance from a range of lines from *B. napus, B. oleracea, B. rapa, B juncea, S. alba, Camelina sativa* and other related species; the authors estimated the resistance based on seedlings emergence and survival but all 122 genotypes were susceptible and only *S. alba* had better performance (Yang and Verma, 1992). Similarly to Acharya et al., they also identified differences in host-plant responses between and within the tested species including *B. napus* and *B. campestris* (Acharya et al., 1984). Furthermore, Babiker et al., tried to assess the responses of different genotypes of *B. napus, B. rapa, B. juncea, B. carinata, S. alba* and *C. sativa* to AG 2-1 but none of the genotypes were

resistant; survival of seedlings, shoot length and fresh weight were significantly reduced compared to the control seedlings (Babiker et al., 2013). Yet they were able to identify tolerance to AG 8 infection in some genotypes and through heritability experiments found that resistance could be improved (Babiker et al., 2013). This is also in correlation with Khangura et al., as they also found that progenies of AG 2-1 inoculated asymptomatic (or with few lesions) plants performed better than their parents (Khangura et al., 1999). Although, these are promising findings it is evident that the lack of genetic resistance remains as a major problem for controlling this pathogen. In the absence of suitable resistance traits in readily accessible germplasm, conventional breeding techniques may not be enough and transgenic approaches or ancestral introgression lines may be required.

1.10 Plant defences

An alternative approach to elucidate the lack of resistance and develop efficient control methods would be to understand the plant-host and pathogen interaction at the molecular level and identify how OSR interacts with AG 2-1. Considering the necrotrophic lifestyle of this pathogen, the understanding of the early stages of infection/interaction are important as they will help to prevent the colonisation by *R. solani* (Okubara et al., 2014). Unfortunately, so far it is not clear how AG 2-1 overcomes/manipulates plant defences and it seems that different mechanisms are used by AG 2-1 compared to other AGs. Studies in *Arabidopsis* showed that different AG induce different plant responses: Perl-Treves et al., indicated induction of glutathione *S*-transferase *GSTF8* gene resulted from the infection of AG 8 but not from AG 2-1, which is very aggressive to *Arabidopsis* (Perl-Treves et al., 2004). The last observation together with the different infection structures that AG 2-1 used during colonisation indicated that this AG is capable of repressing *Arabidopsis's* defence mechanisms (Perl-Treves et al., 2004). Additionally Foley et al., compared AG 8 and AG 2-1 induced defences in different *Arabidopsis* ecotypes; the two AGs differently regulated the expression of several plant-defence related genes with three *PR* genes to show a two-fold induction by AG 2-1 and oxidases to be exclusively induced by AG 8 (Foley et al., 2016). Furthermore, probably the most interesting outcome of their work

was that resistance of *Arabidopsis* to AG 8 was linked with NAPDH oxidases while AG 2-1 through an unknown mechanism is able to overcome or suppress them (Foley et al., 2016). The role of the major plant defence hormones salicylic acid (SA) and jasmonic acid (JA) are also unclear. It is generally known that upon necrotrophic fungi attack, JA related defences are induced whilst SA is related with biotrophic fungi (Glazebrook, 2005). When different *Arabidopsis* mutants for JA, SA, ET (ethylene) and ABA (abscisic acid) were used for the evaluation of the role of those hormones against AG 8 and AG 2-1, it was revealed that they do not have a significant role in defences against these AGs, at least individually (Foley et al., 2016). Moreover, the expression of the Germin-Like Protein gene from sugar beet (*BvGLP-1*) in Arabidopsis resulted in the increase of both H_2O_2 and different marker genes related with SA and JA triggered plant defences. Additionally, in the same study expression of *BvGLP-1* conferred resistance of the transgenic plants to AG 2-1, measured as reduced root colonisation and smaller and fewer lesions on the leaves compared to wild type plants (Knecht et al., 2010). Overall the authors concluded that *BvGLP-1* has an important role in plant defences of *Arabidopsis* against AG 2-1 during the early stages of infection (Knecht et al., 2010). Generally it seems that AG 2-1 has developed an advanced strategy to hijack plant defences in OSR probably via regulation of various genes in both JA and SA pathways. Okubara et al., reviewed the genetic basis of the interaction between *R. solani* and different host plants (rice, wheat and potato) and they concluded that considering the complexity of *Rhizoctonia* spp. and the different pathogenicity mechanisms exploited by the pathogen, it is expected that various components and genes are required for resistance (Okubara et al., 2014). Unfortunately, currently we are lacking a better insight in the molecular aspects of the interaction between AG 2-1 and OSR, hence more work to emphasize towards this direction is needed. Additionally, although *Arabidopsis* as a model plant is a great tool to explore the molecular basis of the interaction with AG 2-1, it is important to study the response of *B. napus* since the pathogen seems to use different strategies towards different hosts.

1.11 Conclusion

In this review we provide an account of current knowledge regarding the interaction between *R. solani* AG 2-1 and OSR and current control strategies. The pathogen has a worldwide distribution but does not appear always to cause a major problem in every region where it is detected. Nonetheless, alterations in agriculture and crop management, including cultivated crops, tillage practices, crop rotation systems and fertilisation application, can change its prevalence in fields with detrimental effects to OSR. Currently, early infection of OSR seedlings could be prevented to some extent with seed treatments and cultural practices, whereas biofumigation and biocontrol methods need further investigation for this pathogen. However, the lack of genetic resistance as well as lack of knowledge of the molecular aspects of the interactions are two major constrains for the control of AG 2-1 and future research should aim to clarify why AG 2-1 is so specialised in *B. napus* and which are the mechanisms used to supress plant defence mechanisms.

1.12 Abbreviations

OSR: oilseed rape, AG: anastomosis group, GSL: glucosinolates, ITC: isothiocyanates, SA: salicylic acid, JA: jasmonic acid, ET: ethylene, ABA: abscisic acid, SDHI: succinate dehydrogenase inhibitors, DMI: demethylation inhibitors

1.13 References

ACHARYA, S. N., VERMA, P. R., DUECK, J. & DOWNEY, R. K. 1984. Screening rapeseed/canola for resistance to damping-off and seedling root rot caused by *Rhizoctonia solani. Canadian Journal of Plant Pathology,* 6**,** 325-328.

AGRIOS, G. N. 2005. *Plant Pathology*, Elsevier Academic Press.

AJAYI-OYETUNDE, O. O., BUTTS-WILMSMEYER, C. J. & BRADLEY, C. A. 2017. Sensitivity of *Rhizoctonia solani* to Succinate Dehydrogenase Inhibitor and Demethylation Inhibitor Fungicides. *Plant Disease,* 101**,** 487-495.

ANDERSON, N. A. 1982. The Genetics and Pathology of *Rhizoctonia Solani*. *Annual Review of Phytopathology,* 20**,** 329-347.

ATANASOVA, L., LE CROM, S., GRUBER, S., COULPIER, F., SEIDL-SEIBOTH, V., KUBICEK, C. P. & DRUZHININA, I. S. 2013. Comparative transcriptomics reveals different strategies of Trichoderma mycoparasitism. *Bmc Genomics,* 14**,** 15.

BABIKER, E., HULBERT, S., SCHROEDER, K. & PAULITZ, T. 2013. Evaluation of Brassica species for resistance to *Rhizoctonia solani* and binucleate *Rhizoctonia* (*Ceratobasidum* spp.) under controlled environment conditions. *European Journal of Plant Pathology,* 136**,** 763-772.

BENITEZ, T., RINCON, A. M., LIMON, M. C. & CODON, A. C. 2004. Biocontrol mechanisms of Trichoderma strains. *International Microbiology,* 7**,** 249-260.

BROWN, M., WOODHALL, J., MOONEY, S. & RAY, R. The occurrence and population dynamics of *Rhizoctonia solani* in soil of winter wheat. The Dundee Conference. Crop Protection in Northern Britain 2014, Dundee, UK, 25-26 February 2014, 2014. The Association for Crop Protection in Northern Britain, 107-112.

BUTTNER, G., PFAHLER, B. & MARLANDER, B. 2004. Greenhouse and field techniques for testing sugar beet for resistance to *Rhizoctonia* root and crown rot. *Plant Breeding,* 123**,** 158-166.

CARLING, D. E., BAIRD, R. E., GITAITIS, R. D., BRAINARD, K. A. & KUNINAGA, S. 2002. Characterization of AG-13, a newly reported anastomosis group of *Rhizoctonia solani*. *Phytopathology,* 92**,** 893-899.

CARRÉ, P. & POUZET, A. 2014. Rapeseed market, worldwide and in Europe. *OCL,* 21**,** D102.

CHALHOUB, B., DENOEUD, F., LIU, S., PARKIN, I. A. P., TANG, H., WANG, X., CHIQUET, J., BELCRAM, H., TONG, C., SAMANS, B., CORRÉA, M., DA SILVA, C., JUST, J., FALENTIN, C., KOH, C. S., LE CLAINCHE, I., BERNARD, M., BENTO, P., NOEL, B., LABADIE, K., ALBERTI, A., CHARLES, M., ARNAUD, D., GUO, H., DAVIAUD, C., ALAMERY, S., JABBARI, K., ZHAO, M., EDGER, P. P., CHELAIFA, H., TACK, D., LASSALLE, G., MESTIRI, I., SCHNEL, N., LE PASLIER, M.-C., FAN, G., RENAULT, V., BAYER, P. E., GOLICZ, A. A., MANOLI, S., LEE, T.-H., THI, V. H. D., CHALABI, S., HU, Q., FAN, C., TOLLENAERE, R., LU, Y., BATTAIL, C., SHEN, J., SIDEBOTTOM, C. H. D., WANG, X., CANAGUIER, A., CHAUVEAU, A., BÉRARD, A., DENIOT, G., GUAN, M., LIU, Z., SUN, F., LIM, Y. P., LYONS, E., TOWN, C. D., BANCROFT, I., WANG, X., MENG, J., MA, J., PIRES, J. C., KING, G. J., BRUNEL, D., DELOURME, R., RENARD, M., AURY, J.-M., ADAMS, K. L., BATLEY, J., SNOWDON, R. J., TOST, J., EDWARDS, D., ZHOU, Y., HUA, W., SHARPE, A. G., PATERSON, A. H., GUAN, C. & WINCKER, P. 2014. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. *Science,* 345**,** 950-953.

CHUNG, W. C., HUANG, J. W., HUANG, H. C. & JEN, J. F. 2002. Effect of ground *Brassica* seed meal on control of *Rhizoctonia* damping-off of cabbage. *Canadian Journal of Plant Pathology,* 24**,** 211-218.

COHEN, M. F., YAMASAKI, H. & MAZZOLA, M. 2005. *Brassica napu*s seed meal soil amendment modifies microbial community structure, nitric oxide production and incidence of *Rhizoctonia* root rot. *Soil Biology & Biochemistry,* 37**,** 1215-1227.

COOK, R. J. 2001. Management of wheat and barley root diseases in modern farming systems. *Australasian Plant Pathology,* 30**,** 119-126.

CUBETA, M. A. & VILGALYS, R. 1997. Population biology of the *Rhizoctonia solani* complex. *Phytopathology,* 87**,** 480-484.

FOLEY, R. C., KIDD, B. N., HANE, J. K., ANDERSON, J. P. & SINGH, K. B. 2016. Reactive Oxygen Species Play a Role in the Infection of the Necrotrophic Fungi, *Rhizoctonia solani* in Wheat. *Plos One,* 11.

GLAZEBROOK, J. 2005. Contrasting Mechanisms of Defense Against Biotrophic and Necrotrophic Pathogens. *Annual Review of Phytopathology,* 43**,** 205-227.

GOLL, M. B., SCHADE-SCHUTZE, A., SWART, G., OOSTENDORP, M., SCHOTT, J. J., JASER, B. & FELSENSTEIN, F. G. 2014. Survey on the prevalence of *Rhizoctonia* spp. in European soils and determination of the baseline sensitivity towards sedaxane. *Plant Pathology,* 63**,** 148-154.

GUILLEMAUT, C., EDEL-HERMANN, V., CAMPOROTA, P., ALABOUVETTE, C., RICHARD-MOLARD, M. & STEINBERG, C. 2003. Typing of anastomosis groups of *Rhizoctonia solani* by restriction analysis of ribosomal DNA. *Canadian Journal of Microbiology,* 49**,** 556-568.

HANDISENI, M., BROWN, J., ZEMETRA, R. & MAZZOLA, M. 2013. Effect of *Brassicaceae* seed meals with different glucosinolate profiles on *Rhizoctonia* root rot in wheat. *Crop Protection,* 48**,** 1-5.

HANNUKKALA, A. O., RASTAS, M., LAITINEN, P. & LATVALA, S. 2016. *Rhizoctonia solani* injuries in oilseed crops in Finland and impacts of different crop management practices on disease incidence and severity. *Annals of Applied Biology,* 169**,** 257-273.

HU, Q., HUA, W., YIN, Y., ZHANG, X., LIU, L., SHI, J., ZHAO, Y., QIN, L., CHEN, C. & WANG, H. 2017. Rapeseed research and production in China. *The Crop Journal,* 5**,** 127-135.

JAAFFAR, A. K. M., PAULITZ, T. C., SCHROEDER, K. L., THOMASHOW, L. S. & WELLER, D. M. 2016. Molecular Characterization, Morphological Characteristics, Virulence, and Geographic Distribution of *Rhizoctonia* spp. in Washington State. *Phytopathology,* 106**,** 459-473.

KARLSSON, M., ATANASOVA, L., JENSEN, D. F. & ZEILINGER, S. 2017. Necrotrophic Mycoparasites and Their Genomes. *Microbiology Spectrum,* 5**,** 21.

KATARIA, H. R. & VERMA, P. R. 1992. *Rhizoctonia solani* damping-off and root rot in oilseed rape and canola. *Crop Protection,* 11**,** 8-13.

KHANGURA, R. K., BARBETTI, M. J. & SWEETINGHAM, M. W. 1999. Characterization and pathogenicity of *Rhizoctonia* species on canola. *Plant Disease,* 83**,** 714-721.

KHARBANDA, P. D. & TEWARI, J. P. 1996. Integrated management of canola diseases using cultural methods. *Canadian Journal of Plant Pathology,* 18**,** 168-175.

KIRKEGAARD, J. A., SARWAR, M., WONG, P. T. W., MEAD, A., HOWE, G. & NEWELL, M. 2000. Field studies on the biofumigation of take-all by *Brassica* break crops. *Australian Journal of Agricultural Research,* 51**,** 445-456.

KLEIN-GEBBINCK, H. W. & WOODS, D. L. 2002. Yield Loss Assessment in Canola: Effects of Brown Girdling Root Rot and Maggot Damage on Single Plant Yield. *Plant Disease,* 86**,** 1005-1010.

KNECHT, K., SEYFFARTH, M., DESEL, C., THURAU, T., SHERAMETI, I., LOU, B. G., OELMULLER, R. & CAI, D. G. 2010. Expression of BvGLP-1 Encoding a Germin-Like Protein from Sugar Beet in *Arabidopsis thaliana* Leads to Resistance Against Phytopathogenic Fungi. *Molecular Plant-Microbe Interactions,* 23**,** 446-457.

KREMEN, C. & MILES, A. 2012. Ecosystem Services in Biologically Diversified versus Conventional Farming Systems: Benefits, Externalities, and Trade-Offs. *Ecology and Society,* 17**,** 25.

LAMICHHANE, J. R., DURR, C., SCHWANCK, A. A., ROBIN, M. H., SARTHOU, J. P., CELLIER, V., MESSEAN, A. & AUBERTOT, J. N. 2017. Integrated management of damping-off diseases. A review. *Agronomy for Sustainable Development,* 37**,** 25.

LAMPRECHT, S. C., TEWOLDEMEDHIN, Y. T., CALITZ, F. J. & MAZZOLA, M. 2011. Evaluation of strategies for the control of canola and lupin seedling diseases caused by *Rhizoctonia* anastomosis groups. *European Journal of Plant Pathology,* 130**,** 427-439.

LE COINTE, R., SIMON, T. E., DELARUE, P., HERVE, M., LECLERC, M. & POGGI, S. 2016. Reducing the Use of Pesticides with Site-Specific Application: The Chemical Control of *Rhizoctonia solani* as a Case of Study for the Management of Soil- Borne Diseases. *Plos One,* 11**,** 18.

MASON, A. S. & SNOWDON, R. J. 2016. Oilseed rape: learning about ancient and recent polyploid evolution from a recent crop species. *Plant Biology,* 18**,** 883-892.

MELZER, M. S., YU, H., LABUN, T., DICKSON, A. & BOLAND, G. J. 2016. Characterization and pathogenicity of *Rhizoctonia* spp. from field crops in Canada. *Canadian Journal of Plant Pathology,* 38**,** 367-374.

O'BRIEN, P. A. 2017. Biological control of plant diseases. *Australasian Plant Pathology,* 46**,** 293-304.

OGOSHI, A. 1987. Ecology and pathogenicity of anastomosis groups of *Rhizoctonia solani* Kühn *Annual Review of Phytopathology,* 25**,** 125-143.

OGOSHI, A., COOK, R. J. & BASSETT, E. N. 1990. *Rhizoctonia* species and anastomosis groups causing root-rot of wheat and barley in Pacific-Northwest *Phytopathology,* 80**,** 784-788.

OKUBARA, P. A., DICKMAN, M. B. & BLECHL, A. E. 2014. Molecular and genetic aspects of controlling the soilborne necrotrophic pathogens *Rhizoctonia* and *Pythium*. *Plant Science,* 228**,** 61-70.

PANNECOUCQUE, J. & HOFTE, M. 2009. Interactions between cauliflower and *Rhizoctonia* anastomosis groups with different levels of aggressiveness. *Bmc Plant Biology,* 9**,** 12.

PAPAVIZAS, G. C., ADAMS, P. B., LUMSDEN, R. D., LEWIS, J. A., DOW, R. L., AYERS, W. A. & KANTZES, J. G. 1975. Ecology and Epidemiology of *Rhizoctonia solani* in Field Soil. *Phytopathology,* 65**,** 871-877.

PERL-TREVES, R., FOLEY, R. C., CHEN, W. Q. & SINGH, K. B. 2004. Early induction of the Arabidopsis GSTF8 promoter by specific strains of the fungal pathogen *Rhizoctonia solani*. *Molecular Plant-Microbe Interactions,* 17**,** 70-80.

SHARMA-POUDYAL, D., PAULITZ, T. C. & DU TOIT, L. J. 2015. Stunted Patches in Onion Bulb Crops in Oregon and Washington: Etiology and Yield Loss. *Plant Disease,* 99**,** 648-658.

SHARON, M., FREEMAN, S. & SNEH, B. 2011. Assessment of resistance pathways induced in *Arabidopsis thaliana* by hypovirulent *Rhizoctonia* spp. isolates. *Phytopathology,* 101**,** 828-38.

SMITH, B. J. & KIRKEGAARD, J. A. 2002. In vitro inhibition of soil microorganisms by 2-phenylethyl isothiocyanate. *Plant Pathology,* 51**,** 585-593.

STAVI, I., BEL, G. & ZAADY, E. 2016. Soil functions and ecosystem services in conventional, conservation, and integrated agricultural systems. A review. *Agronomy for Sustainable Development,* 36**,** 12.

STODART, B. J., HARVEY, P. R., NEATE, S. M., MELANSON, D. L. & SCOTT, E. S. 2007. Genetic variation and pathogenicity of anastomosis group 2 isolates of *Rhizoctonia solani* in Australia. *Mycological Research,* 111**,** 891-900.

STURROCK, C. J., WOODHALL, J., BROWN, M., WALKER, C., MOONEY, S. J. & RAY, R. V. 2015. Effects of damping-off caused by *Rhizoctonia solani* anastomosis group 2-1 on roots of wheat and oil seed rape quantified using X-ray Computed Tomography and real-time PCR. *Frontiers in Plant Science,* 6.

TEO, B. K., YITBAREK, S. M., VERMA, P. R. & MORRALL, R. A. A. 1988. Influence of soil moisture, seeding date, and *Rhizoctonia solani* isolates (AG 2-1 and AG 4) on disease incidence and yield in canola. *Canadian Journal of Plant Pathology,* 10**,** 151-158.

TEWOLDEMEDHIN, Y. T., LAMPRECHT, S. C., MCLEOD, A. & MAZZOLA, M. 2006. Characterization of *Rhizoctonia* spp. Recovered from Crop Plants Used in Rotational Cropping Systems in the Western Cape Province of South Africa. *Plant Disease,* 90**,** 1399-1406.

THOMAS, C. L., GRAHAM, N. S., HAYDEN, R., MEACHAM, M. C., NEUGEBAUER, K., NIGHTINGALE, M., DUPUY, L. X., HAMMOND, J. P., WHITE, P. J. & BROADLEY, M. R. 2016. High-throughput phenotyping (HTP) identifies seedling root traits linked to

variation in seed yield and nutrient capture in field-grown oilseed rape (*Brassica napus* L.). *Annals of Botany*.

TOMASO-PETERSON, M. & TREVATHAN, L. E. 2007. Characterization of *Rhizoctonia*-like fungi isolated from agronomic crops and turfgrasses in Mississippi. *Plant Disease,* 91**,** 260-265.

USDA, N. A. S. S. 2017. *Crop Production Historical Track Records* [Online]. United States Department of Agriculture. Available: http://usda.mannlib.cornell.edu/usda/current/htrcp/htrcp-04-13-2017.pdf [Accessed].

VAN DAM, N. M., TYTGAT, T. O. G. & KIRKEGAARD, J. A. 2009. Root and shoot glucosinolates: a comparison of their diversity, function and interactions in natural and managed ecosystems. *Phytochemistry Reviews,* 8**,** 171-186.

VERMA, P. R. 1996. Biology and control of *Rhizoctonia solani* on rapeseed: A review. *Phytoprotection,* 77**,** 99-111.

VILGALYS, R. & CUBETA, M. A. 1994. Molecular Systematics and Population Biology of *Rhizoctonia*. *Annual Review of Phytopathology,* 32**,** 135-155.

VINALE, F., SIVASITHAMPARAM, K., GHISALBERTI, E. L., MARRA, R., WOO, S. L. & LORITO, M. 2008. *Trichoderma*–plant–pathogen interactions. *Soil Biology and Biochemistry,* 40**,** 1-10.

XUE, A. G., COBER, E., MORRISON, M. J., VOLDENG, H. D. & MA, B. L. 2007. Effect of seed treatments on emergence, yield, and root rot severity of soybean under *Rhizoctonia solani* inoculated field conditions in Ontario. *Canadian Journal of Plant Science,* 87**,** 167-174.

YANG, J. & VERMA, P. R. 1992. Screening genotypes for resistance to pre-emergence damping-off and postemergence seedling root rot of oilseed rape and canola caused by *Rhizoctonia solani* AG-2-1. *Crop Protection,* 11**,** 443-448.

YITBAREK, S. M., VERMA, P. R., GUGEL, R. K. & MORRALL, R. A. A. 1988. Effect of soil-temperature and inoculum density on pre-emergence damping-off of canola caused by *Rhizoctonia solani Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie,* 10**,** 93-98.

YITBAREK, S. M., VERMA, P. R. & MORRALL, R. A. A. 1987. Anastomosis groups, pathogenicity, and specificity of *Rhizoctonia solani* isolates from seedling and adult rapeseed/canola plants and soils in Saskatchewan. *Canadian Journal of Plant Pathology*, 9**,** 6-13.

ZEUN, R., SCALLIET, G. & OOSTENDORP, M. 2013. Biological activity of sedaxane a novel broad-spectrum fungicide for seed treatment. *Pest Management Science*, 69**,** 527-534.

ZHANG, X. Y., HUO, H. L., WANG, W., ZHOU, H. Y., DONG, B. Z. & ZHAO, G. Q. 2016. First Report of Damping-off and Seedling Blight on Oat Caused by *Rhizoctonia solani* AG 2-1 in China. *Plant Disease*, 100**,** 653-654.

Chapter 2. **Development of high-throughput methods to screen disease caused by *Rhizoctonia solani* AG 2-1 in oilseed rape**

This chapter presents the development and comparison of four high-throughput screening methods and initial screening of commercial oilseed rape cultivars. It is published in Plant Methods.

2.1 Author contribution:

The experiments were designed by F. Drizou with the contribution of R. Ray and N. Graham. Experiments were performed by F. Drizou. Data analysed by F. Drizou with the contribution of R. Ray, production of figures and tables was performed by F. Drizou with the contribution of R. Ray and N. Graham. The manuscript was written by F. Drizou with the contribution of R. Ray, N. Graham and T. Bruce. All authors have read and approved the manuscript.

Note: The work in this chapter was performed simultaneously or after the screening in chapter 3.

2.2 Abstract

Rhizoctonia solani (Kühn) is a soil-borne, necrotrophic fungus causing damping off, root rot and stem canker in many cultivated plants worldwide. Oilseed rape (OSR, *Brassica napus*) is the primary host for anastomosis group (AG) 2-1 of *R. solani* causing pre- and post-emergence damping-off resulting in death of seedlings and impaired crop establishment. Presently, there are no known resistant OSR genotypes and the main methods for disease control are fungicide seed treatments and cultural practices. The identification of sources of resistance for crop breeding is essential for sustainable management of the disease. However, a high-throughput, reliable screening method for resistance traits is required. The aim of this work was to develop a low cost, rapid screening method for disease phenotyping and identification of resistance traits.

Four growth systems were developed and tested: 1. nutrient media plates, 2. compost trays, 3. Light Expanded Clay Aggregate (LECA) trays, and 4. a hydroponic pouch and wick system. Seedlings were inoculated with virulent AG 2-1 to cause damping-off disease and grown for a period of 4-10 days. Visual disease assessments were carried out or disease was estimated through image analysis using Image J.

Inoculation of LECA was the most suitable method for phenotyping disease caused by *R. solani* AG 2-1 as it enabled the detection of differences in disease severity among OSR genotypes within a short time period whilst allowing measurements to be conducted on whole plants. This system is expected to facilitate identification of resistant germplasm.

Keywords: *Rhizoctonia solani*, oilseed rape, high-throughput phenotyping, disease, plant characteristics

2.3 Introduction

Rhizoctonia solani (Kühn) [teleomorph *Thanatephorus cucumeris* (Donk)] is a necrotrophic soil-borne fungus belonging to the phylum Basidiomycota. The species is sub-divided into anastomosis groups (AG) based on genetic and biological characteristics, as well as host-specific pathogenicity (Anderson, 1982, Ogoshi, 1987). Among the groups, AG 2-1 is the most destructive to oilseed rape (OSR, *Brassica napus*) and other members of the Brassicaceae (Babiker et al., 2013, Yang and Verma, 1992). Under favourable temperatures, ranging from 18 to 20^{o}C, moist soil conditions and in the presence of the host, the growing hyphae infect young OSR seedlings causing pre- and post-emergence damping-off and root rot (Acharya et al., 1984, Kataria and Verma, 1992, Yang and Verma, 1992). Damping-off is characterised by the formation of brown lesions and eventually rotting of the hypocotyl (Khangura et al., 1999). The infection can also result in root rot and stem rot in older plants (Khangura et al., 1999, Verma, 1996). *Brassica napus* is a widely cultivated crop for oil production for human consumption and biodiesel, as well as for animal fodder. It is an amphiploid species derived from the crossing of *Brassica rapa* and *Brassica oleracea* and has undergone breeding for the optimisation of oil production and yields (Allender and King, 2010). Although many studies have attempted to identify resistant or tolerant genotypes of *B. napus* and related species, currently there are no known resistant OSR genotypes to AG 2-1 (Acharya et al., 1984, Babiker et al., 2013). Babiker et al., assessed the survival of 85 genotypes of *B. napus* and other *Brassica* species four weeks after sowing in inoculated soil (Babiker et al., 2013). Their results showed that all genotypes were susceptible, the majority of seedlings died and only 18 genotypes survived with survival rates ranging from 8.3% to 88.3% (Babiker et al., 2013).

The pathogen can be partially controlled using seed treatments prior to sowing (Lamprecht et al., 2011) and via cultural practices (Verma, 1996, Yang and Verma, 1992). However, these control measures only reduce the inoculum in the soil and thus delay the infection. The use of biofumigation and seed meals, from Brassicaceous plants, that usually suppress soil-borne pathogens (Cohen et al., 2005, Handiseni et al., 2013) or the application of beneficial biological

control organisms such as *Trichoderma* and binucleate *Rhizoctonia* (Verma, 1996), are not effective against *R. solani* AG 2-1. Consequently, the identification of traits and genes associated with resistance to *R. solani* AG 2-1 is an essential step towards the development of sustainable integrative control strategies for this pathogen.

An important factor in developing a method is to consider the epidemiology of the pathogen and the specificity of the pathosystem. In the case of *R. solani* and *B. napus* seed germination, emergence and survival under inoculated conditions can potentially reveal phenotypic differences among genotypes that play a role in susceptibility or resistance towards AG 2-1. The developmental rate of genotypes is likely to influence disease outcome (Kataria and Verma, 1992, Verma, 1996), therefore plants that emerge faster are expected to perform better. Additionally, plant characteristics such as hypocotyl length and root architecture may explain the ability of certain genotypes to escape infection. Furthermore, the progress of disease as well as its severity in different plant organs could potentially indicate genetic differences among different genotypes. At present the most popular method to assess disease severity and classify different genotypes and plant species to their susceptibility to *R. solani* is using pots with soil or soil-free media (Babiker et al., 2013, Khangura et al., 1999, Yang and Verma, 1992). Although screening in soil is realistic and provides an ideal environment for the fungi, it is time consuming, labour intensive and requires extensive controlled environment space. This limits the number of plants that can be screened quickly and cheaply. Another major bottleneck in identification of resistance to soil-borne pathogens, apart from the time and space required when using inoculated soil or compost to cause disease, is the uncertainty and/or reproducibility of moderate disease on which to detect consistent differences between genotypes.

The aim of the present work was to develop a low cost, rapid and high-throughput method to enable the screening of OSR genotypes for identification of *R. solani* AG 2-1 resistance. Four different methods were tested: media nutrient plates, hydroponic growth in pouches and growth in trays with compost or Light Expanded Clay Aggregate (LECA). The methods were evaluated to screen disease and/or assess plant physiological characteristics

within a short period of time during the early stages of infection among different OSR genotypes.

2.4 Methods

2.4.1 Inoculum and seeds

Rhizoctonia solani AG 2-1 (#1934 from the University of Nottingham isolate collection), originally isolated from OSR plants, was used to produce inoculum. The pathogenicity of this isolate to OSR was previously confirmed by Sturrock et al. (2015). The inoculum was grown on Potato Glucose Agar (PGA; Sigma-Aldrich, UK) at room temperature (18-20^{o}C) for a period of 10 to 14 days prior to the inoculation. In order to exclude contamination by other pathogens and ensure their germination, seeds were surface sterilised with 4% sodium hypochlorite (Parazone, Jeyes Limited, UK) for 5 min followed by three rinses with distilled autoclaved water and then pre-germinated on round filter paper (diameter 85 mm, GE Healthcare Whatman, UK) with 3 ml of sterile water and kept in dark at room temperature (18-20^{o}C) for 2 days. A group of eight *B. napus* genotypes, not previously tested for AG 2-1 resistance, was used for the evaluation of the methods to evaluate their performance against AG2-1. The group consisted of seven commercial winter oilseed cultivars 'Temple'(conventional), 'Abaco'(conventional), 'Lioness'(conventional), 'Grizzly'(conventional), 'Galileo'(conventional), 'Sequoia'(semi-dwarf hybrid) and 'ES Betty'(restored hybrid) and one fodder type ('Canard').

2.4.2 Nutrient media plates

Square petri dishes-plates (120 x 120 x 17 mm Greiner Bio-One International) were filled with sterile 50% Hoagland No. 2 Basal Salt Mixture (Sigma-Aldrich, UK), pH= 5.8 and 1% w/v agar (Agar-Agar granular powder, Fisher Scientific, UK). On each plate 3 seedlings of each genotype were placed 2 cm from the top of the plate with equal distances between them. For the inoculation, 1 plug (5 x 5 mm) of *R. solani* AG 2-1 from a colony growing on PGA was placed below each seed and 1 cm above the bottom of the plate (Figure 2.1). The control plates were not inoculated. Inoculated and control plates were sealed with parafilm and kept in an upright position in a controlled

environment room at 18ºC and 12 h light: 12 h dark. Photosynthetically active radiation (PAR) was 218.5 μmol s^{-1} m^{-2} at a height of 4 cm (LI-250A light meter, LI-COR Biosciences).

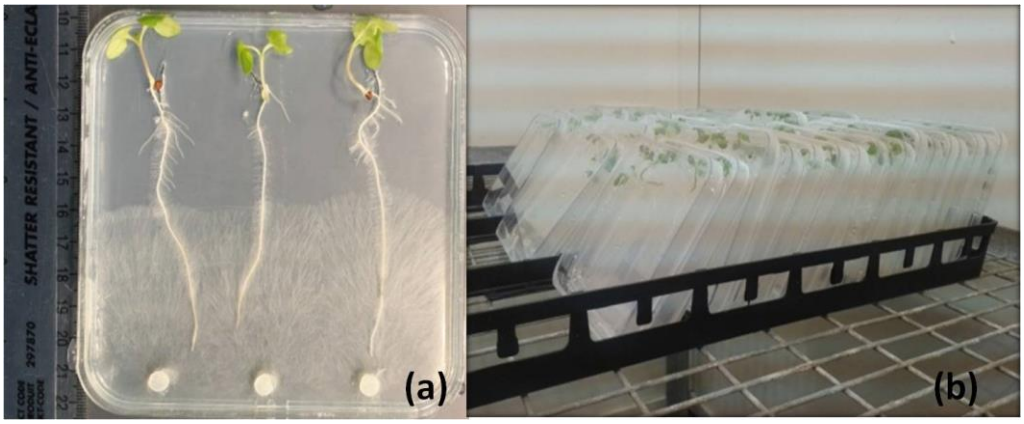


Figure 2.1 Oilseed rape seedlings (a) under inoculation with AG 2-1 in nutrient media plates and (b) plates with seedlings during the experiment.

2.4.3 Hydroponic growth in pouch and wick system

A method previously developed for high-throughput phenotyping of roots in tanks (Atkinson et al., 2015, Thomas et al., 2016b) was modified for screening disease caused by *R. solani* AG 2-1. The construction of the tank consisted of a metal frame with 9 drip trays and 192 growth-pouch positions. Each pouch was made of an acrylic bar, onto which 2 filter papers (Anchor Paper Company, St Paul, MN, USA) were placed on each side and covered with a black polythene sheet (Cransford, Polyethylene Ltd, Suffolk, UK). The filter papers and the sheets were held on the bars with foldback clips (19mm). Prior to sowing, pouches were left to soak overnight in nutrient solution (25% Hoagland's in 2 L of purified water per tray). During the experiment filter papers on growth pouches remained soaked by adding purified water in the trays in equal amounts. Filter papers and clips were autoclaved and acrylic bars were bleached and sprayed with 70% ethanol prior to their use, to eliminate contamination. One seedling was placed in each side of the growth pouch, in the middle and approximately 3 cm from the top of the filter paper and left to grow for 3 days in a controlled environment room (18 ºC, 12 h light: 12 h dark). Then the seedlings were inoculated by adding 1 mycelia PGA plug (5 x 5 mm) 3 cm below the tip of the primary root and another 2 plugs diametrically opposite to each other and 3 cm away from the top of the primary

root. For the control seedlings PGA plugs (5 x 5 mm) without inoculum were used (Figure 2.2).

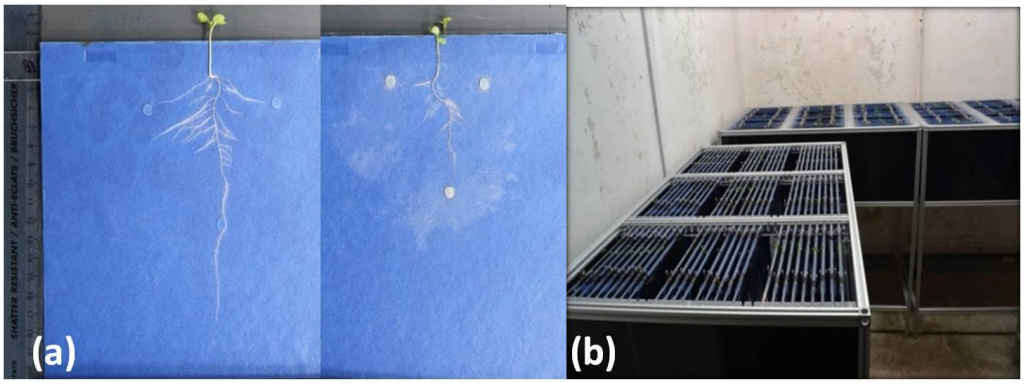


Figure 2.2 (a) Oilseed rape seedlings growing on the filter paper of pouches under control (left) and inoculated (right) conditions. (b) Hydroponic tanks with pouches during experiment.

2.4.4 Growth in compost trays

Plastic trays (6143, Beekenkamp Verpakkingen, Netherlands) with 308 wells (3 x 3 cm) were filled with compost (Levington F2s, Everris Limited, UK) up to 2cm and then each well was inoculated with 1 mycelia PGA plug (5 x 5 mm) of *R. solani* AG 2-1. A layer (0.5 cm) of compost was added above the inoculum and 3 pre-germinated surface sterilised seeds of OSR were placed in each well and covered with compost in order to fill up the well (1.5 cm layer). For the control wells 1 PGA plug without inoculum was added in each well. The trays were left in a controlled environment room (18^{o}C, 12 h light: 12 h dark) (Figure 2.3).

2.4.5 Growth in Light Expanded Clay Aggregate (LECA) trays

Light Expanded Clay Aggregate (LECA) was used to develop a screening method that kept the roots of young seedlings intact. Each compartment of a plastic tray (6143, Beekenkamp Verpakkingen, Netherlands) with 308 wells (3 x 3 cm) was filled with approximately 3 LECA particles (size 4-10 mm; Saint-Gobain Weber Limited, UK) enough to block the bottom and then 1 mycelia PGA plug (5 x 5 mm) of AG 2-1was added for the inoculated treatment or 1 PGA plug for the control treatment. LECA particles were added to fill each compartment up to the 75% of the well volume and then 2 pre-germinated seeds were added. Another layer of LECA was used to fill the wells to the top

(Figure 2.3). An equal amount of 25% Hoagland's in 0.5 L of purified water was supplemented in each well of the tray.

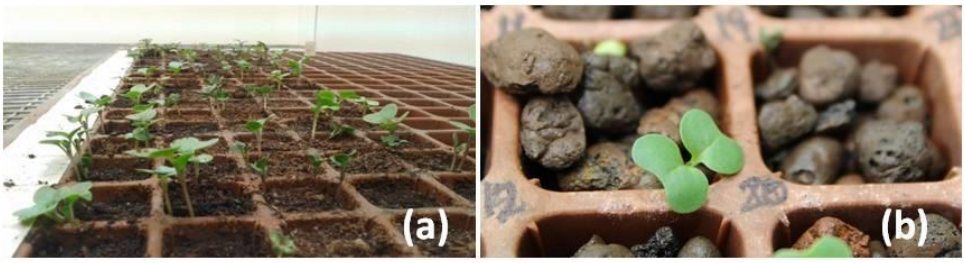


Figure 2.3 (a) Oilseed rape seedlings growing in compost trays and (b) trays with LECA during experiments.

2.4.6 Assessments on disease and plant characteristics

In nutrient media plates and in hydroponic pouches disease as well as plant characteristics (hypocotyl, primary root and lateral root length, lateral root and leaf number) were assessed using the same method but at different time points; Nutrient media plates were assessed at 4, 7 and 10 days post inoculation (dpi) while the seedlings in the hydroponic pouches only at 4 dpi. Disease assessment was made with disease severity categories modified from Khangura et al. (Khangura et al., 1999); for hypocotyl rot the seedlings were categorised on a scale of 0 to 3 (0 = no lesions, 1 = lesions on hypocotyls affecting <25% of the length of the hypocotyl, 2 = lesions covering 26-75% of the length of the hypocotyl, 3 = lesions covering >75% of the length of the hypocotyl), for primary root rot on a 0 to 6 scale (0 = no lesions, 1 = small lesions on primary root, 2 = discoloration up to 50% of primary root, 3 = discoloration 51-75% of the primary root, 4 = discoloration >75% and necrosis covering up to 30% of primary root, 5 = necrosis covering 31-60% of primary root, 6 = necrosis covering >61% or dead root) and for leaf disease on a 0 to 4 scale (0 = no lesions, 1 = disease affecting up to 25% of total leaf area, 2 = disease affecting 25-50% of total leaf area, 3 = disease affecting 51-75% of total leaf area, 4 = completely necrotic leaves of total leaf area). Disease index (DI %) was calculated as: [S (no. plants in disease category) × numerical value of disease category) × 100] / [(no. plants in all categories) × (maximum value on rating scale)]. Plant images were taken from the plates using a digital SLR camera (Canon 1100D, EOS Utility software, Canon Inc., Tokyo, Japan) and

analysed with ImageJ (version 1.4.7, (Schneider et al., 2012)) software and used for the assessment of plant characteristics.

In compost trays, emergence and survival were assessed daily, 2 days after planting and for a period of 5 days. Final counts of emergence and survival were taken on the 10 dpi and then seedlings were removed from the wells, washed and assessed for disease. For non-emerged seedlings, soil was removed and examined to ensure that control seedlings (or seeds) were healthy while the inoculated were heavily infected (dead). For the disease assessments, the above disease scale was modified by including another level for seedlings suffering from pre-emergence damping-off (not emerged) and those that they did not survive due to post-emergence damping-off. Thus for hypocotyl rot, seedlings were rated on a 0 to 4 scale (4 = completely dead or/and not emerged), for primary root rot on a 0 to 7 scale (7 = completely dead or/and not emerged) and for leaf disease on a 0 to 5 scale (5 = not emerged). The percentage of disease index was calculated as described before. Control seedlings that did not emerge were scaled as healthy, as they were found in the compost without any disease symptoms.

Survival of seedlings in trays with LECA was estimated 5 dpi, then the seedlings were removed and images were taken to estimate disease (Canon 1300D, EOS Utility software, Canon Inc., Tokyo, Japan) and analysed with ImageJ (version 1.4.7, software). Seedlings that had not emerged in the control treatment, were assessed in order to ensure that they were viable and not infected, contrary with seedlings that had not emerged in the inoculated treatment which were heavily infected. In contrast to the other methods, disease was estimated as a percentage of the infected plant area to the total plant area for hypocotyls and for roots.

2.4.7 Experimental design and statistical analysis

All statistical analysis was performed using GenStat (15th Edition, VSN International Ltd, Hemel Hempstead, UK). The experiments for each method were designed as randomized blocks with two factors; genotype and inoculum. Where appropriate disease development, seedling emergence, survival and plant characteristics were analysed using analysis of variance (ANOVA) for repeated measures. General ANOVA was used for variables assessed less than

three times. Each method consisted of two replicated experiments, analysed as replicates when there were no significant interactions detected. Disease progress on the genotypes was analysed by excluding the non-inoculated controls in each of the four methods.

2.5 Results

2.5.1 Nutrient media plates

Disease development on the roots of inoculated seedlings in nutrient media plates revealed significant differences during the 10 days of the experiment (P = 0.006; Figure 2.4). Disease developed slower on the genotype 'Grizzly', which had consistently less disease compared to the other genotypes. 'Abaco' followed 'Grizzly' but did not have significantly different disease severity compared to the other genotypes (Figure 2.4). Disease on hypocotyl and leaves was inconsistent between the two replicate experiments (results not shown).

Over time, AG 2-1 significantly reduced the length or the number of assessed plant characteristics apart from hypocotyl length (Table 2.1). Inoculated seedlings had significantly fewer leaves, smaller and fewer lateral roots, shorter primary roots and as a result total length of roots was also reduced (Table 2.1). However, hypocotyl growth was not different between inoculated and control seedlings (P = 0.216). There were no interactions between inoculum and genotype and in both inoculated and un-inoculated seedlings consistent differences were observed in the growth of each of these plant characteristic between the different days (Table 2.2). Lateral root length (P < 0.001) and total root length (P = 0.001) were significantly different between the different genotypes over the 10 days. Hypocotyl length was different among the varieties for each of the 3 days, with 'Grizzly' always having shorter hypocotyl and longest lateral roots (Table 2.2).

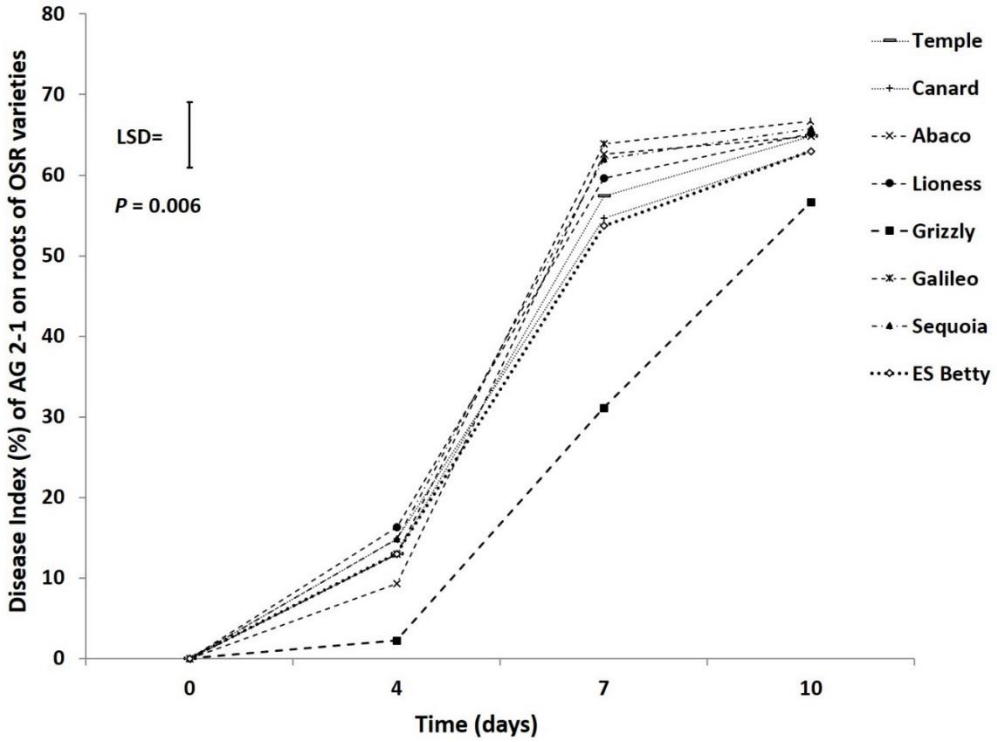


Figure 2.4. Progress of disease caused by AG 2-1 on roots of seedlings of the eight varieties growing in media plates.

Additionally, the number of lateral roots was also significantly different between the genotypes with 'Canard' always having more lateral roots. Significant differences for primary root length between varieties were observed for day 4 and 7 but not on day 10. Seedlings of 'Grizzly' had consistently shorter primary roots (Table 2.2). Significant differences between genotypes in total length of the roots and number of leaves were observed only on the 4th and 7th day, respectively.

Table 2.1 Plant characteristics under inoculated (AG 2-1) and un-inoculated (control) conditions during the 10 days of the experiment in nutrient media plates. RL: root lengths. Lengths are expressed as cm. $P_{(time*inoculum)}$ values and $LSD_{(time*inoculum)}$ (ANOVA) were used for the comparison between the two treatments and $P_{(time)}$ values and $LSD_{(time)}$ for the comparison among different days.

	Hypocotyl Length			Leaf Number			Lateral RL			Lateral Root Number			Primary RL			Total RL		
Treatment	4d	7d	10d	4d	7d	10d	4d	7d	10d	4d	7d	10d	4d	7d	10d	4d	7d	10d
AG 2-1	1.99	2.30	2.37	2	3.14	1.97	1.27	2.26	2.63	12.21	21.64	23.1	7.09	7.57	7.48	8.37	9.83	10.07
Control	1.78	2.06	2.37	1.99	3.12	3.84	1.16	2.37	3.30	10.79	22.42	26.72	7.98	10.60	11.51	9.13	12.97	14.81
$P_{(time*inoculum)}$		0.216			<.001			<.001			0.021			<.001			<.001	
$LSD_{(time*inoculum)}$		0.26			0.23			0.44			3.15			1.07			1.20	
$P_{(time)}$		<0.001			<0.001			<0.001			<0.001			<0.001			<0.001	
$LSD_{(time)}$		0.15			0.15			0.18			1.76			0.30			0.35	

Table 2.2 Plant characteristics of the tested genotypes, in nutrient media plates. RL: root length. Lengths are expressed in cm. Comparisons for each plant characteristic among genotypes were made by using *P* values and LSD (ANOVA).

	Hypocotyl Length			Leaf Number			Lateral RL			Lateral Root Number			Primary RL			Total RL		
Genotype	**4d**	**7d**	**10d**	**4d**	**7d**	**10d**	**4d**	**7d**	**10d**	**4d**	**7d**	**10d**	**4d**	**7d**	**10d**	**4d**	**7d**	**10d**
Temple	2.25	2.34	2.87	2.00	3.25	3.14	1.11	1.61	2.76	9.28	18.94	20.92	7.59	9.46	9.03	8.71	11.06	11.79
Canard	2.11	2.68	2.81	2.00	3.61	3.06	1.34	2.23	2.87	18.19	29.31	30.31	8.79	10.22	10.33	10.13	12.45	13.20
Abaco	1.77	2.08	2.10	2.01	3.24	3.07	1.30	2.09	2.53	14.12	22.70	26.67	6.83	8.19	8.93	8.14	10.28	11.45
Lioness	2.23	1.27	1.35	2.00	3.18	2.81	1.25	2.25	2.72	12.49	20.89	23.65	7.68	8.82	9.23	8.93	11.06	11.95
Grizzly	1.18	1.27	1.35	2.00	2.97	3.07	1.16	3.11	4.23	6.71	18.32	24.00	4.65	7.16	7.92	5.81	10.26	12.16
Galileo	1.73	1.99	2.27	2.00	2.78	2.49	1.04	2.34	2.76	11.78	25.00	29.94	8.08	9.91	10.43	9.12	12.24	13.19
Sequoia	1.79	2.01	2.03	2.00	3.14	2.93	1.29	2.38	3.14	10.75	18.14	23.03	8.91	9.72	10.08	10.20	12.10	13.22
ES Betty	1.99	2.31	2.52	2.00	2.86	2.69	1.23	2.51	2.73	8.69	22.97	20.78	7.74	9.22	9.85	8.97	11.73	12.58
P	<0.001	<0.001	<0.001	0.99	<0.001	0.254	0.781	0.041	0.043	<0.001	0.105	0.001	<0.001	0.021	0.186	<0.001	0.129	0.49
LSD	0.39	0.39	0.59	0.07	0.32	0.57	0.41	1.15	1.02	3.26	8.04	5.35	1.35	1.59	1.86	1.40	1.57	1.88

2.5.2 Hydroponic growth in pouch and wick system

Infection of seedlings with AG 2-1 did not result in significant differences in disease severity between the genotypes for any of the examined plant organs (Table 2.3). Inoculated seedling characteristics were all significantly affected by disease 4 dpi compared to their controls except for lateral root number (P = 0.066; Table 2.4).

Table 2.3 Disease Index on hypocotyls, roots and leaves of the tested genotypes after inoculation with AG 2-1 for four days on the hydroponic growth pouches. For the comparison of disease severity among genotypes within each plant part *P* values and LSD were used (ANOVA).

	Disease Index (%)		
Genotype	**Hypocotyl**	**Root**	**Leaves**
Temple	61.1	54.2	22.9
Canard	69.4	68.1	35.4
Abaco	66.7	54.2	18.8
Lioness	69.4	54.2	18.8
Grizzly	72.2	72.2	47.9
Galileo	77.8	45.8	37.5
Sequoia	75.0	52.8	35.4
ES Betty	58.3	58.3	16.7
P	0.935	0.663	0.533
LSD	32.88	29.28	34.88

Additionally, significant variation was observed between genotypes for some of their morphological characteristics (Table 2.4): hypocotyl length (P < 0.001), lateral root length (P = 0.011) and lateral root number (P = 0.011) were significantly different. The length of the hypocotyl was significantly reduced in infected seedlings with ‘Grizzly’, ‘Galileo’ and ‘Sequoia’ being most affected. ‘Canard’ had the least reduction and ‘ES Betty’ had no reduction in hypocotyl length despite the disease (Table 2.4). In general, ‘Canard’ had

shorter hypocotyls compared to the rest while ‘Abaco’ and ‘Sequoia’ had longer ones. The number of leaves of inoculated seedlings was significantly reduced compared to controls for all genotypes but no differences were observed among the genotypes. Lateral roots of genotypes were significantly shorter under inoculation with ‘ES Betty’ and ‘Grizzly’ being more affected with reduction of length of 72.2% and 88.1% respectively. Although lateral root length was significantly reduced in infected seedlings, lateral root number was not affected. Nevertheless, genotypes differed in the number of lateral roots with ‘Canard’ having more lateral roots. The length of the primary roots was significantly reduced due to infection of AG 2-1 in all genotypes with more pronounced reduction in ‘Grizzly’ (61.8%), ‘Sequoia’ (55.9%) and ‘ES Betty’ (48.5%). The total length of roots was also significantly reduced due to the infection with AG 2-1 with ‘ES Betty’, ‘Sequoia’ and ‘Grizzly’ having the greatest reduction of length. Despite the effect of AG 2-1 infection the genotypes did not significantly differ in primary and total root lengths (Table 2.4).

Table 2.4 Comparison of plant characteristics between inoculated (AG 2-1) and un-inoculated (Control) seedlings of different OSR genotypes 4 days after inoculation on hydroponic growth pouches. RL: root length. Lengths are expressed in cm. $P_{(genotype)}$ and $LSD_{(genotype)}$ were used for the comparison among genotypes and $P_{(inoculum)}$ and $LSD_{(inoculum)}$ for the comparison between treatments (ANOVA).

	Hypocotyl Length		Leaf Number		Lateral RL		Lateral Root Number		Primary RL		Total RL	
Genotype	AG 2-1	Control	AG 2-1	Control	AG 2-1	Control	AG 2-1	Control	AG 2-1	Control	AG 2-1	Control
Temple	1.87	2.19	1.63	1.99	0.52	1.29	2.00	4.85	1.40	2.44	1.92	3.73
Canard	1.31	1.40	1.50	2.08	1.15	1.62	4.67	5.83	2.09	2.83	3.24	4.45
Abaco	2.79	2.93	2.00	2.00	0.49	0.91	2.33	3.17	1.85	2.40	2.34	3.30
Lioness	2.13	2.62	1.33	2.08	0.54	0.93	3.92	3.58	1.94	2.98	2.48	3.91
Grizzly	1.48	2.41	1.46	1.99	0.15	1.22	0.42	1.65	0.68	1.78	0.82	3.00
Galileo	1.81	2.54	1.54	2.00	0.32	0.49	1.33	1.08	1.39	2.55	1.70	3.04
Sequoia	2.50	3.16	1.54	2.00	0.33	1.18	2.33	3.25	1.50	3.40	1.83	4.58
ES Betty	2.50	2.34	1.71	2.00	0.47	1.69	2.75	5.08	1.74	3.38	2.21	5.07
$P_{(genotype)}$	<0.001		0.721		0.011		0.011		0.299		0.115	
$LSD_{(genotype)}$	0.56		0.32		0.49		2.34		1.13		1.37	
$P_{(inoculum)}$	0.005		<0.001		<0.001		0.066		<0.001		<0.001	
$LSD_{(inoculum)}$	0.28		0.16		0.25		1.17		0.57		0.68	

2.5.3 Growth in Compost trays

Inoculation of seedlings in compost trays with AG 2-1 resulted in significant differences in disease severity between the genotypes on hypocotyls (P = 0.003) and leaves (P < 0.001) but not in roots (P = 0.073; Figure 2.5). 'ES Betty' and 'Canard' were consistently least affected, followed by 'Abaco' and 'Sequoia', 'Lioness' and 'Grizzly' (Figure 2.5). 'Galileo' and 'Temple' were the genotypes with significantly more disease (Figure 2.5).

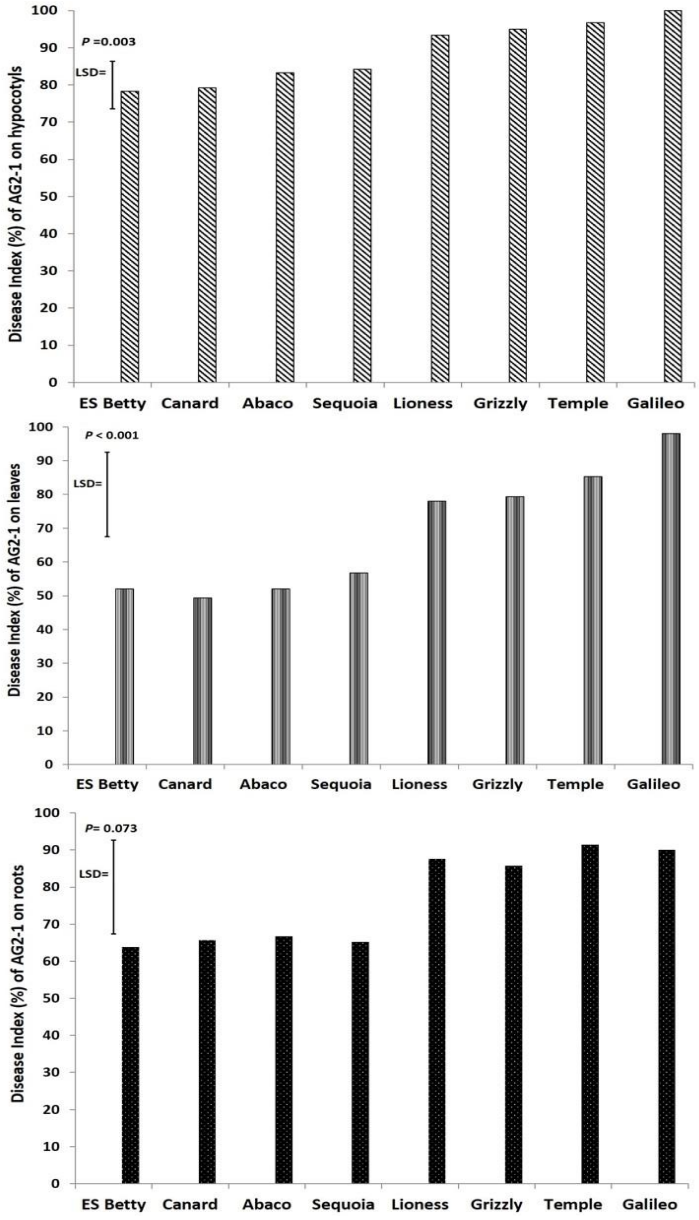


Figure 2.5 Disease on hypocotyls, leaves and roots of the tested genotypes 10 days after inoculation in compost trays.

Emergence of seedlings was significantly different between genotypes ($P < 0.001$) and inoculation with AG 2-1 reduced seedling emergence in almost all varieties apart from 'Canard', 'Grizzly' and 'ES Betty' ($P < 0.001$). However, there was no significant interaction between genotypes and treatment ($P = 0.186$) (Table 2.5).

Table 2.5 Comparison of emergence between inoculated (AG 2-1) and un-inoculated (Control) seedlings of different OSR genotypes 10 dpi in compost trays. $P_{(inoculum)}$ and LSD $_{(inoculum)}$ were used for the comparison between treatments and $P_{(inoculum*genotype)}$ and LSD $_{(inoculum*genotype)}$ for the interaction between genotypes and treatments (ANOVA).

	Emergence (%)	
Genotype	AG 2-1	Control
Temple	30.0	67.2
Canard	83.9	88.3
Abaco	60.0	98.3
Lioness	43.3	88.9
Grizzly	42.8	57.8
Galileo	11.7	64.4
Sequoia	63.3	98.9
ES Betty	53.3	77.2
$P_{(genotype)}$	< 0.001	
$LSD_{(genotype)}$	18.586	
$P_{(inoculum)}$	< 0.001	
$LSD_{(inoculum)}$	9.293	
$P_{(inoculum*genotype)}$	0.186	
$LSD_{(inoculum*genotype)}$	26.284	

Infection of seedlings with AG 2-1 enabled us to detect differences in survival between inoculated and non-inoculated control seedlings ($P < 0.001$) and there were significant differences between genotypes in seedling survival ($P = 0.004$; Figure 2.6). 'Canard' was the genotype with significantly greater survival and the only one with no significant differences between inoculated

and control seedlings (Figure 2.6). ‘Sequoia’, ‘Abaco’, ‘ES Betty’ and ‘Grizzly’ followed, with the first two not being significantly different from ‘Canard’. The poorest survival was observed for ‘Galileo’, ‘Temple’ and ‘Lioness’ (Figure 2.6).

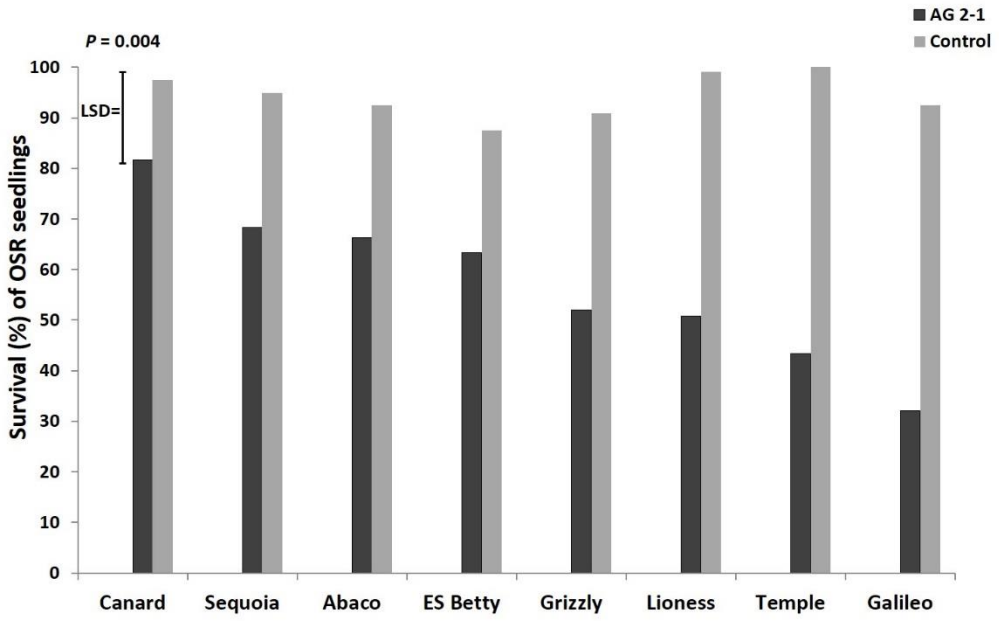


Figure 2.6 Percentage of survival of different OSR genotypes 10 days post inoculation in compost trays. Comparisons for the interaction between treatment and genotype were made with *P* values and LSD (ANOVA).

2.5.4 Growth in LECA trays

AG 2-1 was able to grow and infect seedlings grown in trays filled with LECA. The inoculation resulted in disease symptoms 5 days post inoculation ($P < 0.001$) and enabled assessment through image analysis. Screening for disease revealed significant differences between the tested genotypes for both disease on hypocotyls ($P = 0.002$) and on roots ($P = 0.006$). ‘Sequoia’ was the genotype with consistently less disease on both roots and hypocotyls followed by ‘ES Betty’ (Figure 2.7). ‘Canard’ and ‘Lioness’ ranked in the middle and had significantly lower disease than ‘Grizzly’ ($P = 0.002$). ‘Galileo’, ‘Temple’, ‘Abaco’ and ‘Grizzly’ were the genotypes with the highest disease levels (Figure 2.7). Disease severity on roots indicated that genotypes had similar responses to AG 2-1 infection: ‘Sequoia’ was the genotype with the least disease followed by ‘ES Betty’ and ‘Lioness’; ‘Canard’ ranked in the middle, and ‘Temple’ was the genotype with the most severe disease symptoms on roots ($P = 0.006$; Figure 2.7).

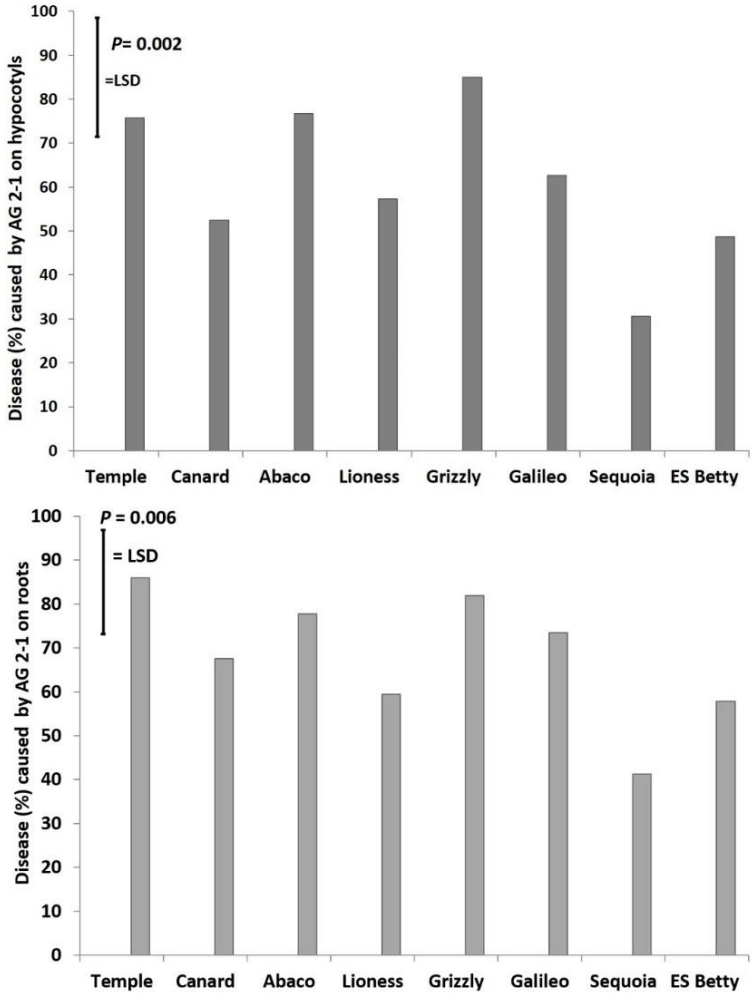


Figure 2.7 Disease on hypocotyls and roots of the tested genotypes 5 dpi in trays with LECA.

Inoculation with AG 2-1 reduced seedling survival ($P < 0.001$) 5 dpi but survival was not significantly different between genotypes ($P = 0.107$) and no significant interaction was observed between genotypes and treatment ($P = 0.716$).

2.6 Discussion

The primary aim of this study was to develop a high throughput method for evaluation of OSR resistance to disease caused by *R. solani* AG 2-1, as a first step towards the identification of traits that could be used in future breeding programs. Early infection of OSR by *R. solani* AG 2-1 leads to pre- and post-

emergence damping off which reduces crop establishment, but infection in later stages towards the maturity of plant is less damaging (Verma, 1996). Therefore, our objective was to develop methods to enable assessment of the early stages of disease progression. A key aspect of our work was to develop a low cost, rapid method that would enable screening of a large number of different OSR genotypes. The four developed methods here (nutrient media plates, hydroponic growth in pouches, trays with compost or LECA) lasted no more than 10 days and enabled the screening of up to 240 seedlings. We used a simple and cheap inoculation technique with mycelial plugs, which allows the induction of disease symptoms and minimises the time for inoculum production to 7 days.

Plant growth in media plates is a commonly used method for the evaluation of seedling growth and root architecture phenotyping. We aimed to further test this for the assessment of initial infection and disease development. Our results indicated that nutrient media plates are a good method for disease phenotyping of roots: both fungal hyphae and root systems grew successfully on the surface of the media. All the steps of infection and disease development could be observed and differences in disease severity amongst different genotypes were detected. Also, due to the horizontal growth of the root system, root architecture was easily measured. Unfortunately, in contrast to roots, this method is not suitable for assessing disease in hypocotyls and leaves. There was no consistency in disease severity among genotypes between the two replicate experiments with hypocotyls. In many cases, hypocotyls escaped hyphae and tended to grow towards the lids of the plates. In the same way the leaves of these plants were also escaping the pathogen. Consequently, this variation in growth led to the uneven and inconsistent infection among genotypes and between experiments. Nonetheless, disease significantly affected both leaves and roots of inoculated seedlings compared to controls, with reduction of healthy leaf area, root length (both primary and lateral) and lateral root number. The results are in agreement with a recent study showing that AG 2-1 causes severe disease by significantly reducing root length and density of inoculated OSR plants and is capable of killing the seedling within 6 dpi (Sturrock et al., 2015). The analysis of plant characteristics showed that

genotypes differ in lateral root and total root length as well as their growth rates. Among the genotypes, 'Grizzly' was the only one that consistently had significantly lower disease but also shorter hypocotyl and primary root compared to other genotypes. Therefore, it might be that the slower growth rate contributed to delay in infection and thus resulted in lower disease levels observed on plates. 'Grizzly' is a winter hybrid known to carry genes for stem canker resistance and for that reason is included in breeding programs (Jestin et al., 2015), however in our tests with 56.6% of root disease 'Grizzly' was susceptible to AG 2-1.

Advanced high-throughput methods have been developed to screen the root system (Hund et al., 2009) and to quantify traits and identify Quantitative Trait Loci (QTLs) (Atkinson et al., 2015). Atkinson et al., screened a mapping population of wheat seedlings aiming to identify QTLs linked with root traits in hydroponic pouch and wick system (Atkinson et al., 2015). Also Thomas et al., used this approach for screening a range of OSR genotypes under control environment and field conditions (Thomas et al., 2016b). Here we modified the method for screening disease caused by AG 2-1 in OSR. Our results showed that *R. solani* was able to grow on filter paper and infect young OSR seedlings causing disease symptoms 4 dpi. Within this time, disease developed on hypocotyls, roots and leaves and resulted in their reduction in inoculated plants compared to controls. However, no differences were detected between genotypes for disease and all were observed to be highly susceptible under this method of inoculation. It is likely that the tested genotypes are characterized by only minor differences and the present screening method could not detect them under the tested conditions. However, this is in contrast with the results of the other two methods, where significant differences on disease severity were observed. Different inoculum densities and length of inoculation periods were tested (results not shown) prior to the present experimental procedure, which appeared to be the most consistent. Possibly the moist environment of the filter paper and the polythene sheet as well as the lack of the soil environment altered hyphal growth and the infection process. *Rhizoctonia solani* is a soil-borne pathogen, thus the presence of soil with nutrients, organic matter and aeration play a pivotal role in its epidemiology. In this growing system the

polythene sheet was attached to the filter paper but in the position of the seedlings, small aerate cavities were formed possibly enabling the pathogen to grow better. As a result, pathogen hyphae were denser close to the seedling and eventually led to greater disease on plants, whilst in the other methods pathogen growth was more even. Nonetheless, this method enabled us to detect differences in plant characteristics between inoculated and un-inoculated control seedlings, as well as differences among genotypes in a short period of time.

Soil and compost are most commonly used for the evaluation of plant resistance against soil-borne pathogens. In the case of *R. solani,* the vast majority of studies focussing on plant responses to pathogen exposure, have used soil (Acharya et al., 1984, Babiker et al., 2013), soil free media (Yang and Verma, 1992) or a combination of both (Lamprecht et al., 2011). In this way, the experiments simulate more realistic conditions that occur in the field and a better evaluation of the plants response to the pathogen is observed. Therefore we decided as a suitable alternative that the third method should be developed with compost. In contrast to other studies, we used multiple cell-trays which save space and time by enabling us to screen more than 100 different genotypes per tray in a single experiment. The trays were also ideal to assess the early stages of infection in young seedlings that are less than 10 days old. An additional benefit of this method is that it enabled the recording of emergence and survival of seedlings and hence record pre- and post-emergence damping off. Low emergence of inoculated seedlings compared to controls, indicated susceptibility of those cultivars to pre-emergence damping off and confirmed the detrimental effect of AG 2-1 to OSR during early growth stages. Disease screening on hypocotyls and leaves was easily conducted, but in contrast the extraction and assessment of the delicate roots of seedlings damaged by root rot was difficult and time consuming. Despite meticulous work, it was hard to keep the roots intact. We were unable to detect significant differences in root disease between cultivars in this method but we were able to detect differences in disease severity of hypocotyls and leaves. ‘ES Betty’ and ‘Canard’ were consistently the two genotypes with the lowest disease while ‘Temple’ and ‘Galileo’ were the most susceptible. This is in agreement with

emergence and survival data and it can be an indication that these genotypes may carry both quantitative and qualitative traits allowing them to perform better against AG 2-1. In this research all genotypes were pre-germinated in order to standardise our methods, and therefore their germination rates under inoculated conditions were not assessed. However, it is possible that some genotypes are able to germinate and emerge faster and therefore escape and/or be less affected by the infection. Indeed, Sturrock et al., suggested that rapid germination of OSR seedlings may enable the early establishment of a strong root system allowing better nutrient uptake and growth and consequent recovery from AG 2-1 infection (Sturrock et al., 2015).

We aimed to improve the method by eliminating high inoculum pressure and most importantly by reducing damage to roots to be able to better discriminate the genotypes in our disease assessments. Therefore we decided first to reduce the time that the seedlings were exposed to the pathogen from 10 to 5 dpi. Secondly we used a medium that would not affect seedling growth but would minimise the damage to the root system upon removal. In this respect, LECA particles with the addition of nutrient solution appeared to be an appropriate medium. LECA has been receiving a growing acceptance as an environmental friendly natural material with great benefits in civil engineering and gardening. Currently there is a limited number of published studies examining the use of LECA as a growing medium (Graber and Junge, 2009, Laznik et al., 2011, Trdan et al., 2007) and to the best of our knowledge only one study has examined the growth of a fungi in LECA (Douds et al., 2014). In this study the authors showed that arbuscular mycorrhizal fungi (AMF) were not able to colonise their tested plant, *Paspalum notatum*, when grown in LECA and consequently concluded that LECA was not colonised effectively by AMF (Douds et al., 2014). However, the results of the current study show that the necrotrophic pathogen *R. solani* AG 2-1 was able to grow on the surface of LECA particles, observed as hyphal mass and infect OSR seedlings. The inoculation period of 5 days was sufficient to induce disease symptoms without killing the seedlings. At the same time differences in disease severity of the tested genotypes were detected for both hypocotyls and roots. The use of LECA preserved the roots intact during their collection from the trays and

therefore allowed more accurate disease assessments. Taking images of the seedlings and analysing them with Image J not only allowed us to complete the experiments faster but also to estimate the disease more objectively compared to more subjective visual assessments which are not taking into account differences in growth and development of the seedlings. The OSR genotypes had different responses to AG 2-1 infection: 'Sequoia' was the least affected for both damping off and root rot, followed by 'ES Betty'. Although disease affected the survival of inoculated OSR seedlings compared to the controls, we were not able to detect significant differences in survival of seedlings between the different genotypes at 5 dpi.

Comparison of different methods

Assessing the severity of disease caused by AG 2-1 on hypocotyls and/or roots of young seedlings is the most important measure for the identification of active genetic resistance. Nonetheless, other traits related to rapid development and growth for crop establishment such as root architecture and emergence or survival are important for the identification of disease escape. Each of the four methods we developed has positive and negative aspects: Nutrient media plates enabled the recording of the infection progress and the collection of data on root traits but were not suitable for disease screening of hypocotyls and leaves. Growth in hydroponic pouches can be high-throughput, fast screening method but the moist environment altered *R. solani* growth and we could not detect any difference in disease severity among the tested OSR genotypes. Screening on trays with compost was more realistic approach that makes available holistic disease screens for the plant as well as measurements of emergence and survival. Nevertheless, damage to the root system prevented accurate disease assessment and measurements of root architecture traits and a longer time was required to detect differences. However, the use of LECA holds the benefits of screening in compost trays but also enables the roots to be intact and detect differences between genotypes in root rot disease. We were unable to detect differences in survival most likely due to short infection period of 5 dpi. Most importantly 5 dpi screening in LECA resulted in moderate disease of seedlings compared to screening in compost and this might be the reason that we have small differences in in the ranking of genotypes between the two

methods. Considering the severity of disease 5 dpi and the lack of resistance in the tested genotypes, further screening for a longer period for detection of differences in survival using this method was not pursued here. In Table 2.6 we provide a basic estimation of the cost of screening 100 genotypes by each method, based on the cost of consumables and equipment used; the hydroponic pouch and wick system was the most expensive method as the requirements for building the system were high compared to the other methods that use petri dishes and well trays. As mentioned previously, the choice of method should be based on the scientific aim; in the present study we aimed to identify a low cost high-throughput screening method which would enable the detection of potential resistant OSR genotypes to root diseases such as AG 2-1. Therefore, we required a method that allowed the detection of differences in disease severity and resultant changes to plant morphological characteristics. Screening in trays with LECA fulfilled these criteria it enables fast and high-throughput screening with the assessment of early infection stages. Therefore it is an applicable method for the detection of resistant OSR cultivars to AG 2-1.

Table 2.6 Estimation of cost for the screen of 100 genotypes in the developed methods. The estimation excludes the cost for the camera that was used in the hydroponic pouch and wick system, on nutrient media plates and trays with LECA.

Method	Cost (£) for 100 genotypes
Hydroponic pouch and wick system	348
Nutrient media plates	27.3
Trays with compost	1.05
Trays with LECA	2.14

2.7 Conclusion

The present study provides a new low cost, high-throughput screening method for the identification of potential OSR cultivars that are resistant to root diseases such as *R. solani* AG 2- 1. This method can be used as an early step for the evaluation of germplasm prior to testing under field conditions.

Additionally, it confirms that AG 2-1 is an extremely pathogenic isolate to OSR (Acharya et al., 1984, Babiker et al., 2013, Kataria and Verma, 1992, Yang and Verma, 1992); the inoculum density used resulted in low survival of young seedlings 10 dpi in compost trays and high disease levels ranked from 30% to 85% 5 dpi in trays with LECA. None of the genotypes tested in the current study were resistant. Future screening of diverse populations of *B. napus* and *Brassica* species is essential to elucidate if there is any resistance against this destructive pathogen.

2.8 Abbreviations

OSR: oilseed rape, AG: anastomosis group, LECA: light expanded clay aggregate, PGA: potato glucose agar, dpi: days post inoculation, DI: disease index, LSD: least significant difference of means, RL: root length, QTL: quantitative trait loci, AMF: arbuscular mycorrhizal fungi

2.9 References

ACHARYA, S. N., VERMA, P. R., DUECK, J. & DOWNEY, R. K. 1984. Screening rapeseed/canola for resistance to damping-off and seedling root rot caused by *Rhizoctonia solani*. *Canadian Journal of Plant Pathology,* 6**,** 325-328.

ALLENDER, C. J. & KING, G. J. 2010. Origins of the amphiploid species Brassica napusL. investigated by chloroplast and nuclear molecular markers. *BMC Plant Biology,* 10**,** 1-9.

ANDERSON, N. A. 1982. The Genetics and Pathology of *Rhizoctonia solani*. *Annual Review of Phytopathology,* 20**,** 329-347.

ATKINSON, J. A., WINGEN, L. U., GRIFFITHS, M., POUND, M. P., GAJU, O., FOULKES, M. J., LE GOUIS, J., GRIFFITHS, S., BENNETT, M. J., KING, J. & WELLS, D. M. 2015. Phenotyping pipeline reveals major seedling root growth QTL in hexaploid wheat. *Journal of Experimental Botany*.

BABIKER, E., HULBERT, S., SCHROEDER, K. & PAULITZ, T. 2013. Evaluation of Brassica species for resistance to *Rhizoctonia solani* and binucleate *Rhizoctonia* (*Ceratobasidum* spp.) under controlled environment conditions. *European Journal of Plant Pathology,* 136**,** 763-772.

COHEN, M. F., YAMASAKI, H. & MAZZOLA, M. 2005. Brassica napus seed meal soil amendment modifies microbial community structure, nitric oxide production and incidence of *Rhizoctonia* root rot. *Soil Biology & Biochemistry,* 37**,** 1215-1227.

DOUDS, D. D., LEE, J., UKNALIS, J., BOATENG, A. A. & ZIEGLER-ULSH, C. 2014. Pelletized Biochar as a Carrier for AM Fungi in the On-Farm System of Inoculum Production in Compost and Vermiculite Mixtures. *Compost Science & Utilization,* 22**,** 253-262.

GRABER, A. & JUNGE, R. 2009. Aquaponic Systems: Nutrient recycling from fish wastewater by vegetable production. *Desalination,* 246**,** 147-156.

HANDISENI, M., BROWN, J., ZEMETRA, R. & MAZZOLA, M. 2013. Effect of *Brassicaceae* seed meals with different glucosinolate profiles on *Rhizoctonia* root rot in wheat. *Crop Protection,* 48**,** 1-5.

HUND, A., TRACHSEL, S. & STAMP, P. 2009. Growth of axile and lateral roots of maize: I development of a phenotying platform. *Plant and Soil,* 325**,** 335-349.

JESTIN, C., BARDOL, N., LODE, M., DUFFE, P., DOMIN, C., VALLEE, P., MANGIN, B., MANZANARES-DAULEUX, M. J. & DELOURME, R. 2015. Connected populations for detecting quantitative resistance factors to phoma stem canker in oilseed rape (*Brassica napus* L.). *Molecular Breeding,* 35**,** 16.

KATARIA, H. R. & VERMA, P. R. 1992. *Rhizoctonia solani* damping-off and root rot in oilseed rape and canola. *Crop Protection,* 11**,** 8-13.

KHANGURA, R. K., BARBETTI, M. J. & SWEETINGHAM, M. W. 1999. Characterization and pathogenicity of *Rhizoctonia* species on canola. *Plant Disease,* 83**,** 714-721.

LAMPRECHT, S. C., TEWOLDEMEDHIN, Y. T., CALITZ, F. J. & MAZZOLA, M. 2011. Evaluation of strategies for the control of canola and lupin seedling diseases caused by *Rhizoctonia* anastomosis groups. *European Journal of Plant Pathology,* 130**,** 427-439.

LAZNIK, Z., ZNIDARCIC, D. & TRDAN, S. 2011. Control of *Trialeurodes vaporariorum* (Westwood) adults on glasshouse-grown cucumbers in four different growth substrates: an efficacy comparison of foliar application of *Steinernema feltiae* (Filipjev) and spraying with thiamethoxamn. *Turkish Journal of Agriculture and Forestry,* 35**,** 631-640.

OGOSHI, A. 1987. Ecology and pathogenicity of anastomosis groups of *Rhizoctonia solani* Kühn *Annual Review of Phytopathology,* 25**,** 125-143.

SCHNEIDER, C. A., RASBAND, W. S. & ELICEIRI, K. W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat Meth,* 9**,** 671-675.

STURROCK, C. J., WOODHALL, J., BROWN, M., WALKER, C., MOONEY, S. J. & RAY, R. V. 2015. Effects of damping-off caused by *Rhizoctonia solani* anastomosis group 2-1 on roots of wheat and oil

seed rape quantified using X-ray Computed Tomography and real-time PCR. *Frontiers in Plant Science,* 6.

THOMAS, C. L., GRAHAM, N. S., HAYDEN, R., MEACHAM, M. C., NEUGEBAUER, K., NIGHTINGALE, M., DUPUY, L. X., HAMMOND, J. P., WHITE, P. J. & BROADLEY, M. R. 2016. High-throughput phenotyping (HTP) identifies seedling root traits linked to variation in seed yield and nutrient capture in field-grown oilseed rape (*Brassica napus* L.). *Annals of Botany*.

TRDAN, S., ZNIDARCIC, D. & VIDRIH, M. 2007. Control of *Frankliniella occidentalis* on glasshouse-grown cucumbers: an efficacy comparison of foliar application of S*teinernema feltiae* and spraying with abamectin. *Russian Journal of Nematology,* 15**,** 25-34.

VERMA, P. R. 1996. Biology and control of *Rhizoctonia solani* on rapeseed: A review. *Phytoprotection,* 77**,** 99-111.

YANG, J. & VERMA, P. R. 1992. Screening genotypes for resistance to pre-emergence damping-off and postemergence seedling root rot of oilseed rape and canola caused by *Rhizoctonia solani* AG-2-1. *Crop Protection,* 11**,** 443-448.

Chapter 3. **Screening of germplasm for resistance to *Rhizoctonia solani* AG 2-1**

3.1 Author contribution

Experimental work was designed by F Drizou with the contribution of R Ray and performed by F Drizou with the help of S Grange for the screening of the TCDH population. Analysis of data was performed by F Drizou. The chapter was composed by F Drizou with the contribution of N Graham, T Bruce and R Ray.

Note: The screening in this chapter was performed simultaneously or before the development of methods in chapter 2. Therefore it was performed in trays with compost and not with LECA.

3.2 Abstract

Oilseed rape (*Brassica napus*, OSR) is an important crop which has undergone intensive breeding for improved yield and oil quality. The soil-borne pathogen *Rhizoctonia solani* Anastomosis Group (AG) 2-1 is extremely aggressive to OSR seedlings, causing damping-off disease and eventually death of seedlings. OSR genotypes with resistance to AG 2-1 have not yet been identified. In the current study, we aimed to identify resistance by screening a range of OSR germplasm. The screening was conducted in multi-well trays filled with compost for 5 or 10 days post inoculation. The pathogenicity of AG 2-1 to seedlings was assessed by measuring emergence, survival and disease index on hypocotyls and roots. Additionally, we also tried to identify if resistance could be induced in the next generation through an epigenetic stress response, by comparing multigenerational seedling performance to AG 2-1 under inoculation with AG 2-1. Our results indicated that within the OSR germplasm tested there is no resistance to AG 2-1, because all genotypes had high disease levels and reduced emergence and survival.

Keywords

Oilseed rape, *Rhizoctonia solani* AG 2-1, resistance, screening, inheritance

3.3 Introduction

The plant family of Brassicaceae comprises important species for agriculture including *Brassica napus*, oilseed rape (OSR). This species is probably the youngest member of the family and the result of hybridisation between *Brassica rapa* and *Brassica oleracea*. Oilseed rape consists of two subgenomes; subgenome A from *B. rapa* and subgenome C from *B. oleracea.* (Chalhoub et al., 2014). As with other members of the Brassicaceae family, it is known for its glucosinolates (GSL) secondary metabolites, which when hydrolysed produce active compounds that are used as defensive weapons against herbivorous insects (Bruce, 2014). Due to this unique chemistry, plant material from oilseeds also has a potential in biofumigation against soil-borne pathogens (Kirkegaard et al., 2000, Kirkegaard et al., 1996). OSR is a profitable crop and has undergone intensive breeding for optimisation of yield, oil quality and resistance against pathogens and pests (Gupta, 2012, Li et al., 2016). Nonetheless, to date there is no reported resistant variety against *Rhizoctonia solani* anastomosis group (AG) 2-1 (Acharya et al., 1984, Babiker et al., 2013). This species consists of different AGs, each genetically and morphologically different and with different pathogenicity against different host plants (Anderson, 1982, Cubeta and Vilgalys, 1997). Oilseed rape is the primary host for AG 2-1 during the early seedling stage, whilst at later growth stages it is more susceptible to AG 4 (Verma, 1996). Infection of young seedlings causes damping-off disease which eventually leads to death of seedlings, impairment of crop establishment and consequently yield losses (Stodart et al., 2007). Currently the most common methods to eliminate the pathogen are cultural practices and chemical seed treatments usually with fungicides including sedaxane, penflufen and ipconazole (Ajayi-Oyetunde et al., 2017, Ghorbani et al., 2009, Lamprecht et al., 2011, Zeun et al., 2013). However, considering the increase in demand for more sustainable control methods against pathogens and pest, the identification of resistant or tolerant traits in oilseed rape against AG 2-1 is essential.

Another aspect for the development of advanced pest management and plant breeding that gains a lot of interest the last decades is the understanding of transgenerational defence induction. Holeski et al., in their review define

transgenerational induction as *'A change in offspring phenotype that is cued by an environmental signal in the parental generation, and it is expressed independently of changes in the offspring genotype.'* (Holeski et al., 2012). Many studies have shown that exposure of a parental plant to an attacker or a compound results in increased resistance of the progeny plant (reviewed in Bruce et al., 2007, Hématy et al., 2009, Holeski et al., 2012). One of the possible mechanisms that leads to this outcome are epigenetic changes that mainly include DNA methylation and histone modification (Bruce et al., 2007, Hématy et al., 2009, Holeski et al., 2012). For example Luna et al., showed that systemic acquired resistance (SAR), a type of induced defence mechanism, after inoculation with *Pseudomonas syringae* bacteria, was carried on to the next generation of *Arabidopsis* plants. In precise, progeny of inoculated parents were less colonised by *Hyaloperonospora arabidopsidis* compared to progeny of control plants (Luna et al., 2012). Additionally, the authors found that this phenomenon was epigenetically controlled via hypothelation and histone modifications of marker genes of jasmonic and salicylic acid (Luna et al., 2012). Yang, and Verma, stated that resistance to AG 2-1 could be improved through selection (Yang and Verma, 1992). In their study, symptomless plants 21 dpi were collected. Seeds were sown with seeds from the original parental lines under growth room and field conditions. Progenies appeared to have improved emergence in growth room conditions and for some of them also in the field. Nonetheless, no further research was published to show if resistance could have been gained as a transgenerational defence induction effect.

We aimed to i) identify resistant or tolerant traits by screening a number of OSR lines including commercial cultivars, selected genotypes from diversity sets and a mapping population; ii) evaluate the findings of Yang and Verma (1992) and examined if previous infection of *B. napus* and *Arabidopsis thaliana* plants with AG 2-1, resulted in better performance of their progeny as a result of transgenerational defence induction. We assessed emergence and survival of seedlings, as this could indicate the ability of the plant to escape the disease and also damping-off disease on hypocotyls and roots.

3.4 Material and Methods

3.4.1 Inoculum and seeds

Rhizoctonia solani AG 2-1, originally isolated from OSR plants was used to produce inoculum (Isolate collection at the University of Nottingham: #1934). For the experiments 10 to 14 days old inoculum was used, growing on Potato Glucose Agar (PGA; Sigma-Aldrich, UK) at room temperature (18-20^{o}C). MINELESS and Westar seeds obtained from Ishita Ahuja (Norwegian University of Science and Technology). Seeds were sown and plants left to flower, self-pollinate and produce seeds. All seeds were surface sterilised with 4% sodium hypochlorite (Parazone, Jeyes Limited, UK) for 5 min followed by three rinses with distilled autoclaved water and then pre-germinated on round filter paper (diameter 85mm, GE Healthcare Whatman, UK) with 3 ml of sterile water and kept in dark at room temperature (18-20^{o}C) for 2 days.

Arabidopsis thaliana, Columbia 0 (Col.0), seeds were surface sterilised with of 5% sodium hypochlorite (Parazone, Jeyes Limited, UK) for 3min followed by three washes with distilled autoclaved water. The seeds were kept in 1.5 ml of distilled autoclaved water in dark, at 4 ^{o}C for 3 days, to break their dormancy. For the screening of the progeny, seeds were pre-germinated on round filter paper (85mm, GE Healthcare Whatman, UK) with 2 ml of distilled autoclaved water for 2 days prior to the experiment.

3.4.2 Phenotyping different germplasm groups

The screening was separated into four different groups of germplasm: 1. germplasm including commercial cultivars from (Tantal, Nugget, Verona, Westar, Tapidor and Comet), a choice of genotypes from two diversity panels: ASSYST-224 from the ERANET-ASSYST consortium diversity population (Bus et al., 2014, Bus et al., 2011, Körber et al., 2015, Körber et al., 2012) and DFFS-68 from the Diversity Fixed Foundation Set (DFFS) (Pink et al., 2008). Also, a genetically modified plant of cv. Westar, named MINELESS, in which the specialist myriosin cells and myriosinase enzyme have been removed making the plant unable to hydrolyse glucosinolates (GSL) to the active defensive compounds (Borgen et al., 2010) and one genotype of *B. rapa*. 2. a selection of genotypes from the ASSYST diversity population (ASSYST

genotypes: 209, 210, 194, 279, 269, 224, 447, 187) and Westar as susceptible control. 3. the TCDH (Temple x Canard Doubled Haploid) mapping population.

3.4.2.1 Phenotyping of group 1- Commercial cultivars and group 2- Selection of ASSYST population

The screening was performed in 308-well plastic trays (6143, Beekenkamp Verpakkingen, Netherlands) with compost (Levington F2s, Everris Limited, UK) as described in Drizou et al. (2017). For the inoculated treatment 1, mycelia PGA plug (5 x 5mm) of *R. solani* AG 2-1 was added in each well (Drizou et al., 2017) whilst for the control treatment 1 PGA plug without inoculum was added in each well. The trays were left in a controlled environment room (18 ºC, 12h light: 12h dark).

Emergence and survival were assessed daily, 2 days after planting and for a period of 5 days on the final day (10 dpi) seedlings were removed from the wells, washed and assessed for disease with the following scale (Drizou et al., 2017): for hypocotyl rot the seedlings were categorised on a scale of 0 to 4 (0 = no lesions, 1 = lesions on hypocotyls affecting <25% of the length of the hypocotyl, 2 = lesions covering 26-75% of the length of the hypocotyl, 3 = lesions covering >75% of the length of the hypocotyl, 4 = completely dead or/and not emerged), for primary root rot on a 0 to 7 scale (0 = no lesions, 1 = small lesions on primary root, 2 = discoloration up to 50% of primary root, 3 = discoloration 51-75% of the primary root, 4 = discoloration >75% and necrosis covering up to 30% of primary root, 5 = necrosis covering 31-60% of primary root, 6= necrosis covering >61% or dead root, 7 = completely dead or/and not emerged). Disease index (DI %) was calculated as: [S (no. plants in disease category) × numerical value of disease category) × 100]/ [(no. plants in all categories) × (maximum value on rating scale)]. Control seedlings that did not emerge were scaled as healthy, as they were found in the compost without any disease symptoms.

3.4.2.2 Phenotyping group 3 -TCDH population

The screening of this population was first performed for 10 days as described above on groups 1 and 2. Due to high disease levels, it was difficult to compare lines and therefore a collection of genotypes (TCDH: 130, 124, 42,

150, 11, 48, 100, 92 and the parents Temple and Canard) of this population were screened again for a period of 5 dpi using the same protocol. Following this, genotypes TCDH 42 and TCDH 24 were screened again with the parents but with a different protocol for disease assessments: On the fifth day post inoculation (dpi) seedlings were removed and images were taken (Canon 1300D, EOS Utility software, Canon Inc., Tokyo, Japan) and analysed with ImageJ (version 1.4.7, software). Instead of estimating disease as DI%, disease was estimated as follow: Disease% = [(plant area affected by disease)/(total plant area)] * 100.

3.4.3 Identifying if there is a transgenerational induction of resistance to AG 2-1

From the available germplasm we chose cultivars that were also screened by Yang and Verma: Westar, which was used in their study as a susceptible control and Nugget. Comet which appeared to be highly susceptible to AG 2-1 during the screening of group 1 in the present work and ES Betty, a hybrid that in previous screening shown to be relatively tolerant (Drizou et al., 2017). We chose to include experiments with *A. thaliana* firstly because plant's life cycle is significantly quicker compared to *B. napus*, so we would obtain progenies much earlier and secondly because as a model plant would enabled us to investigate if our hypothesis was supported by changes in transcriptional level.

3.4.3.1 Phenotyping OSR and *A. thaliana*

Oilseed rape cultivars were screened in trays with compost, as in group 1 and 2, but only emergence and survival were assessed for a period of 10 days. Then seedlings that had survived from inoculation with AG 2-1 were removed from the trays and transplanted in pots with compost (Levington F2s, Everris Limited, UK). Plants were left to grow in a glasshouse with controlled environmental conditions (20 ^{o}C day and 15 ^{o}C night, 16h light: 8h dark). Winter OSR cultivars were vernalised for a period of 6 weeks at 6 ^{o}C, 12h light: 12h dark and then moved to the glasshouse and left to flower, self-pollinate and produce seeds. Seeds were harvested to proceed with the screening of their progeny. The screening of progeny plants was performed in the same way as has been stated before for a period of 10 dpi.

To obtain seeds of progeny of *A. thaliana* seeds were sown in plastic trays (6143, Beekenkamp Verpakkingen, Netherlands) and each of the 308 well compartments was half filled with compost (Levington M3 Everris Limited, UK) and then 1 mycelia PGA plug (5 x 5mm) of *R. solani* AG 2-1, or a PGA plug for the control treatment, was added and covered with compost to fill all the well compartment. Approximately 50 seeds were placed on the top of the compost by pipetting and allowed to germinate and grow in a room (18 ^{o}C, 12h light: 12h dark). Plants that survived from AG 2-1 inoculation 21 dpi, as well as control plants were removed from the wells and transplanted to pots with compost and allowed to grow, produce flowers, self-pollinate and set seeds. Harvested seeds of those plants were used to proceed with the experiments.

For the screening of progeny, seeds were separated into two groups; those produced from inoculated plants (P+) and those produced from control plants (P-) and exposed in both treatments (AG 2-1 inoculation and control). The screening performed as described before in trays with compost but in each well compartment 10 pre-germinated seeds were placed on the top of the compost using forceps. Emergence was assessed 10 and 13 dpi and survival 21 dpi.

3.4.4 Experimental design and statistical analysis

All statistical analysis was performed using GenStat (17th Edition, VSN International Ltd, Hemel Hempstead, UK). The experiments were designed as randomized blocks with two factors; genotype and inoculums or origin and inoculum for group 4 with *A. thaliana*. Seedling emergence and survival were analysed using analysis of variance (ANOVA) for repeated measures or general ANOVA if were analysed for individual days. Seedling survival on the TCDH population lines 5 dpi was analysed with generalised linear model (GLM) with binomial distribution and logit link function. General ANOVA was used to analyse disease. For comparisons between the parents Temple and Canard, two-sample t-test with group factor was performed. To evaluate the affect origin in OSR on group 4, survival and emergence means from ANOVA were analysed with two sample t-test for each treatment. Each method consisted of two replicated experiments, analysed as replicates when there were no significant interactions detected. Disease on the genotypes was analysed by excluding the non-inoculated controls.

3.5 Results

3.5.1 Group 1 – OSR germplasm

Inoculation with AG 2-1 significantly reduced emergence ($P< 0.001$, LSD= 10.696) and survival ($P< 0.001$, LSD= 7.686) of seedlings compared to non-inoculated control on group 1 (Table 3.1); *B. rapa*, Comet and DFFS- 68 Conqueror Bronze top, were the genotypes with the lowest emergence and survival under inoculation, while Tantal had the highest emergence (75.83%) and survival (73.75%). Verona was the second best emerged genotype (71.11%) and ASSYST 224 wild accession had the second highest survival (67.5%) and very similar to Verona (67.29%) which was third. The tested genotypes differed significantly in their emergence and survival independently from the inoculation treatment (emergence $P= 0.002$, LSD= 23.917, survival $P= 0.015$, LSD= 17.187), with *B. rapa* having the lowest followed by Comet and ASSYST 224, Verona and Tantal have the highest emergence. However, no significant interactions were observed neither in emergence between genotype and inoculum treatment ($P= 0.119$, LSD= 33.824) nor in survival ($P= 0.182$, LSD= 24.307) with all genotypes being similarly affected in their survival under inoculation with AG 2-1 at 10 dpi.

Table 3.1 Emergence (%) and survival (%) of seedlings of different genotypes, in group 1, under inoculation for 10 days. Comparison between genotypes were made with $P_{(genotype)}$ and LSD $_{(genotype)}$, comparison between treatments with $P_{(inoculum)}$ and $LSD_{(inoculum)}$ and comparison between genotypes and treatments with $P_{(genotype*inoculum)}$ and $LSD_{(genotype*inoculum)}$ (ANOVA).

	Emergence (%)		Survival (%)	
Genotype	**AG 2-1**	**Control**	**AG 2-1**	**Control**
ASSYST-224	56.67	90	67.5	92.5
Tantal	75.83	80	73.75	81.87
DFFS-68	43.61	82.22	53.54	86.67
Nugget	52.5	73.33	60.83	80.62
Verona	71.11	84.44	67.29	90.42
MINELESS	49.17	64.72	55.42	81.25
Westar	65	62.78	62.92	73.75
Tapidor	60.28	70	59.17	77.5
Comet	10	48.89	30	67.5
B. rapa	8.33	79.44	29.37	88.54
$P_{(genotype)}$	0.002		0.015	
LSD $_{(genotype)}$	23.917		17.187	
$P_{(inoculum)}$	< 0.001		< 0.001	
$LSD_{(inoculum)}$	10.696		7.686	
$P_{(genotype*inoculum)}$	0.119		0.182	
$LSD_{(genotype*inoculum)}$	33.824		24.307	

Disease levels on hypocotyl of seedlings at 10 dpi was significantly different between genotypes (*P*= 0.004, LSD= 10.81); ASSYST 224, Tantal and DFFS 68 had the lowest disease levels (79.6 %, 86.7% and 89.6% respectively) compared to the other genotypes and Tapidor, Comet and *B. rapa* the highest; 98.7% for Tapidor and 100% for Comet and *B. rapa* (Figure 3.1). Nonetheless, the DIH was extremely high even for the least affected varieties. Similar results obtain from disease screening of roots, with significant differences between genotypes (*P*= 0.002, LSD= 18.77). DIR, ASSYST 224 and Tantal had the least disease (62.9% and 76.9% respectively) and Comet and *B. rapa* the highest (100%) (Figure 3.1).

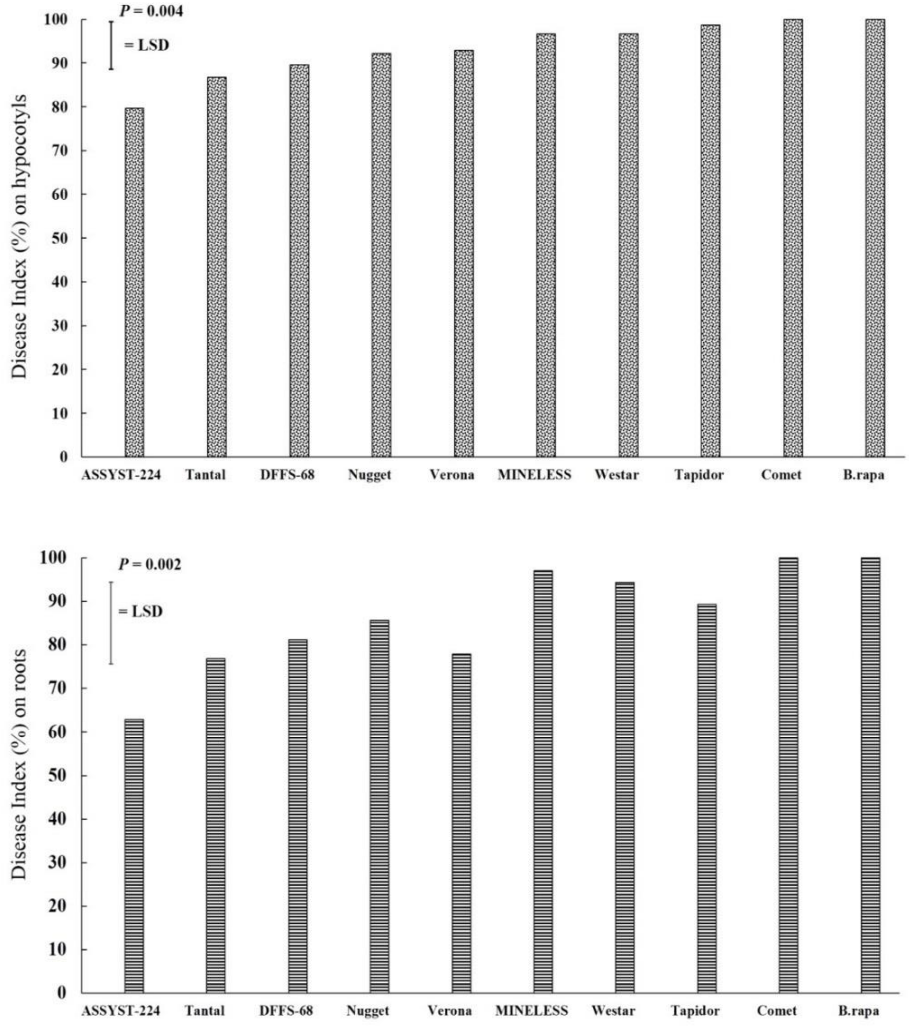


Figure 3.1 Disease Index (%) on hypocotyls (top) and roots (bottom) of different genotypes in group 1, 10 dpi with AG 2-1. For the comparison between genotypes *P* values and LSD were used (ANOVA).

3.5.2 Group 2 – ASSYST population

Significant interactions were observed for seedling emergence between genotypes and treatments (*P*= 0.034, LSD= 31.636) on the selection of the ASSYT population; inoculation with AG 2-1 significantly reduced seedlings emergence in all genotypes except ASSYST-194, -269 and -279 (Table 3.2).

Contrary to that, with seedlings survival there was no significant interaction between genotypes and inoculation treatment (*P* = 0.164, LSD= 21.224) and only ASSYST 269 Tantal had similar survival between treatments (Table 3.2). Survival was significantly reduced in all tested genotypes (*P*< 0.001, LSD= 7.075) with ASSYST 447 Rotabaggeue and ASSYST 209 Ragged Jack to be the most affected by AG 2-1 10 dpi (Table 3.2). No significant interactions were observed between genotypes under inoculation treatment during disease assessments for both hypocotyls (*P*= 0.75, LSD= 18.05) and roots (*P*= 0.86, LSD= 20.87) (Supplementary Table 3.1). On hypocotyls DI ranged from 83.3% (ASSYST 224) to 98.6% (ASSYST 209, 210) and for roots from 83.3% (ASSYST 224) to 97.6% (ASSYST 209, 210). Westar was also susceptible with 100% of DI in both hypocotyls and roots (Supplementary Table 3.1).

Table 3.2 Emergence (%) and survival of seedlings during 10 days of inoculation, in ASSYST population with Westar as test. For the comparison between genotypes $P_{(genotype)}$ and LSD $_{(genotype)}$ were used, for the comparison between different treatments $P_{(inoculum)}$ and $LSD_{(inoculum)}$ were used and for the comparison of between genotypes and different treatments $P_{(genotype*inoculum)}$ and $LSD_{(genotype*inoculum)}$ were used (ANOVA).

	Emergence (%)		Survival (%)	
Genotype	**AG 2-1**	**Control**	**AG 2-1**	**Control**
ASSYST 209	27.8	96.3	39.6	90.3
ASSYST 210	36.1	78.7	45.1	84.7
ASSYST 194	76.8	84	68.8	100
ASSYST 279	71.3	90.7	70.8	95.8
ASSYST 269	71.3	75.9	68.1	87.5
ASSYST 224	45.4	79.6	52.1	87.5
ASSYST 447	21.3	95.4	39.6	100
ASSYST 187	52.8	85.2	67.4	96.5
Westar	50	86.1	45.1	91.7
$P_{(genotype)}$	0.246		0.034	
LSD $_{(genotype)}$	22.37		15.008	
$P_{(inoculum)}$	<0.001		< 0.001	
$LSD_{(inoculum)}$	10.545		7.075	
$P_{(genotype*inoculum)}$	0.034		0.164	
$LSD_{(genotype*inoculum)}$	31.636		21.224	

3.5.3 Group 3: Temple x Canard population

Screening of the TCDH population for 10 days under inoculation with AG 2-1 showed significant differences between genotypes for both emergence (*P*= 0.03, LSD= 38.25) and survival (*P*= 0.016, LSD= 24.17) (Table 3.3). Emergence ranged from 5.56 - 89.92%, although Canard emerged better than Temple, this was not statistically significant (Table 3.3). Similarly, survival ranged from 28.47-87.5%, again mean survival of Canard was better than Temple but this was not significantly different (Table 3.3). However, no differences were detected for disease either on hypocotyls (*P*= 0.906, LSD= 7.98) or roots (*P*= 0.686, LSD= 12.81) (Table 3.3).

Table 3.3 Emergence (%) and survival (%) and Disease index (%) on hypocotyls (DIH) and roots (DIR), of TCDH population and parental lines Temple and Canard under inoculation with AG 2-1 for 10 days. For the comparison between the parents and between different genotypes *P* values and LSD were used (ANOVA).

Trait	Parents		TCDH population				
	Temple	Canard	Mean	Range	*P*	LSD	d.f.
Emergence	33.33	49.07	38.85	5.56-89.82	0.030	38.25	122
Survival	38.19	46.53	48.76	28.47-87.5	0.016	24.17	122
DIH	95.83	93.06	96.97	91.7-100	0.906	7.98	122
DIR	96.03	91.27	94.86	83.3-100	0.686	12.81	122

Temple had more disease compared to Canard for both traits but not statistically different (DIH: t= 0.45, d.f.= 10, *P*= 0.661 and DIR: t= 0.70, d.f.= 10, *P*= 0.501). When the population was screened under non-inoculated conditions for 10 days, significant differences were observed between genotypes for emergence (*P*< 0.001, LSD= 17.41) and survival (*P*< 0.001, LSD= 11.16), emergence varied from 30.53-100% and survival from 54.17-100% (Table 3.4).

As it is explained in the material and methods section (Phenotyping group 3 - TCDH population), due to increased disease levels under inoculation with AG 2-1 for 10 days, a random selection of genotypes was screened again for 5

days. Although, emergence as well survival of seedlings for 5 days was not significantly different between genotypes, were on the limit to be significant (emergence: *P*= 0.058, LSD= 37.338, survival: *P*= 0.055, LSD= 21.56) (Table 3.5).

Table 3.4 Emergence (%) and survival (%) of TCDH population and parental lines Temple and Canard under control (non-inoculated) conditions for 10 days. For the comparison between the parents and between different genotypes *P* values and LSD were used.

Trait	Parents		TCDH population				
	Temple	Canard	Mean	Range	*P*	LSD	d.f.
Emergence	85.19	96.37	90.34	30.56-100	< 0.001	17.41	116
Survival	99.31	100	96.68	54.17-100	<0.001	11.16	116

Table 3.5 Emergence (%) and survival (%) of different genotypes of TCDH population and their parental genotypes, Temple and Canard, under inoculation with AG 2-1 for 5 days. For the comparison between genotypes P values and LSD were used (ANOVA).

Genotype	Emergence (%)	Survival (%)
TCDH 100	44.44	65.6
TCDH 92	48.15	61.1
TCDH 11	55.56	68.9
TCDH 48	72.22	81.1
TCDH 124	83.33	87.8
TCDH 150	83.33	86.7
TCDH 42	92.59	90
TCDH 130	100	92.2
Temple	66.67	75.6
Canard	77.78	76.7
P	0.058	0.055
LSD	37.338	21.56

However, inoculation for 5 days revealed significant differences between the tested genotypes for both disease in hypocotyls (P< 0.001, LSD= 15.85) and roots (P= 0.004, LSD= 22.75) (Figure 3.2). Disease on hypocotyls was lower on genotypes TCDH 130 (58.3 %), -124 (59.7 %) and -42 (70.8 %) while genotype TCDH 92 had the highest level (94.4 %). The parents, Temple and Canard were not significantly different from each other (t= 0.82, d.f.= 10, P= 0.430). Disease on roots was similar; TCDH 130, -124, -42 and -48 had the least disease (49.2 %, 53.2 %, 61.9 % and 58.7 % respectively) and genotype TCDH 92 the most (96 %). Again Temple and Canard were not significantly different from each other (t= -1.20, d.f.= 10, P= 0.259) (Figure 3.2).

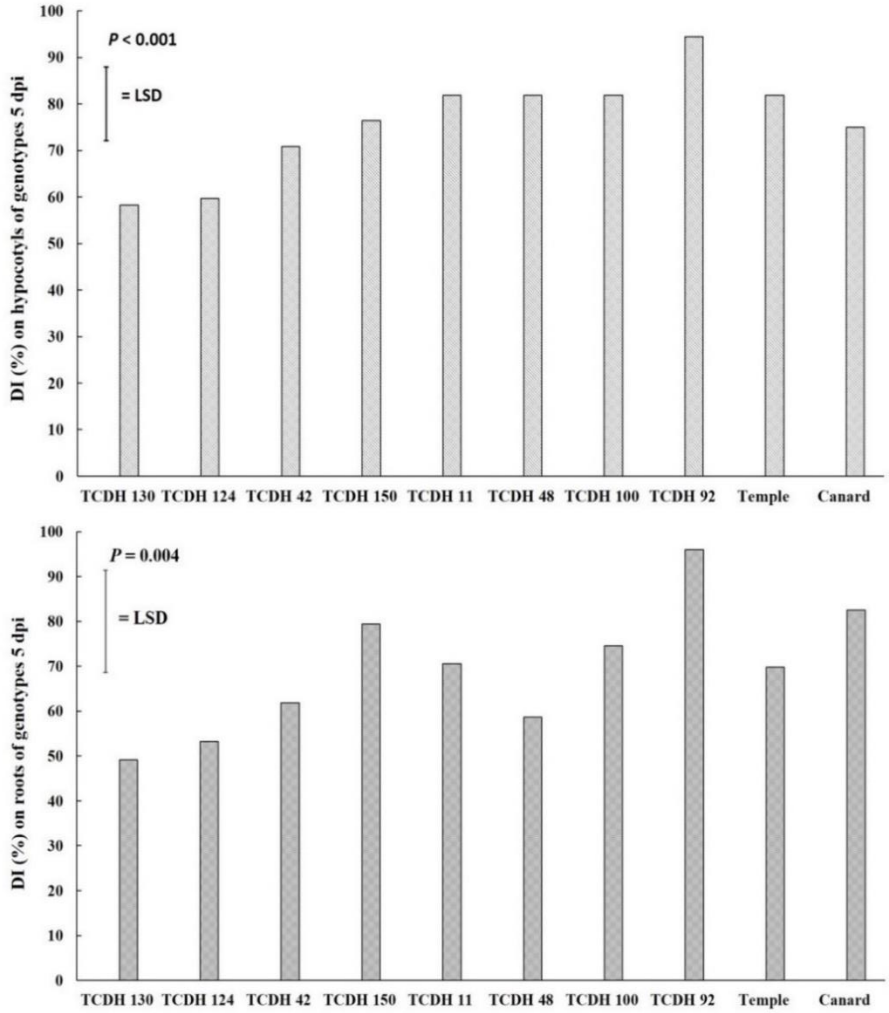


Figure 3.2 Disease index (DI) (%) on hypocotyls and roots of TCDH genotypes and their parents, Temple and Canard, 5dpi with AG 2-1. For comparison between genotypes *P* values and LSD were used (ANOVA).

When Temple and Canard were screened for 5 dpi with genotypes TCDH 24 and TCDH 42, with altered protocol on disease assessment (material and methods, Phenotyping group 3 -TCDH population), neither disease on hypocotyls (*P*= 0.23, s.e.d.= 3.43, d.f.= 227) nor roots (*P*= 0.18, s.e.d.= 5.46, d.f.= 227) (Table 3.6) was significantly different between them.

Table 3.6 Percentage of disease on hypocotyls (DH) and roots (DR) and survival 5 dpi with AG 2-1. For identification of differences between genotypes P values and standard errors of differences (s.e.d) were used (disease: ANOVA, survival: GLM)

Genotype	DH (%)	DR (%)	Survival
TCDH 24	94.4	89.7	0.15
TCDH 42	93.5	84.4	0.28
Temple	90.6	86.3	0.22
Canard	88	77.9	0.40
P	0.23	0.18	0.04
s.e.d	3.43	5.46	0.52
d.f.	227	227	286

However, survival was different between genotypes and parents (*P*= 0.04, s.e.d= 0.52, d.f.= 286). Genotype TCDH 24 (0.15) had the lowest survival 5 dpi, TCDH 42 (0.28) and Temple (0.22) had similar survival and Canard had the best survival (0.40) and statistically significant different from Temple (t= -1.98, d.f.= 118, *P*= 0.05) (Figure 3.3). Temple and Canard were not significantly different for disease on hypocotyls (t= 0.67, d.f.= 118, *P*= 0.507) and roots (t= 1.47, d.f.= 118, *P*= 0.143).

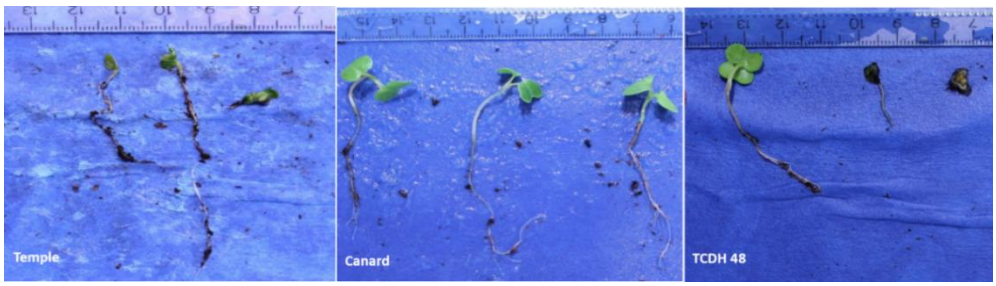


Figure 3.3 Seedlings of Temple (left), Canard (middle) and TCDH 48 (right) 5 dpi with AG 2-1.

3.5.4 OSR and *A. thaliana* in inherited resistance experiment

Screening of the 4 OSR genotypes for 10 dpi did not show significant interactions between genotypes and treatments for emergence (*P*= 0.79, LSD= 18.56). Seedlings emergence in each genotype was similar between the two treatments (Figure 3.4) (*P*= 0.76, LSD= 9.28) but significant differences were observed between genotypes (*P*< 0.001, LSD= 13.12) with Comet having the lowest emergence compared to the other 3 genotypes (Table 3.7). Survival of seedlings over 10 dpi did not reveal any significant interaction between genotypes and treatments (*P*= 0.085, LSD= 16.66), with genotypes having similar survival (Table 3.7). However, when we analyse the survival on the last day of the experiment, it was clear that inoculation with AG 2-1 had significantly reduced seedlings survival by 37% (*P*= 0.0013, LSD= 19.23).

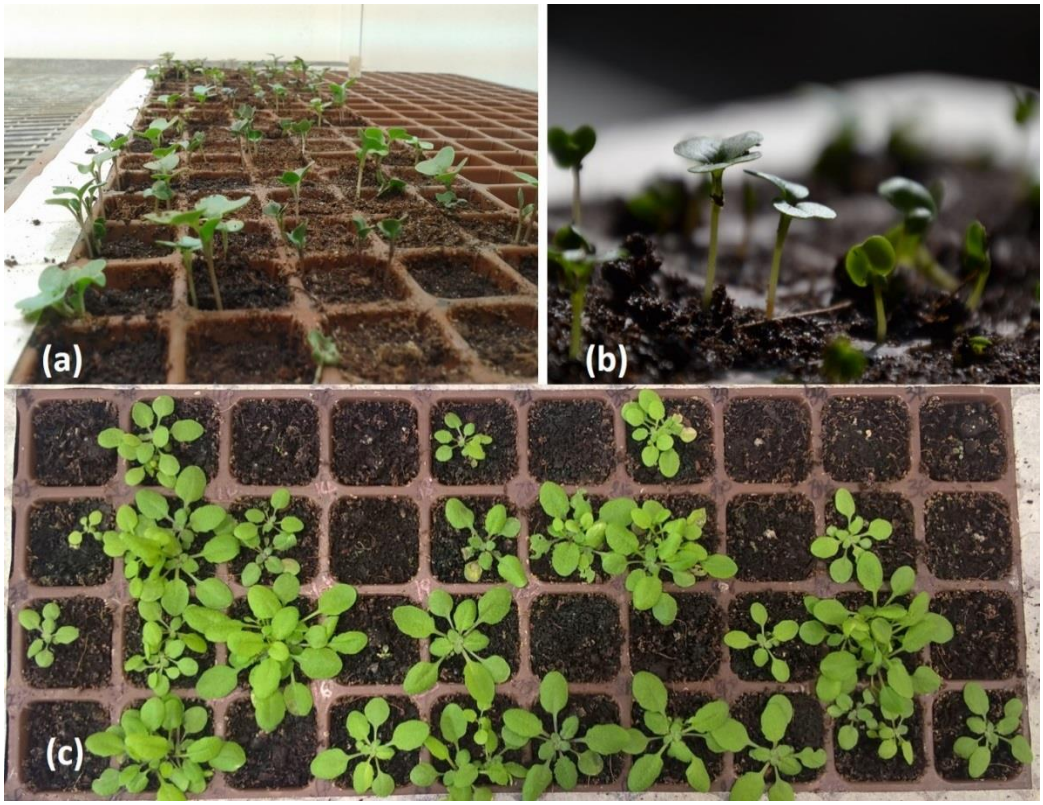


Figure 3.4 OSR seedling under inoculation and control conditions during the first experiment of group 4 (in order to obtain survivors) (a). OSR seedling with hypocotyl rot 10 dpi next to healthy seedling (b). *A. thaliana* seedlings on 21st dpi during the screening of progeny and parents (c).

Table 3.7 Emergence and survival of OSR seedlings under inoculation with AG 2-1 for 10 days. Responses of parental plants or plants derived from plants previously inoculated with AG 2-1 (P+) under inoculation (AG 2-1) or control treatment. For the comparisons *P* values and LSD were used (ANOVA).

	Emergence (%)				**Survival (%)**			
Genotype	**Parents**		**Progeny of (P+)**		**Parents**		**Progeny of (P+)**	
	AG2-1	**Control**	**AG2-1**	**Control**	**AG2-1**	**Control**	**AG2-1**	**Control**
Westar	99.31	99.31	8.33	30.56	94.79	95.83	29.17	46.87
Nugget	95.83	95.83	71.53	73.61	85.42	94.79	73.44	77.6
Comet	49.31	52.78	48.61	75.69	55.73	65.62	53.65	81.25
ES Betty	83.33	74.31	42.36	48.61	76.56	85.42	53.65	61.46
$P_{(genotype)}$	< 0.001		0.004		0.001		0.008	
$LSD_{(genotype)}$	13.12		27.84		11.78		21.02	
$P_{(inoculum)}$	0.76		0.144		0.083		0.058	
$LSD_{(inoculum)}$	9.28		19.68		8.33		14.86	
$P_{(inoculum*genotype)}$	0.79		0.75		0.085		0.66	
$LSD_{(inoculum*genotype)}$	18.56		39.37		16.66		29.73	

Screening of plants derived from the inoculated survivors, showed no interaction between genotypes and treatments neither on emergence (*P*= 0.75, LSD= 39.37) nor on survival (*P*= 0.66, LSD= 29.73) (Table 3.7). Although survival was on the limit to be significant between treated and inoculated seedlings over 10 dpi (*P*= 0.058, LSD= 14.86), when was analysed only for the last day of the experiment significant difference was observed between treatments, with AG 2-1 to significantly reduce survival of seedlings by 59.6% (*P*= 0.006, LSD= 19.94).

When we compared emergence and survival of progeny plants to their ancestors no significant interaction was observed between treatment and origin for emergence (*P*= 0.507, LSD= 35.58) and survival (*P*= 0.673, LSD= 25.16) (Supplementary Table 3.2). However, origin alone did affect emergence of seedlings and survival with progeny seedlings having 38.63% reduced emergence (*P*= 0.019, LSD= 25.16) and 27.2% reduced survival (*P*= 0.018, LSD= 17.68) compared to their parents (Supplementary Table 3.2). Emergence between progeny and parental plants on AG 2-1 treatment was significantly different (t= 2.26, d.f.= 6, *P*= 0.032) with progeny having reduced emergence compared to parental plants. However, this effect was lost on the control treatment where both had similar emergence (t= 1.54, d.f.= 6, *P*= 0.088). Under inoculation with AG 2-1 survival of progeny was significantly reduced (t= 2.08, d.f.= 6, *P*= 0.041) while on the control treatment there was no significant difference between them (t= 1.76, d.f.= 6, *P*= 0.064).

Similarly to OSR, no significant interaction was observed between treatment and origin of *A. thaliana* seedlings neither for emergence (Figure 3.4) (*P*= 0.579, LSD= 8.54) nor for survival (*P*= 0.813, LSD= 7.819) (Table 3.8). Survival on the last day (21 dpi) (Figure 3.4) show that inoculation with AG 2-1 reduces seedling survival compare to control seedlings (*P*= 0.009, LSD= 6.82) but it is independent from seedlings origin (*P*= 0.219, LSD= 6.82).

Table 3.8 Emergence and survival of *A. thaliana* seedlings from plants derived from plants under inoculation (Parent +) or non-inoculated control (Parent -) under inoculation (AG 2-1) and control treatment. For the comparisons P values and LSD were used (ANOVA).

	Emergence (%)		Survival (%)	
Origin	AG2-1	Control	AG2-1	Control
Parent (+)	13.62	12.5	33.5	37
Parent (-)	16	18.25	37	41.25
P	0.579		0.893	
LSD	8.54		7.819	

3.6 Discussion

Identification of resistant OSR germplasm to *R. solani* AG 2-1 has been a challenge for many years. *Brassica* species are characterized by close genetic relations and therefore many studies have also included other cultivated or wild species such as *B.rapa*, *B. oleracea*, *B. carinata*, *B. juncea*, *Sinapis alba*, *Camelina sativa* (Acharya et al., 1984, Babiker et al., 2013, Yang and Verma, 1992). However, no complete resistance has been identified in OSR but only differences between and within species susceptibility to AG 2-1 (Acharya et al., 1984, Yang and Verma, 1992). Here we attempted to identify resistance/tolerance of OSR to AG 2-1 by screening a number of different commercial cultivars, a selection of germplasm from diversity sets and a mapping population.

Our screening was separated into different groups; we first screened (group 1) a range of different genotypes that have been in the recommended lists for UK in addition with two genotypes from two populations, one *B. rapa* genotype, MINELESS and Westar. All of these genotypes were highly susceptible to AG 2-1. Emergence and survival of seedlings was negatively affected by AG 2-1 during the 10 dpi and although genotypes were different on their emergence and survival, we were unable to detect significant interactions between them under inoculation. Additionally, disease symptoms were severe in hypocotyls and in roots, although in general roots seem to have less disease compared to hypocotyls. There was variation between genotypes with ASSYST 224 having the least disease and Comet and *B. rapa* having the highest. Westar has been

used previously as a control cultivar during screening against AG 2-1 (Yang and Verma, 1992), here was also susceptible. The choice for screening MINELESS was made based on the fact that the direct role of GSL as defensive compounds against *R. solani* is debatable; in in vitro experiments, both aliphatic and aromatic isothiocyanates (ITCs) were toxic to five cereal pathogens, including *R. solani* (although the authors did not specify which AG was tested) and *Gaeumanomyces graminis* var. *tritici* which were very sensitive (Sarwar et al., 1998). Also seed coating with seed meal of *B. juncea* and a chemical carrier was sufficient to eliminate damping-off disease in *B. oleracea* caused by AG 4 (Chung et al., 2002). Nonetheless, Smith and Kirkegaard showed that the variability within *R. solani* is also reflected on its responses to 2-phenylethyl ITC in vitro (Smith and Kirkegaard, 2002). Thus, the response of a certain AG to GSL compounds is vague. That said, there is no study focusing on the effect of GSL from OSR on AG 2-1. Here we aimed to identify if GSL have a role the interaction with AG 2-1 and we hypothesised that GSL will enhance defence towards AG 2-1, therefore we expected that the transgenic genotype would be more susceptible compared to the background Westar. Unfortunately, Westar's susceptibility to AG 2-1 infection was a drawback and no differences were observed between Westar and MINELESS, thus we did not proceed with further experiments with these genotypes.

ASSYST 224 had the least disease for both hypocotyls and roots and the highest survival of seedlings. This genotype is from a well-defined population of diverse *B. napus* genotypes, hence screening other genotypes of this population was a logical step forwards, as the potential identification of tolerance or resistance could lead to the discovery of quantitative trait loci (QTL). We proceeded with screening of group 2, a selection of different ASSYST genotypes with Westar and ASSYST 224 as control genotypes. However, all genotypes were highly susceptible to infection of AG 2-1 when exposed for 10 days. Disease symptoms in hypocotyls and roots were severe and no differences were detected between genotypes. ASSYST 224 was the genotype with the least disease, although still high (83.3% for both hypocotyls and roots) and Westar was the genotype with the most disease reaching 100% both in hypocotyls and roots. Nevertheless, emergence of seedlings between

genotypes was different and genotypes ASSYST 269, -194 and -279 emerged equally well with their control seedlings. However, these genotypes had significant lower survival compare to their controls, despite the fact that these genotypes had the highest survival under inoculation compare to other genotypes. Regarding ASSYST 224 and Westar emergence and survival was slightly altered compare to their previous screening but with similar responses to AG 2-1 infection.

In previous screening (Drizou et al., 2017) we identified that genotypes Temple and Canard had contrasting survival under inoculation with AG 2-1 at 10 dpi. These genotypes have been used to produce the doubled haploid mapping population (TCDH: Temple Canard Doubled Haploid) (https://www.jic.ac.uk/staff/ian-bancroft/mapping.html). Considering that the parental genotypes had contrasting responses we decided to screen the population under the same conditions. Screening for 10 dpi showed significant differences between genotypes' emergence from 5.7 % to 89.8 % and similar survival from 28.5 % to 87.5 %. Nonetheless, Temple and Canard had similar emergence and survival. We also screened the genotypes under non-inoculate conditions to obtain any genotype differences on emergence and survival. Genotypes differed in emergence and survival but in general their performance was good with mean emergence of 90.3% and mean survival of 96.7 %, compared to 38.8 % and 48.8 % respectively under inoculation. Similarly to groups 1 and 2, inoculation with AG 2-1 for 10 days resulted in high disease level and we were unable to discriminate any tolerance. Thus, we decided to reduce the inoculum pressure by eliminating the time that the seedlings were exposed to the pathogen.

Minimising the days of exposure to the pathogen resulted in better emergence and survival and less disease. Although, we could not detect significant differences in emergence and survival of the selected genotypes, there was a variation of 55.6 % and 31.2 % respectively and genotypes TCDH 130 and -42 had the best performance. Disease, however was significantly reduced compared to the 10 dpi screening, with rates starting from <60% for disease on hypocotyls and 50% for disease on roots. Tested genotypes responded significantly different to inoculation with AG 2-1, with TCDH 130 and -124

having the least disease on hypocotyls and roots, followed by TCDH 42. In contrast, TCDH 92 (DIH= 94.4 % and DIR= 96 %). Temple and Canard were not significantly different from each other for neither DIH nor DIR. Although in hypocotyls Temple had more severe symptoms compared to Canard this was different when we looked at disease symptoms on roots. These results showed that screening for 5 dpi is a more suitable method for the detection of differences in disease symptoms and therefore we decided to screen all the population with this protocol. By the time that the first experimental replicate completed, we were acknowledged that the two thirds of the population were not segregating and that they were identical with the parent Temple, thus we only present the results from this experimental replicate (Supplementary Table 3.3). Nevertheless, we used these results and selected TCDH 24 and TCDH 42, belonging to the one third of the TCDH population that was segregating and screened them with the parents. From this screening, no significant differences were observed between the two genotypes and the parents for disease both on hypocotyls and roots. Survival of seedlings however, was significantly different, with Canard having the best survival, two times better than Temple (Canard: 0.40, Temple: 0.22). TCDH 42 survived similar to Temple and TCDH 24 had the worst survival (0.15).

Overall, screening of genotypes in these groups confirmed previous studies that AG 2-1 is extremely pathogenic to OSR and there is no resistance (Acharya et al., 1984, Babiker et al., 2013, Kataria and Verma, 1992, Khangura et al., 1999, Sturrock et al., 2015, Yang and Verma, 1992). In these studies screening was conducted with different methods for both inoculation and assessment of response to infection. Here exposure of seedlings for 10 days in combination with the highly pathogenic AG 2-1 resulted in increased disease level. Our choice to reduce the exposure to AG 2-1 to 5 dpi, during the screening of TCDH population, appeared to be good. Probably screening of genotypes in the other two groups for 5 days would have enabled us to identify more striking differences on group 1 and significant differences between genotypes on group 2. Even with this possibility, the current data prove that there is lack of resistance in the tested germplasm. Nonetheless, the significant differences in emergence (group 1 and group 3) and survival (group 3) of

tested germplasm may indicate the existence of traits that may be help to escape disease by AG 2-1. It is known that infection by AG 2-1 prevents seedlings germination (pre-emergence damping-off) and seedlings development due to root and hypocotyl rot (post-emergence damping-off) (Kataria and Verma, 1992). An infection process that has been shown, using X-ray computed tomography, to result in complete maceration of roots and hypocotyls within 6 dpi (Sturrock et al., 2015). Therefore, it is expected faster emergence and growth of seedlings to benefit OSR to escape infection by AG 2-1.

In order to evaluate the findings from Yang and Verma (1992) and our hypothesis that resistance to AG 2-1 could be a transgenerational defence induction, we screened four OSR cultivars: Westar, Nugget, Comet and ES Betty. Initial screening of the genotypes, detected no significant difference on their emergence and survival 10 dpi and all they performed equally well to their non-inoculated controls, also all had better emergence and survival compare their screening on group 1. Although these results implied that inoculation did not affect their survival, the analysis on the last day show that AG 2-1 infection actually had negative impact by reducing it significantly compared to the controls. When progenies were screened again, no significant interactions were observed between genotypes and treatments but survival was significantly reduced on the last day of the experiment on inoculated seedlings. When we compared the results of the progenies to the original parental lines, we did not detect any significant interaction between treatment and origin of the seeds on emergence and survival. However, origin of seeds alone appeared to be a significant factor for both seedlings emergence and survival; seedlings originating from previously AG 2-1 infected plants had significantly reduced emergence and survival.

Rhizoctonia solani AG 2-1 has been shown to be highly pathogenic to *A. thaliana*; a range of 36 ecotypes had been screened previously and all appeared to be susceptible to AG 2-1 7 dpi with survival rates less than 33% (Foley et al., 2013). However, similar to OSR, *A. thaliana* seedlings did not show improved performance as the result of previous infection of parental plants. Our results do not support neither our hypothesis, that AG 2-1 infection can

result in improved performance/ transgenerational defence induction nor the argument that selection could potentially result in improved resistance to AG 2-1, at least for the tested OSR germplasm and *A. thaliana* Col. 0 under these experimental conditions. A recent study showed that although exposure of *A. thaliana* plants to volatile organic compounds (VOCs) from *R. solani* AG 2-2 IIB primes plant growth, does not affect disease resistance to the pathogen (Cordovez et al., 2017). Also, this priming effect of VOC-triggered plant growth was not transgenerational, as seedlings originated from VOC-exposed parents had not different root weight compared to seedlings from control parents and had slightly reduced shoot weight (Cordovez et al., 2017). Although this study partly supports the finding of the present work, it is important to take into account that they examined the effect of VOCs on priming and resistance and not the direct contact with the pathogen.

The fact that the 4 OSR genotypes were susceptible from the first screening and they had only minor differences between them in addition to the increased inoculum pressure, as we stated before, probably also affected this outcome. Although, the parental plants were survivors, they were suffering due to infection even in later stages and probably this affected their ability to set healthy seeds; seeds of progenies were smaller compare to parental seeds. It is known that small seed size is linked with lower emergence and seedlings survival (Wei and Darmency, 2008), so it is likely that OSR progenies performed worse compared to their parents because of the quality of the seeds. Following this we need to point out that a transgenerational induction also can be a result of maternal effect (Holeski et al., 2012), where maternal plants affect the seed quality and therefore the offspring. If this is the case it seems that we have a transgenerational induction of susceptibility rather resistance, although such a conclusion is largely hypothetical. Nonetheless, the fact that in both OSR and *A. thaliana*, there was no interaction between treatment and seeds' origin leads to the conclusion that previous infection with AG 2-1 does not result in better performance of progenies and therefore induced defence in this case seems not to be an inherited trait. Future work could confirm the strength of this observation by testing more generations.

3.7 Conclusions

The present study confirms that AG 2-1 is a very pathogenic isolate for OSR germplasm and results in high disease level and reduced seedling performance. However, we consider that the increased inoculum pressure probably prevented us to detect differences that could indicate tolerance. Future work could be benefited from this and perform screenings under 5 dpi. Additionally, it seems that resistance of OSR to AG 2-1 is unlikely to be the result of a transgenerational defence induction, inherited as result of epigenetic stress responses.

3.8 Abbreviations

OSR: oilseed rape, AG: anastomosis group, TCDH: Temple x Canard Doubled Haploid, cv: cultivar, PGA: potato glucose agar, dpi: days post inoculation, GSL: glucosinolates, P+: plants produced from inoculated plants, P- : plants produced from control plants, ANOVA: analysis of variance, GLM: generalised linear model, s.e.d: standard errors of differences, d.f.: degrees of freedom, ITCs: isothiocyanates, QTL: quantitative trait loci, VOCs: volatile organic compounds.

3.9 References

ACHARYA, S. N., VERMA, P. R., DUECK, J. & DOWNEY, R. K. 1984. Screening rapeseed/canola for resistance to damping-off and seedling root rot caused by *Rhizoctonia solani. Canadian Journal of Plant Pathology,* 6**,** 325-328.

AJAYI-OYETUNDE, O. O., BUTTS-WILMSMEYER, C. J. & BRADLEY, C. A. 2017. Sensitivity of *Rhizoctonia solani* to Succinate Dehydrogenase Inhibitor and Demethylation Inhibitor Fungicides. *Plant Disease,* 101**,** 487-495.

ANDERSON, N. A. 1982. The Genetics and Pathology of *Rhizoctonia solani*. *Annual Review of Phytopathology,* 20**,** 329-347.

BABIKER, E., HULBERT, S., SCHROEDER, K. & PAULITZ, T. 2013. Evaluation of *Brassica* species for resistance to Rhizoctonia solani and binucleate Rhizoctonia (Ceratobasidum spp.) under controlled environment conditions. *European Journal of Plant Pathology,* 136**,** 763-772.

BORGEN, B. H., THANGSTAD, O. P., AHUJA, I., ROSSITER, J. T. & BONES, A. M. 2010. Removing the mustard oil bomb from seeds: transgenic ablation of myrosin cells in oilseed rape (*Brassica napus*) produces MINELESS seeds. *Journal of Experimental Botany,* 61**,** 1683-1697.

BRUCE, T. J. A. 2014. Glucosinolates in oilseed rape: secondary metabolites that influence interactions with herbivores and their natural enemies. *Annals of Applied Biology,* 164**,** 348-353.

BRUCE, T. J. A., MATTHES, M. C., NAPIER, J. A. & PICKETT, J. A. 2007. Stressful "memories" of plants: Evidence and possible mechanisms. *Plant Science,* 173**,** 603-608.

CHALHOUB, B., DENOEUD, F., LIU, S., PARKIN, I. A. P., TANG, H., WANG, X., CHIQUET, J., BELCRAM, H., TONG, C., SAMANS, B., CORRÉA, M., DA SILVA, C., JUST, J., FALENTIN, C., KOH, C. S., LE CLAINCHE, I., BERNARD, M., BENTO, P., NOEL, B., LABADIE, K., ALBERTI, A., CHARLES, M., ARNAUD, D., GUO, H., DAVIAUD, C., ALAMERY, S., JABBARI, K., ZHAO, M.,

EDGER, P. P., CHELAIFA, H., TACK, D., LASSALLE, G., MESTIRI, I., SCHNEL, N., LE PASLIER, M.-C., FAN, G., RENAULT, V., BAYER, P. E., GOLICZ, A. A., MANOLI, S., LEE, T.-H., THI, V. H. D., CHALABI, S., HU, Q., FAN, C., TOLLENAERE, R., LU, Y., BATTAIL, C., SHEN, J., SIDEBOTTOM, C. H. D., WANG, X., CANAGUIER, A., CHAUVEAU, A., BÉRARD, A., DENIOT, G., GUAN, M., LIU, Z., SUN, F., LIM, Y. P., LYONS, E., TOWN, C. D., BANCROFT, I., WANG, X., MENG, J., MA, J., PIRES, J. C., KING, G. J., BRUNEL, D., DELOURME, R., RENARD, M., AURY, J.-M., ADAMS, K. L., BATLEY, J., SNOWDON, R. J., TOST, J., EDWARDS, D., ZHOU, Y., HUA, W., SHARPE, A. G., PATERSON, A. H., GUAN, C. & WINCKER, P. 2014. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. *Science,* 345**,** 950-953.

CHUNG, W. C., HUANG, J. W., HUANG, H. C. & JEN, J. F. 2002. Effect of ground *Brassica* seed meal on control of *Rhizoctonia* damping-off of cabbage. *Canadian Journal of Plant Pathology,* 24**,** 211-218.

CORDOVEZ, V., MOMMER, L., MOISAN, K., LUCAS-BARBOSA, D., PIERIK, R., MUMM, R., CARRION, V. J. & RAAIJMAKERS, J. M. 2017. Plant Phenotypic and Transcriptional Changes Induced by Volatiles from the Fungal Root Pathogen *Rhizoctonia solani*. *Frontiers in Plant Science,* 8.

CUBETA, M. A. & VILGALYS, R. 1997. Population biology of the *Rhizoctonia solani* complex. *Phytopathology,* 87**,** 480-484.

DRIZOU, F., GRAHAM, N. S., BRUCE, T. J. A. & RAY, R. V. 2017. Development of high-throughput methods to screen disease caused by *Rhizoctonia solani* AG 2-1 in oilseed rape. *Plant Methods,* 13**,** 45.

FOLEY, R. C., GLEASON, C. A., ANDERSON, J. P., HAMANN, T. & SINGH, K. B. 2013. Genetic and Genomic Analysis of Rhizoctonia solani Interactions with Arabidopsis; Evidence of Resistance Mediated through NADPH Oxidases. *Plos One,* 8.

GHORBANI, R., WILCOCKSON, S., KOOCHEKI, A. & LEIFERT, C. 2009. *Soil Management for Sustainable Crop Disease Control: A Review,* New York, Springer.

GUPTA, S. K. 2012. *Brassicas,* New York, Springer.

HÉMATY, K., CHERK, C. & SOMERVILLE, S. 2009. Host–pathogen warfare at the plant cell wall. *Current Opinion in Plant Biology,* 12**,** 406-413.

HOLESKI, L. M., JANDER, G. & AGRAWAL, A. A. 2012. Transgenerational defense induction and epigenetic inheritance in plants. *Trends in Ecology & Evolution,* 27**,** 618-626.

KATARIA, H. R. & VERMA, P. R. 1992. *Rhizoctonia solani* damping-off and root rot in oilseed rape and canola. *Crop Protection,* 11**,** 8-13.

KHANGURA, R. K., BARBETTI, M. J. & SWEETINGHAM, M. W. 1999. Characterization and pathogenicity of *Rhizoctonia* species on canola. *Plant Disease,* 83**,** 714-721.

KIRKEGAARD, J. A., SARWAR, M., WONG, P. T. W., MEAD, A., HOWE, G. & NEWELL, M. 2000. Field studies on the biofumigation of take-all by *Brassica* break crops. *Australian Journal of Agricultural Research,* 51**,** 445-456.

KIRKEGAARD, J. A., WONG, P. T. W. & DESMARCHELIER, J. M. 1996. In vitro suppression of fungal root pathogens of cereals by *Brassica* tissues. *Plant Pathology,* 45**,** 593-603.

LAMPRECHT, S. C., TEWOLDEMEDHIN, Y. T., CALITZ, F. J. & MAZZOLA, M. 2011. Evaluation of strategies for the control of canola and lupin seedling diseases caused by *Rhizoctonia* anastomosis groups. *European Journal of Plant Pathology,* 130**,** 427-439.

LI, L. X., LUO, Y. J., CHEN, B. Y., XU, K., ZHANG, F. G., LI, H., HUANG, Q., XIAO, X., ZHANG, T. Y., HU, J. H., LI, F. & WU, X. M. 2016. A Genome-Wide Association Study Reveals New Loci for Resistance to Clubroot Disease in Brassica napus. *Frontiers in Plant Science,* 7**,** 12.

SARWAR, M., KIRKEGAARD, J. A., WONG, P. T. W. & DESMARCHELIER, J. M. 1998. Biofumigation potential of *brassicas* - III. In vitro toxicity of isothiocyanates to soil-borne fungal pathogens. *Plant and Soil,* 201**,** 103-112.

SMITH, B. J. & KIRKEGAARD, J. A. 2002. In vitro inhibition of soil microorganisms by 2-phenylethyl isothiocyanate. *Plant Pathology,* 51**,** 585-593.

STODART, B. J., HARVEY, P. R., NEATE, S. M., MELANSON, D. L. & SCOTT, E. S. 2007. Genetic variation and pathogenicity of anastomosis group 2 isolates of *Rhizoctonia solani* in Australia. *Mycological Research,* 111**,** 891-900.

STURROCK, C. J., WOODHALL, J., BROWN, M., WALKER, C., MOONEY, S. J. & RAY, R. V. 2015. Effects of damping-off caused by *Rhizoctonia solani* anastomosis group 2-1 on roots of wheat and oil seed rape quantified using X-ray Computed Tomography and real-time PCR. *Frontiers in Plant Science,* 6.

VERMA, P. R. 1996. Biology and control of *Rhizoctonia solani* on rapeseed: A review. *Phytoprotection,* 77**,** 99-111.

WEI, W. & DARMENCY, H. 2008. Gene flow hampered by low seed size of hybrids between oilseed rape and five wild relatives. *Seed Science Research,* 18**,** 115-123.

YANG, J. & VERMA, P. R. 1992. Screening genotypes for resistance to pre-emergence damping-off and postemergence seedling root rot of oilseed rape and canola caused by *Rhizoctonia solani* AG-2-1. *Crop Protection,* 11**,** 443-448.

ZEUN, R., SCALLIET, G. & OOSTENDORP, M. 2013. Biological activity of sedaxane a novel broad-spectrum fungicide for seed treatment. *Pest Management Science,* 69**,** 527-534.

3.10 Supplementary data

Supplementary Table 3.1 Disease index in hypocotyls (DIH) and roots (DIR) 10 dpi of tested genotypes on ASSYST population and cultivar Westar. For the comparison between the genotypes *P* values and LSD were used (ANOVA).

Genotype	DIH	DIR
ASSYST 209	98.6	97.6
ASSYST 210	98.6	97.6
ASSYST 194	93.1	92.9
ASSYST 279	93.1	89.7
ASSYST 269	94.4	92.1
ASSYST 224	83.3	83.3
ASSYST 447	97.2	89.7
ASSYST 187	94.4	92.9
Westar	100	100
P	0.75	0.86
LSD	18.05	20.87

Supplementary Table 3.2 Emergence and survival of parental OSR seedlings and progenies of inoculated parents (progeny) under inoculation with AG 2-1 for 10 days. For the comparisons *P* values and LSD were used (ANOVA).

	Emergence (%)		Survival (%)	
Origin	AG2-1	Control	AG2-1	Control
Parent	81.9	80.6	78.1	85.4
Progeny	42.7	57.1	52.5	66.8
$P_{(origin)}$	0.019		0.0018	
$LSD_{(origin)}$	25.16		17.68	
$P_{(treatment)}$	0.583		0.208	
$LSD_{(treatment)}$	25.16		17.68	
$P_{(origin*treatment)}$	0.507		0.673	
$LSD_{(origin*treatment)}$	35.58		25	

Supplementary Table 3.3 Percentage of disease on hypocotyls (DH) and roots (DR) and survival 5 dpi with AG 2-1. For identification of differences between genotypes *P* values and standard errors of differences (s.e.d) were used (disease: ANOVA, survival: GLM)

Trait	Parents		TCDH population				
	Temple	Canard	Mean	Range	*P*	s.e.d	d.f.
Survival	0.725	0.726	-	0-1	0.055	67.5	108
DH (%)	91.33	80.44	86.34	58.65-100	<0.001	8.95	108
DR (%)	83.51	91.22	90.56	71.34-100	<0.001	8.18	108

Chapter 4. **Infestation by *Myzus persicae* increases susceptibility of *Brassica napus* cv. 'Canard' to *Rhizoctonia solani* AG 2-1**

This chapter presents the indirect interaction between AG 2-1 and *M. persicae* on OSR cultivars 'Temple' and 'Canard'. Also examines plant defences in cv 'Canard' against AG 2-1 and *M. persicae* estimated as induced changes on gene expression. This chapter is in preparation to be submitted.

4.1 Author contribution

Practical work was designed and performed by F Drizou under the supervision of N Graham, R Ray and T Bruce. The manuscript was composed by F Drizou with the contribution of N Graham, R Ray and T Bruce.

4.2 Abstract

Plants have the ability to defend themselves against herbivorous insects and plant pathogens. Understanding the complicated interactions triggering plant defence mechanism is of great interest as it may allow the development of more effective and sustainable disease control methods. *Myzus persicae* and *Rhizoctonia solani* anastomosis group (AG) 2-1 are two important organisms attacking oilseed rape (OSR), causing disease and reduced yields. At present, is unclear how these two interact with each other and with OSR defences and therefore the aim of the present study was to gain a better insight into the indirect interaction between aphids and pathogen. In separate experiments, we assessed the effect of AG 2-1 infection on aphid performance, measured as growth rate and population increase and then the effect of aphid infestation on AG 2-1 by quantifying disease and the amount of fungal DNA in plant stems and compost for two OSR varieties, 'Canard' and 'Temple'. Additionally, we examined the expression of genes related to jasmonic acid (JA) and salicylic acid (SA) defence pathways. There was no significant effect of AG 2-1 infection on *M. persicae* performance. However, aphid infestation in one of the varieties, 'Canard', resulted in significantly increased disease symptoms caused by AG 2-1, although, the amount of fungal DNA was not significantly different between treatments. Expression of *LOX3* and *MYC2* was elevated under AG 2-1 treatment but downregulated in plants with both aphids and pathogen. Therefore it seems plausible that alterations in the JA signalling due to aphid infestation resulted in the increased susceptibility to AG 2-1.

Key words: *R. solani* AG 2-1, *Myzus persicae*, oilseed rape, indirect interaction, plant defences, jasmonic acid, salicylic acid

4.3 Introduction

Plants are exposed to a variety of attacking organisms aboveground and belowground, including pathogens and herbivorous insects. Due to natural selection, host plants and their enemies have coevolved and are subjected to a constant arms race for their survival (Occhipinti, 2013). Plants are able to defend themselves either with constitutive or with more energy-effective inducible defences, additionally in response to plant defence mechanisms, enemies have also evolved counteracting defences (Bruce, 2015, Glazebrook, 2005, Pieterse and Dicke, 2007). The interactions between an attacker and a host plant embrace the recognition of herbivore associated molecular patterns (HAMPs) or pathogen associated molecular patterns (PAMPs) (for herbivorous insects and pathogens respectively) by the plant which lead to plant triggered immunity (PTI). However, herbivores and pathogens are able to overcome this first layer of plant defences, by the secretion of effectors and plants respond with a second layer of defences named effectors triggered immunity (ETI) (Bruce, 2015, Jaouannet et al., 2014, Wang et al., 2014). Chemical defences and secondary metabolites also have a crucial role in plant defences (Bruce, 2015). Plant hormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) are known to play a key role. JA and SA signalling are thought to be the most important with JA to be activated against herbivory by chewing insects, wounding and necrotrophic pathogens, whilst SA against biotrophic pathogens and phloem feeding insects (Glazebrook, 2005, Vos et al., 2013). Although SA and JA often act antagonistically through a cross-talk, recent studies provide evidence that SA and JA can also act in a synergistic way (Liu et al., 2016) and their activation is highly dependent on the nature of the attacker (feeding guild of herbivore and lifestyle of the pathogen) as well as the plant species (Bari and Jones, 2009, Glazebrook, 2005). More complex interactions are taking place when different attackers share the same host as these organisms interact with each other indirectly through the induced changes in host-plant's biochemistry, chemistry and nutritional status (Lazebnik et al., 2014, Schultz et al., 2013). A study showed that when *Aphis fabae* fed on *Vicia faba* plants infected with *Botrytis cinerea* (necrotroph) individual aphids development was negatively affected but when the plants

were infected with *Uromyces viciae-fabae* (biotroph) aphids had a better performance, nonetheless during simultaneously infection aphids performed equally well as on the control plants (Al-Naemi and Hatcher, 2013). These results were related with the induced-alterations in nitrogen content after pathogen infection and the authors also speculated that possibly a cross talk occurred between JA botrytis-induced defences and SA *U. viciae fabae*-induced defences (Al-Naemi and Hatcher, 2013). Another study by Drakulic et al., showed that when *Sitobion avenae* aphid and *Fusarium graminearum* fungi share the same wheat plant, the fungi is benefited whilst the aphids are not: the authors detected that during dual attack disease severity was increased but aphid survival was decreased (Drakulic et al., 2015). However, in both studies the authors did not examine the changes in the JA and SA signalling pathways and their role in the observed interactions.

Considering that in agroecosystems plants are exposed to multiple attackers, the fine-tuning of their defences is a key factor determining their fitness (Vos et al., 2013). Understanding the fundamental mechanisms and evolution of plant defences is a crucial step for the development of sustainable control methods in agriculture. This is of great importance considering that chemical control methods are either failing, due to the ability of pests to gain resistance against them (Bass et al., 2011, Puinean et al., 2010) or are restricted due to their harmful effects on non-target beneficial organisms in the ecosystem (Simon-Delso et al., 2015).

The plant family Brassicaceae consists of many important agricultural crops including, oilseed rape (OSR), *Brassica napus*, a polyploid species result of crossing *Brassica rapa* and *Brassica oleracea* (Chalhoub et al., 2014). OSR is one of the most cultivated and profitable crops worldwide (FAOSTAT). Additionally, as with the other members of this plant family, OSR has specialised chemistry due to the production of glucosinolates (GSL) and their breakdown products that are involved in plant defences against herbivorous insects and pathogens (Schoonhoven et al., 2005, van Dam et al., 2009). OSR is the host for the soil-borne necrotrophic pathogen *Rhizoctonia solani* (Kühn). This pathogen is characterized by great genetic variability: it is divided into 13 anastomosis groups (AG), each specialised to a certain plant host (Ogoshi,

1987, Parameter, 1970). Isolates belonging to AG 2-1 are the most pathogenic for OSR; under favourable environmental conditions they infect seedlings and cause damping off disease (Kataria and Verma, 1992, Khangura et al., 1999). Disease in this early stage leads to reduced crop establishment and consequently yield loss. Although, many studies have attempted to identify resistance traits in *B. napus*, resistant germplasm has not been identified and it remains a mystery how AG 2-1 suppresses or avoids plant defences (Babiker et al., 2013, Sturrock et al., 2015). Oilseed rape is one of the secondary hosts of the peach-potato aphid *Myzus persicae* (Sulzer). This aphid is a particularly important pest, not only because of the direct damage it causes but also because it is the vector for more than 100 plant viruses (Blackman and Eastop, 2000). It is a very effective plant herbivore, able to gain resistance against plant defences and even the most effective insecticides, including neonicotinoids (Bass et al., 2011). Currently it is unknown how and if *M. persicae* and *R. solani* AG 2-1 indirectly interact with each other when they share the same host-OSR and how host-plant responds to this dual attack.

The aim of the present study was to identify if there is an interaction between herbivory by *M. persicae* and infection by AG 2-1 in *B. napus* and consequently gain a better insight into OSR defences against two major attacking organisms. We first explored if infection with AG 2-1 had a negative effect on aphid performance, measured in relation to growth and population increase. Secondly, we examined if infestation of *M. persicae* affects the plant's ability to defend itself against AG 2-1 infection, by assessing the disease level and quantifying fungal DNA in plants and compost. Plant performance was estimated by measuring the fresh weight. Additionally, in order to obtain a better insight of the interaction, we examined the expression of genes involved JA and SA signalling.

4.4 Materials and Methods

4.4.1 Plant growth

Brassica napus plants of cultivars (cv) 'Temple' and 'Canard', were grown in a controlled environment room (18 ^{o}C ± 2, 12 h light: 12 h dark) for 3-4 weeks prior to experiments. Seeds originally sown in a mixture of 50% perlite

standard (Sinclair Pro UK) and 50% traysubstrat (Klasmann-Deilmann GmbH, Germany), a week later transplanted in pots (9 cm) with Levington M3 compost (Everris Limited UK).

4.4.2 Aphids and inoculum

Peach-potato aphid, *M. persicae* (ISIL clone), originally obtained from a colony maintained at Rothamsted Research was reared on oilseed rape plants, cultivar 'Westar' under controlled conditions (18 ^{o}C ± 2, 12 h light: 12h dark). *Rhizoctonia solani* AG 2-1 (#1934 from the isolate collection at the University of Nottingham), with known pathogenicity to OSR (Sturrock et al., 2015), was used to produce inoculum. The inoculum was grown on Potato Glucose Agar (PGA; Sigma-Aldrich, UK) for a period of 10-14 days prior to inoculation, at room temperature (18-20^{o}C).

4.4.3 Effect of AG 2-1 infection of plants on *M. persicae*

In order to assess if AG 2-1 infection affects aphid performance, one inoculum plug (5 mm) was used to inoculate each plant. The plug was cut into two equal parts and each of them was placed 1.5 cm away from the stem, opposite to each other at a depth of ~6 cm. For the control treatment, plants were not inoculated. Inoculated (PA) and control (A) plants were kept in a controlled environment room with 18 ^{o}C ± 2, 12 h light: 12 h dark. A week later, three alate (winged) adult aphids were placed with a fine brush on a developed leaf of each of the inoculated and control plants and then a clip cage was adjusted on each leaf to ensure that the aphids were kept on the leaves. Plants were watered every two days.

4.4.3.1 Aphid performance and reproduction

One day after infestation, adult aphids were removed and any nymphs laid were counted. If no nymphs had been laid or the adults had died, new adults were used to replace them. The young nymphs were collected and weighed on a micro balance (Precisa XB 120A, Presica Instruments Ltd Switzerland) and then placed back on the plants. Seven days later they were collected and weighed again in order to estimate their Mean Relative Growth Rate (MRGR) (Leather and Dixon, 1984, Radford, 1967):

$MRGR = (\ln W_2 - \ln W_1) / 7$

Where W_1 is the weight at birth and W_2 is the weight at 7^{th} day.

In order to estimate the intrinsic rate of population increase (r_m), the bigger nymph (or adult) from each clip cage was placed back on the plant to lay new nymphs. For a period of a week, the number of new nymphs was recorded daily. The nymphs were removed from the plant to prevent crowding in the clip cage and to allow the adult to lay more nymphs. Intrinsic rate of population increase was estimated by the following formula, where D = the time taken from the birth of the aphid to the production of the first nymph, which was kept constant in the present study as 7 days, FD = the number of nymphs produced over a period equal to time D, 0.74 constant of Wyatt and White (Wyatt and White, 1977):

$r_m = 0.74 (\ln(FD) / D)$

On the last day (14^{th} day), the above ground plant part was collected and fresh weight was measured (Precisa 12.400 DG-FRSCS, Precisa Instruments Ltd Switzerland) to estimate if there was any difference between treatments and varieties. All AG 2-1 inoculated plants were checked for disease symptoms.

4.4.4 Effect of *M. persicae* on plant susceptibility to AG 2-1

For this experiment OSR plants, (cv 'Canard' and 'Temple') were first infested with aphids and then, three days later, infected with AG 2-1 (AP) in the same way as described above and kept in a room with controlled conditions (18 ^oC ± 2, 12 h light: 12 h dark). For the control (P) treatment, plants without previous aphid infestation were inoculated with AG 2-1. Thirteen days post inoculation (dpi) with AG 2-1, plants from both treatments were removed from the compost and the above ground plant parts were washed and disease on plant stems was visually assessed using a scale of 0-3 (0= no symptoms, 1= light disease less than lesions occupying <50%, 2= moderate disease 50-70%, and 3= severe >70%) and weighed (Precisa 12.400 DG-FRSCS, Precisa Instruments Ltd Switzerland). For the extraction of fungal DNA, stems of each plant were cut and freeze dried (at -40 ^oC for 4 days). Additionally, the compost was left to dry at room temperature (18 ^oC ± 2) for a period of 6-8 days and then kept in sealed bags in a cold room (5 ^oC ± 2) until extraction.

4.4.4.1 Extraction of fungal DNA from compost

The method of Woodhall et al., for extraction of fungal DNA from soil (Woodhall et al., 2012) was adjusted for the present study: compost from two plants was combined into one sample for each treatment of each variety. For homogenization of the sample and extraction of fungal DNA each sample was placed in a 50 ml falcon tube with three 1/4 in. ceramic spheres (MP Biomedicals, USA), 15 ml of CTAB buffer (cetyltrimethylammonium bromide) and 0.45 ml of Antifoam B in a FastPrep-24™ homogeniser (MP Biomedicals, USA). Further extraction was performed as described in the technical protocol of Wizard Magnetic DNA Purification System for Food (Promega Wizard Food Kit, Southampton, UK).

4.4.4.2 Extraction of fungal DNA from plant material

Freeze dried stems were cut into small pieces with scissors and weighed. They were milled by adding some (approximately a volume of 0.2 ml) Lysing matrix D Bulk (MP Biomedicals, USA) to each sample tube and mechanically shaken in a FastPrep-24™ homogeniser (MP Biomedicals, USA). For the extraction, the method described by Ray et al. was used (Ray et al., 2004); because the weight of the stem samples were less than 2 g of the amount of CTAB (15 ml for 2 g of plant sample) was adjusted.

4.4.4.3 Quantification of fungal DNA

Before quantitative Real-time PCR, using species-specific primers (Budge et al., 2009) (Supplementary Table 4.1) for *R. solani* AG 2-1 all DNA samples were amplified in an ITS (Internal Transcribed Spacer) PCR (White, 1990) to ensure that fungal and plant DNA was present and amplifiable within a sample. Amplification using 2x MangoMix (Bioline, UK) was performed in Gene Amp PCR System 9700, Applied Biosystems, USA programmed for: 94°C for 1min and 15 sec, followed by 35 cycles of 94°C for 15 sec, 50°C for 15 sec and 72°C for 45 sec and finished on 72°C for 4 min and 25 sec and hold at 10°C. Amplicon gel electrophoresis was carried out in 1% agarose gels stained with ethidium bromide (0.05%). PCR products were viewed on a Gel Doc 2000 system (Bio-Rad, Buckinghamshire, UK) under UV light. Real-time PCR was performed using SsoAdvanced™ Universal SYBR® Green Supermix (BioRad, USA) with primers specific for *R. solani* AG 2-1 (Budge et al., 2009)

(Supplementary Table 4.1). The amplification protocol was 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 30 sec at 64°C and then followed by 5 sec at 60°C and 95°C (1000 Thermo Cycle, BioRad Laboratories Ltd., UK). For the quantification of the amplified DNA, a standard curve was created using 6 standard dilutions from 10 to 10^{-5} ng/μl. The concentration of DNA on pure samples was calculated using a nanodrop (NanoDrop®) at the ratios of wavelengths 260 nm /230 nm and 260 nm /280 nm and estimated as ng/μl (Nanodrop 1000 V3.8.1 software). DNA samples from OSR stems were diluted (10^{-1} ng/μl) in TE Buffer and their concentration was calculated by estimating the absorbance on wavelengths 260 nm, 280 nm, 328 nm and 360 nm on a spectrophotometer (Cary 50 Probe, Varian, Australia). Based on pure sample's concentration 20 ng/μl dilutions were prepared and used for RT PCR as described previously.

4.4.4.4 Gene expression

4.4.4.4.1 Selection of target genes

The choice of the target genes was based on their role as marker genes in the two major signalling pathways JA and SA and/or on their role in plant defences against *M. persicae* and necrotrophic fungi. Five genes were selected: *LIPOXYGENASE 3* (*LOX3*), *MYC2*, *NON EXPRESSOR OF PATHOGENESIS-RELATED GENES 1* (*NPR1*), *PATHOGENESIS RELATED 1* (*PR1*) and *WRKY38*. The *LOX* family is known to comprise marker genes for JA signalling and also to be related with MYC2 transcription factor, which has a unique role between the two branches of JA signalling in Arabidopsis (Lorenzo et al., 2004). Moreover, *NPR1* is a receptor of SA and regulates the expression of *PR1* marker gene in Arabidopsis (Wu et al., 2012). The family of WRKY transcription factors is also known to play diverse roles in basal plant defenses and it has been shown that *WRKY38* negatively regulates SA responses in Arabidopsis (Kim et al., 2008).

4.4.4.4.2 Collection of samples

Based on our results obtained from the experiments of *M. persicae* effect on plant susceptibility to AG 2-1, we decided to proceed with cv 'Canard' for this experiment. Plants were grown, infested with *M. persicae* and inoculated with AG 2-1 as described above. Samples were collected from five different

treatments: Aphid (A), pathogen (P) and aphid with pathogen (AP) and two control treatments (control 1 and control 2), with two different time points per treatment. We chose two time points to examine an early and a later stage of infection (personal observations showed that at 72 h AG 2-1 hyphae reaches the plant). Based on the sampling times for the pathogen we estimated the time points after aphid infestation. The selection of time points had 24 h intervals to exclude the effect of circadian cycle in the expression of genes. Hence, control 1 samples were collected from plants prior to aphid infestation. Aphid samples were collected at 52 and 76 h post infestation. Control 2 samples were collected from plants, 3 days older than control 1, prior to AG 2-1 infection. Pathogen samples were collected at 72 and 120 h post infection with AG 2-1. For the AP treatment, plants were harvested at 72 and 120 h post AG 2-1 infection (Supplementary Figure 4.1)

4.4.4.4.3 RT-qPCR (Real Time Quantitative PCR)

For each sample one fully developed leaf was collected, immediately frozen in liquid nitrogen and stored at –80^{o}C. Five biological samples were collected for each time point /treatment. Leaf samples were ground to fine powder and RNA was isolated using RNeasy® Plant Mini Kit (QIAGEN, Germany) and treated with DNAase I (RNase-free) (New England Biolabs, UK) following manufacturers' instructions. For assessing the purity of the RNA, samples used for RT-PCR (program: 3 min at 95°C followed by 35 cycles of 30 sec at 95°C, 30 sec at 60°C, followed by 30 sec at 72°C and then for 100 mins at 72°C) (T100™ Thermal Cycle, BioRad Laboratories Ltd., UK) and the amplifications were used to run a 1.5% agarose gel and visualised in InGenius3 with GeneSys image acquisition software (Syngene, Synoptics Ltd.). The amount of the RNA in the samples was quantified in a nanodrop (NanoDrop®). First strand of cDNA synthesis was performed using qScript™ cDNA SuperMIX (Quanta BioSciences, USA) and the obtained cDNA was quantified using a nanodrop (NanoDrop®). RT-qPCR was carried out with three technical replicates per sample, using LuminoCt® SYBR© Green qPCR Ready Mix™ (Sigma-Aldrich, UK), in the following program; 1 min at 95°C followed by 60 cycles of 5 sec at 95°C, 8 sec at 62°C and then followed by 30 sec on 72°C (1000 Thermo Cycle, BioRad Laboratories Ltd., UK). For each of

the target genes primers were designed and tested (Supplementary Table 4.1). *ACTIN* was used as reference gene (Körber et al., 2015).

4.4.5 Statistical analysis

For each of the two experiments 10 plants of each variety were used as biological replicates in each of the treatments. When testing the effect of AG 2-1 infection on *M. persicae*, two experimental replicates were used. When testing the effect of *M. persicae* on AG 2-1, three experimental replicates were used for disease assessments, plants fresh weight and the extraction of fungal DNA from plants and two experimental replicates for the extraction of fungal DNA in compost. General ANOVA (GenStat 17th Edition) was used to detect significant interactions between treatments and varieties for MRGR of aphids, fungal DNA in compost and fresh weight. Two sample t-test was used for the detection of any significant differences within varieties and within treatments for disease, fungal DNA in plant stems and the intrinsic rate of population increase and fresh weight. Fungal DNA data were logarithmically transformed prior to the analysis. For the gene expression analysis, for each treatment point four to two biological replicates were used. The expression of the target genes and *ACTIN* was estimated individually for each using the technical replicates and then an average for each biological replicate in each treatment was calculated. Then the given value of each gene was expressed in relation to the value of the *ACTIN* for the same treatment. General ANOVA was used to estimate if they were significant differences different treatments and time points. In order to detect if there was an interaction between pathogen and aphid-pathogen treatment at the two tested time points, a general ANOVA with two factors (treatment and time) was performed.

4.5 Results

4.5.1 Effect of AG 2-1 plant infection to *M. persicae*

Although, the growth of nymphs during the first week after their birth, measured as MRGR, was not different between varieties and treatments (P= 0.848; Table 4.1), significant differences were observed in population increase between varieties in both treatments (Figure 4.1). *M. persicae* adults laid more nymphs on ‘Temple’ compared to ‘Canard’ both for plants that had been

previously inoculated with AG 2-1 (PA), 89% more nymphs (two sample t-test: t= -2.94, d.f. = 24.81, *P* = 0.0007) and for the non-inoculated control plants (A), 32% more nymphs (two sample t-test: t= -2.56, d.f. = 35, *P* = 0.0015; Figure 4.1). The intrinsic population growth of *M. persicae* aphids was not different between treatments either on 'Canard' (two sample t-test: t= 1.39, d.f. = 25.59, *P* = 0.178) or 'Temple' (two sample t-test: t= -0.08, d.f. = 34, *P* = 0.938; Figure 4.1). However, more nymphs were laid in 'Temple' compared to 'Canard'.

Table 4.1 Mean Relative Growth Rate (MRGR) and Fresh Weight (F.W) of Canard and Temple under pathogen and aphid inoculation (PA) and only aphid infestation (A). For the comparison between treatments and varieties *P* value and LSD were used (ANOVA).

		MRGR	F.W (g)
Canard	PA	0.26	7.96
	A	0.25	11.54
Temple	PA	0.20	8.47
	A	0.18	9.01
$P_{(t*v)}$		0.848	0.111
$LSD_{(t*v)}$		0.114	2.891

No interaction was detected between treatments and varieties for the fresh weight of above ground plant parts (*P*= 0.111, ANOVA; Table 4.1). However, when we used two-sample t-test to detect if there were differences within each treatment, an effect was observed in 'Canard' with AG 2-1 inoculated plants being significantly lighter (31%) compared to aphid-only control plants (two-sample t-test: t= 2.36, d.f. = 26.06, *P* = 0.026). Additionally, a significant difference was observed between the two varieties in the control plants, with 'Canard' being heavier compared to 'Temple' (two-sample t-test: t= 2.86, d.f. = 37, *P* = 0.003).

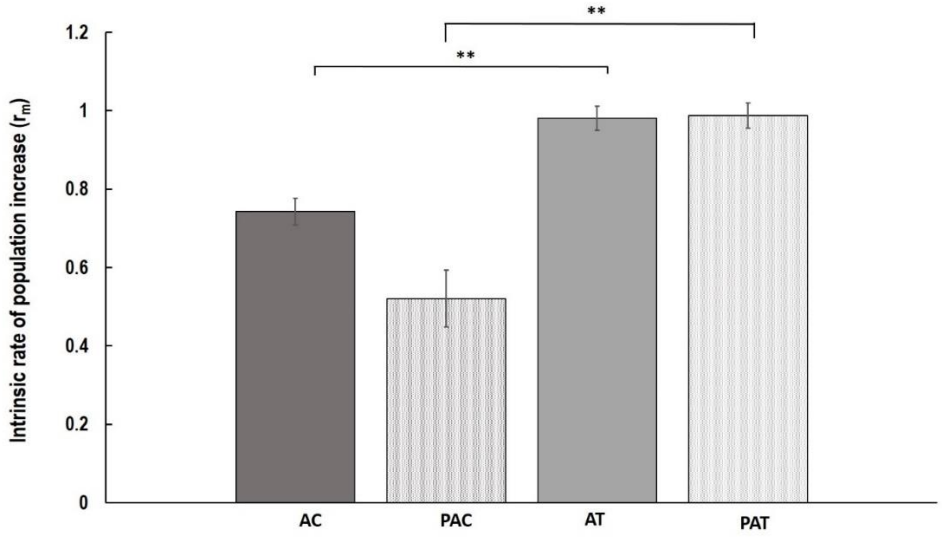


Figure 4.1 Mean of intrinsic rate of population increase (rm) (±SE) of *M. persicae* aphids in Canard (C) and Temple (T), previously inoculated with AG 2-1 (PA) or non- inoculated controls (A). ** $P \leq 0.01$ (two-sample t-test).

4.5.2 *Myzus persicae* effect on plants susceptibility to the AG 2-1

Disease assessment on stems of OSR plants revealed significant differences between treatments and varieties (Figure 4.2). Aphid infestation prior to AG 2-1 infection (AP) resulted in significant higher disease severity (48.7% increase) in 'Canard' plant stems compared to AG 2-1 only infected (P) controls (two-sample t-test: t= 3.11, d.f. = 58, P = 0.001; Figure 4.2). In addition to this, in the aphid-pathogen treatment, 'Canard' plants had significantly more disease (45.2% increase) compared to 'Temple' plants (two-sample t-test: t= 3.02, d.f. = 58, P = 0.002). Nonetheless, disease severity between the two varieties was not different in the controls (P) (two-sample t-test: t= 0.31, d.f. = 58, P = 0.380; Figure 4.2). Also, no differences were detected in disease between the two treatments in Temple plants (two-sample t-test: t= 0.47, d.f. = 58, P = 0.320).

Fungal DNA was significantly higher with a 56.7% increase in 'Canard' plants compared to 'Temple' under aphid infestation (AP) (two-sample t-test: t= 1.73, d.f. = 50.17, P = 0.045) but no significant differences were detected when we compared the varieties in the control (P) treatment (two-sample t-test: t= 0.85, d.f. = 57, P = 0.20; Figure 4.3). Also, no significant differences were observed between the two treatments within either 'Canard' (two-sample t-test: t= 0.651,

d.f. = 58, *P* = 0.306) or 'Temple' (two-sample t-test: t= -0.21, d.f. = 57, *P* = 0.58; Figure 4.3).

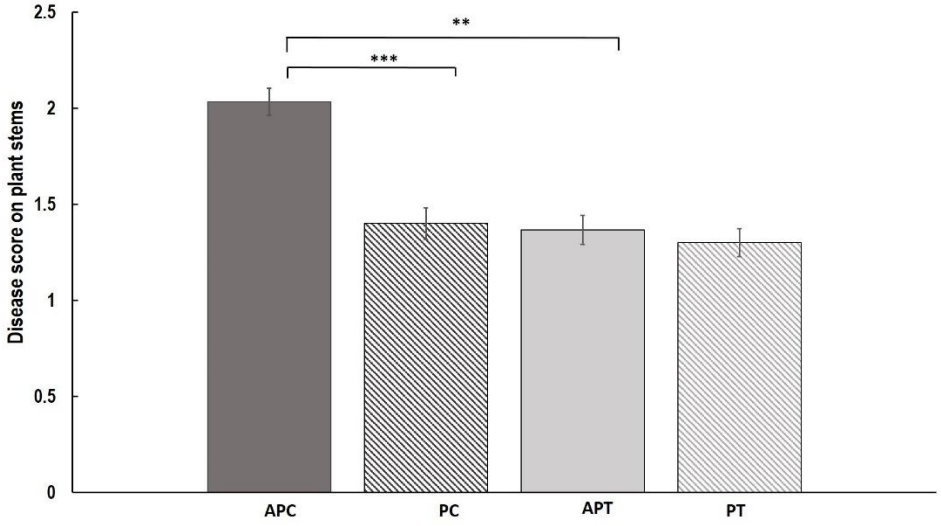


Figure 4.2 Mean of disease (±SE) caused by AG 2-1 13 dpi on OSR stems (n=30) under the following treatments AP: aphid and pathogen infection, P: only pathogen infection. The letters C and T next to treatments indicate Canard and Temple respectively. ** *P* ≤ 0.01, * *P* ≤ 0.001 (two-sample t-test).**

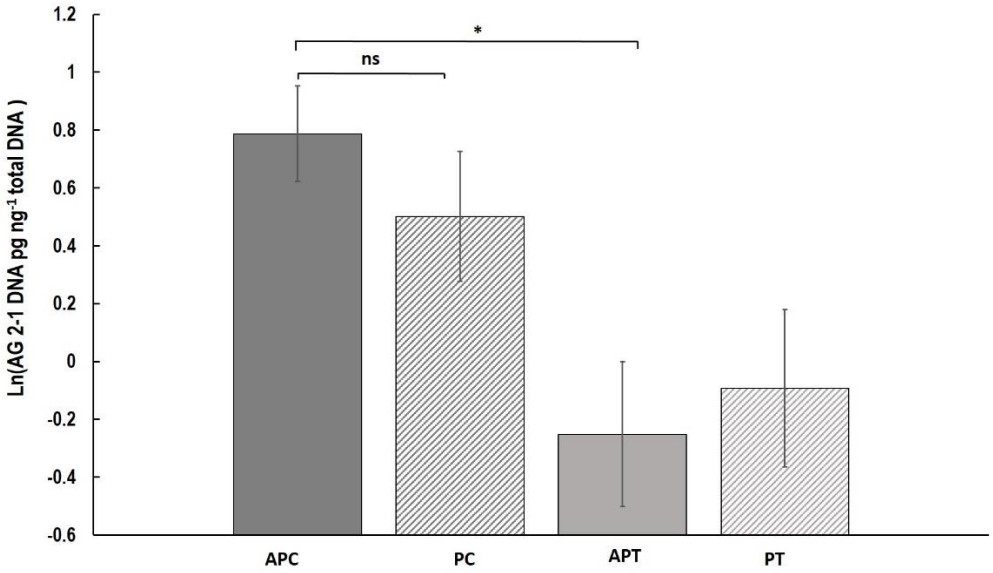


Figure 4.3 Mean of *R. solani* DNA (ln (DNA pg ng^{-1} total DNA)) (±SE) extracted from stems of OSR plants 13 dpi. Treatments; AP: aphid and pathogen infection, P: only pathogen infection, with C: Canard and T: Temple. * *P* ≤ 0.05, (two-sample t-test).

The amount of AG 2-1 extracted from the compost of the tested plants was not different either between varieties or between treatments and there were no significant interaction between them (*P* = 0.669, LSD = 0.446; Supplementary

Table 4.2). Similarly, the fresh weight of above ground plants was not significantly between treatments, varieties and neither was their interaction (*P* = 0.693, LSD = 1.53; Supplementary Table 4.2).

4.5.3 Gene expression

Both infestation with *M. persicae* and inoculation with AG 2-1 induced several alterations in the expression of the tested genes (Figure 4.4). *M. persicae* infestation downregulated the expression of *LOX3* 76 h post infestation (*P* <0.001, LSD = 0.0004) while AG 2-1 infection significantly increased *LOX3* expression 72 h post infection (*P* <0.001, LSD= 0.0004; Figure 4.4). In the presence of both *M. persicae* and AG 2-1 *LOX3* expression was downregulated at 120 h (*P* <0.001, LSD = 0.0004). The expression of the two controls and aphid treatment at 52 h, pathogen treatment and aphid-pathogen at 72 h was similar (Figure 4.4). AG 2-1 infection significantly upregulated *MYC2* at 72 h and expression was further increased 120 h post infection (*P* <0.001, LSD= 0.00006), while *MYC2* expression during *M. persicae* infestation alone or aphid and pathogen infection was similar to that of the controls (Figure 4.4). The expression of *NPR1* was only significantly increased in the presence of both *M. persicae* and AG 2-1, 72 h after infection (*P* <0.001, LSD= 0.0005). *NPR1* expression with aphid infestation at 52 h was significantly higher compared to control 2 and significantly lower compared to pathogen infection at 72 h (*P* <0.001, LSD= 0.0005) but there were no other significant differences between the other treatments, or between the two controls (Figure 4.4). The expression of *PR1* was significantly upregulated under pathogen and aphid-pathogen treatments at both 72 h and 120 h (*P* = 0.014, LSD= 0.0223) while expression during aphid treatment was similar to both controls and the other treatments (Figure 4.4). The expression of *WRKY38* was similar and only significantly different between aphid treatment at 76 h and aphid-pathogen treatment at 120 h with aphid-pathogen treatment at 72 h (with the latter to be significantly lower) (Figure 4.4).

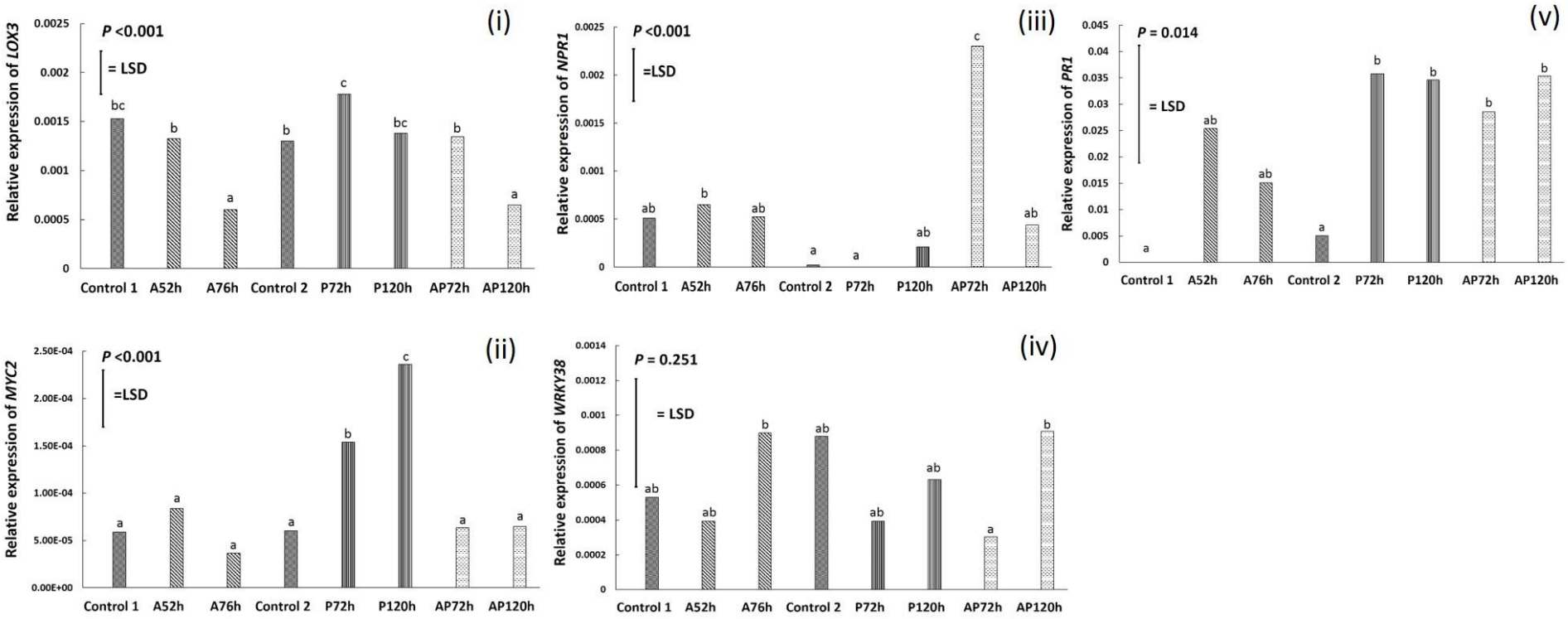


Figure 4.4 Relative expression of (i) *LOX3*, (ii) *MYC2*, (iii) *NPR1*, (iv) *PR1* and (v) *WRKY38* at different treatments and time points: control 1 and control 2, aphid (A); 52 and 76 hours post infestation with aphids, pathogen (P); 72 and 120 hours post inoculation with AG 2-1 and aphid and pathogen (AP); at 72 and 120 hours post infection with AG 2-1. For the comparison between the different treatments *P* value and LSD were used, different letters indicate significant differences (ANOVA).

The comparison between pathogen and aphid-pathogen treatment at 72 h and 120 h revealed that for *LOX3* there was a significant interaction between treatments at 120 h; with expression of the gene downregulated in the presence of *M. persicae* (P = 0.009, LSD= 0.00057). Following this, significant interaction was found between the two different time points tested within aphid-pathogen treatment, where the expression of *LOX3* was significantly reduced at 120 h compared to 72 h (P = 0.0013, LSD= 0.0004). Additionally, significant differences were observed in the expression of *MYC2* between treatments at both 72 h and 120 h with the aphid-pathogen treatment having significantly lower expression compared to that of the pathogen treatment (P <0.001, LSD= 0.000056). Lastly, significant interaction was also observed between treatment and time at the expression of the *NPR1* gene (P <0.001, LSD= 0.00065): at 72 h there was significantly increased expression under aphid-pathogen treatment compared to pathogen treatment (P <0.001, LSD= 0.00046). The expression of *NPR1* within aphid-pathogen treatment was also changed over time, decreasing at 120 h compared to 72 h (P = 0.003, LSD= 0.00046).

4.6 Discussion

Inducible plant defences embrace a combination of strategies against a range of pathogens and herbivores that are activated in a species specific manner (van Loon et al., 2006). These defences involve three main pathways with three phytohormones playing a major role: JA, SA and ET (Bari and Jones, 2009, Dicke and van Poecke, 2002, Dicke et al., 2003). There is a complex network of interactions between JA, ET, SA and other hormones such as ABA that allows composition of effective plant defence strategies (Vos et al., 2013). Plant-pathogen and insect interactions become more complicated if we consider that pests and pathogens are able to take advantage of these defences for their own benefit, for example by inducing SA to supress JA. At the same time evidence is building that plants are able to fine-tune their defences with co-activation of those phytohormone signalling pathways (Li et al., 2006, Liu et al., 2016, Novakova et al., 2014). In the present study, we have investigated how *B. napus* responds to belowground infestation by AG 2-1 and aboveground herbivory by *M. persicae* and how each attacker affects the other

when sharing the same host. We also tested the role of JA and SA in these interactions by analysing alterations in expression of genes involved in those signalling pathways.

Our results illustrate that infection with AG 2-1 seemed not to have an effect on aphid performance as no significant differences were observed for both their growth (MRGR) and their population increase (r_m). The peach-potato aphid is a generalist herbivore able to supress defence mechanisms of its host plants and interfere with both SA and JA responses (De Vos et al., 2005, Thompson and Goggin, 2006). Hence, it is possible that the aphids were able to overcome the defence responses induced by AG 2-1. Nonetheless, as no differences were observed between pathogen-aphid (PA) and aphid treatment, it seems that infection with AG 2-1 and changes induced do not affect *M. persicae*.

The fact that *M. persicae* adults laid more nymphs on 'Temple' plants, regardless of the treatment, compared to 'Canard' implies that 'Temple' serves as a better host for this aphid. It might be that these cultivars differ in their GSL profile and therefore there is a difference in their suitability for the generalist *M. persicae*. 'Temple' is a commercial oilseed cultivar and as such is expected to have lower GSL concentration compare to 'Canard', which is fodder type. However, in a study assessing *M. persicae* performance in different brassicaceous plants, population growth was not related with GSL, as aphids had significantly lower population growth in *B. napus* which has the lowest GSL levels compare to other Brassica species (Le Guigo et al., 2011). The lack of information on the GSL profile of the tested varieties does not allow us to draw an accurate conclusion.

Regarding the fresh weight of the aboveground plant tissues of the controls, 'Temple' plants were about 22% lighter compare to 'Canard'. However, under the presence of both AG 2-1 and *M. persicae* 'Canard' plants weighed less than their controls, which probably implies that this variety is more susceptible to AG 2-1 infection at this growth stage. Previous screening of these varieties has shown contrasting responses regarding survival and disease during the early seedling stage (Drizou et al., 2017). In the present study, the tested plants

were 3-4 weeks old during inoculation which probably alters their ability to defend themselves against AG 2-1. It is known that, AG 2-1 virulence differs based on growing stage of the plant, and that is less pathogenic to older plants (Teo et al., 1988, Yitbarek et al., 1988).

When we looked on the reverse effect, with *M. persicae* infestation prior to AG 2-1, we were able to detect significant differences; although, the two varieties had similar disease levels when they were exposed only to AG 2-1 (control treatment), when aphids were included in the treatment (AP) 'Canard' plants had significantly more disease compared to their controls. This result implies that aphid infestation alters the ability of plants of this variety to defend themselves effectively against AG 2-1. However, this was not observed with 'Temple' plants which had similar disease levels in both aphid–pathogen (AP) and P treatments. Continually, in aphid-pathogen treatment, 'Canard' plants also had significantly more disease compared to 'Temple'. Therefore it seems that this effect is more pronounced in 'Canard'.

Extracted AG 2-1 DNA from the compost did not show any significant interaction. Hence, we can conclude that the possible induced changes are not expressed as alterations in the rhizosphere. It is known that aboveground herbivory results in translocation of nutrients as well as changes to the root exudate profile which consequently affect belowground communities (Bardgett et al., 1998). In the present study, the amount of fungal DNA was the same between treatments, so it is unlikely that alteration of exudates, if any, is stimulating AG 2-1 accumulation in the rhizosphere. Nonetheless, the extracted fungal DNA from plant stems show that although there was a tendency with the AP treatment having more fungal DNA compared to P treatment in 'Canard', this was not statistically significantly different. Consequently, it seems that the main reason for increased disease in 'Canard' under aphid-pathogen treatment is the induced changes by *M. persicae* rather the actual amount of AG 2-1 in the plant. Between the two varieties, 'Canard' tended to have more fungal DNA compared to 'Temple', something that could be probably explained by the fact that in general 'Canard' had more disease compared to 'Temple' and as a result higher amount of AG 2-1. Although, the difference between the two varieties was not statistically significant within the

control treatment, in the AP treatment ‘Temple’ had significantly less fungal DNA compared to ‘Canard’.

In order to gain a better insight into which factors altered ‘Canard’ response to AG 2-1 under aphid infestation, we decided to examine the expression of genes related to JA and SA signalling. *M. persicae* induces both SA- and JA-related defences. Moran and Thompson, showed that *M. persicae* infestation in Arabidopsis resulted in the transcription of *PR1* and *LOX2* but not *LOX1* (Moran and Thompson, 2001). Moreover, although herbivory by *M. persicae* did not alter SA, JA and ET levels, it induced changes in the expression of 2,181 genes in Arabidopsis, including *PR1* (De Vos et al., 2005). However, in *Brassica oleracea*, *M. persicae* did not induce the expression of *BoLOX*, a cloned *LOX* gene from *B. oleracea* sharing similarities with *AtLOX2* in *Arabidopsis thaliana* and *BnLOX2fl* in *B. napus* (Zheng et al., 2007). In the present work, *M. persicae* downregulated the expression of *LOX3* 76 h after infestation but the expression of the other genes was not significantly different compared to control 1, although there were small differences in the actual amounts of the genes between different times. It is tempting to speculate that *M. persicae* induced changes suppress or overcome defences in *B. napus* such as *LOX3*. In this regard, the peach-potato aphid is known to have the ability to deploy host plant defences for its own benefit by effectors in saliva secretions (Elzinga et al., 2014); it is suggested that depending on its host plant, *M. persicae* changes the expression of these effectors to overcome defences (such as GSL compounds of *Brassica* species) to enable colonisation of the plant. Therefore it might be the case that similar activation of salivary effectors resulted in the observed gene expression in the present study.

There is limited work focusing on the molecular interaction between *R. solani* and its hosts. In a recent study the authors discovered that VOCs from *R. solani* AG 2-2 IIB primed *A. thaliana* plants for improved growth but did not affect disease resistance while improved *Mamestra brassicae* caterpillars performance above ground (Cordovez et al., 2017). To understand the underlying molecular mechanism of these observations they performed wide transcriptome analysis and found that AG 2-2 IIB VOCs triggered the upregulation of genes involved with auxin and ABA but downregulated ET-

and JA- responsive genes, indicating that the observed growth-promoting effect by VOCs is facilitated by other signalling pathways (Cordovez et al., 2017).

Screening of different Arabidopsis ecotypes and mutants in signalling pathways with AG 8 and AG 2-1 by Foley et al., revealed that resistance to AG 8 and susceptibility to AG 2-1 was not related to the major signalling pathways (Foley et al., 2013). The authors argued that the final outcome of the interaction between *Arabidopsis* and these AGs should be due to the combination of JA, SA and ET (Foley et al., 2013). Additionally, in the same work both AGs induced changes in the expression of several genes including several *PR* genes (with only AG 2-1 to induce *PR1*) and transcription factors. The major finding of that study was that although NAPDH oxidases played a key role for resistance to AG 8, this was not the case with AG 2-1 which probably overcomes host defences (Foley et al., 2013). Another study in *Arabidopsis* (Perl-Treves et al., 2004) showed that plants respond to *R. solani* infection by inducing the glutathione *S*-transferase *GSTF8* gene promoter independently from SA signalling and this induction was only mediated by the least pathogenic AG 8. AG 2-1 did not induce the promoter and actually killed the plants. The authors stated that AG 2-1 might be able either to escape or suppress plant defence mechanism (Perl-Treves et al., 2004). From those two studies it becomes evident that AG 2-1 is a particularly interesting pathogen which possibly has an ability to manipulate plant host defences.

In the present study AG 2-1 induced the expression of three genes: *LOX3* 72 h post infection, and *MYC2* and *PR1* at both 72 h and 120 h after infection. *MYC2* is known to negatively regulate the expression of the ERF (ETHYLENE RESPONSE FACTOR) branch of the JA signalling pathway that is responsible for defence against necrotrophic pathogens (Dombrecht et al., 2007) and Foley et al. showed that ERF transcription factors were induced by *R. solani* (Foley et al., 2013). Additionally, MYC is known to regulate the increase of wounding/herbivory induce genes such as *LOX* (Dombrecht et al., 2007, Lorenzo et al., 2004). The increase of *MYC2* in our experiments does not correlate with these findings. However, we have to take into account that this

study is in *B. napus* and not in *Arabidopsis* hence different interactions in the signalling pathways may occur.

Additionally, it might be the case that AG 2-1 actually induces the expression of *MYC2* and in this way interferes with the ERF branch of JA and escapes an efficient plant defence against necrotrophic fungi. There is some evidence supporting this hypothesis from studies with other necrotrophic fungi: *Alternaria brassicola* is known to deploy defences of the susceptible host *Brassica juncea* and induce SA-regulated responses and block JA responses, while in the resistant *Sinapis alba* induction of ABA leads to JA response and efficient plant resistance (Mazumder et al., 2013). In another pathosystem, *Sclerotinia sclerotiorum* induced responses in *B. napus* that were related to both JA and SA signalling pathways; there was an increase in the level of plant hormones and the expression of marker genes including *LOX3* and *PR1* (Novakova et al., 2014). Moreover, the WRKY family of transcription factors is known to have a role in basal plant defences and AG 2-1 and AG 8 are known to induce the expression of this family in Arabidopsis (Foley et al., 2013). Here the expression of *WRKY38* was similar and not significantly different from the controls. *WRKY38* has been shown to negatively regulate SA-related defence and result in susceptibility of Arabidopsis to *Pseudomonas syringae* bacteria (Kim et al., 2008). However, the induced expression of *PR1* in our experiments contrasts with that, so it seems that either this effect is not present in our study system or that unknown interactions within the signalling pathways resulted in this outcome.

Furthermore, when OSR plants were exposed to both attackers, we found that although *LOX3* expression was similar to controls 72 h post inoculation, it was downregulated at 120 h, whereas *MYC2* had no significant induction at either 72 h or 120 h post inoculation. Expression of *NPR1* was significantly increased at 72 h but was reduced and was similar to the control at 120 h post inoculation. Expression of *PR1* increased at both examined time points and *WRKY38* had an increase only at 120 h. So there was a differentiation in gene expression when plants were under dual attack compared to when attacked by aphids or pathogen alone. As our main aim was to understand how *M. persicae* affects plant responses to AG 2-1, we compared the P treatment with the AP

treatment; the expression of *LOX3* and *MYC2* was significantly downregulated by the AP treatment compared to the P treatment at both examined times indicating that *M. persicae* induces changes that suppress the expression of AG 2-1 induced genes. Considering the hypothesis that AG 2-1 increases *MYC2* in order to block the ERF branch and overcome plant defences, we would expect that in the presence of aphids disease symptoms would be reduced and not increased. Therefore it seems that the interactions that are taking place and shape the final outcome are more complicated. The increased expression of *NPR1* 72 h post-inoculation under dual attack is also interesting as this gene is known to be a SA receptor regulating the expression of many defence-induced genes (Wu et al., 2012). Taking these results together, we can conclude that during dual attack *M. persicae* infestation suppresses JA-responsive genes and promotes the increase of SA- related genes through unknown interactions which make *B. napus* more susceptible to AG 2-1.

4.7 Conclusion

This work provides, for the first time, information about the interaction between two major enemies of OSR: *M. persicae* and *R. solani* AG 2-1. Our data show that aphid infestation induced changes in OSR that increased susceptibility of 'Canard' plants to AG 2-1 infection, likely due to the suppression of JA signalling pathway. Additionally, I found that *R. solani* AG 2-1 induced the activation of both JA- and SA-responsive genes. However, due to the complexity between the signalling pathways we cannot draw any further conclusion. Future studies should focus on the transcriptomic analysis of marker genes as well as the quantification of all major plant hormones and test the possible role of ET and ABA in the interaction.

4.8 Abbreviations

OSR: oilseed rape, AG: anastomosis group, JA: jasmonic acid (JA), SA: salicylic acid, ET: ethylene, ABA: abscisic acid, HAMPs: herbivore associated molecular patterns, PAMPs: pathogen associated molecular patterns, ETI: effectors triggered immunity, GSL: glucosinolates, cv: Cultivars, PGA: Potato Glucose Agar, PA: pathogen infected plants followed by aphid infestation, A: aphid infested plants, P: pathogen inoculated plants, AP: aphid infested plants

followed by pathogen inoculation, MRGR: Mean Relative Growth Rate, r_m: population increase, h: hours, dpi: days post inoculation, RT-qPCR: Real Time Quantitative PCR, ITS: Internal Transcribed Spacer, ANOVA: Analysis of variance, LSD: least significant difference, d.f.: degrees of freedom, FW: fresh plant weight, VOCs: volatile organic compounds.

4.9 References

AL-NAEMI, F. & HATCHER, P. E. 2013. Contrasting effects of necrotrophic and biotrophic plant pathogens on the aphid Aphis fabae. *Entomologia Experimentalis Et Applicata,* 148**,** 234-245.

BABIKER, E., HULBERT, S., SCHROEDER, K. & PAULITZ, T. 2013. Evaluation of *Brassica* species for resistance to *Rhizoctonia solani* and binucleate *Rhizoctonia* (*Ceratobasidum* spp.) under controlled environment conditions. *European Journal of Plant Pathology,* 136**,** 763-772.

BARDGETT, R. D., WARDLE, D. A. & YEATES, G. W. 1998. Linking above-ground and below-ground interactions: How plant responses to foliar herbivory influence soil organisms. *Soil Biology & Biochemistry,* 30**,** 1867-1878.

BARI, R. & JONES, J. 2009. Role of plant hormones in plant defence responses. *Plant Molecular Biology,* 69**,** 473-488.

BASS, C., PUINEAN, A. M., ANDREWS, M., CUTLER, P., DANIELS, M., ELIAS, J., PAUL, V. L., CROSSTHWAITE, A. J., DENHOLM, I., FIELD, L. M., FOSTER, S. P., LIND, R., WILLIAMSON, M. S. & SLATER, R. 2011. Mutation of a nicotinic acetylcholine receptor β subunit is associated with resistance to neonicotinoid insecticides in the aphid Myzus persicae. *BMC Neuroscience,* 12**,** 51.

BLACKMAN, R. L. & EASTOP, V. F. 2000. *Aphids on the world's crops An identification and information guide*, John Willey & sins Ltd.

BRUCE, T. J. A. 2015. Interplay between insects and plants: dynamic and complex interactions that have coevolved over millions of years but act in milliseconds. *Journal of Experimental Botany,* 66**,** 455-465.

BUDGE, G. E., SHAW, M. W., COLYER, A., PIETRAVALLE, S. & BOONHAM, N. 2009. Molecular tools to investigate *Rhizoctonia solani* distribution in soil. *Plant Pathology,* 58**,** 1071-1080.

CHALHOUB, B., DENOEUD, F., LIU, S., PARKIN, I. A. P., TANG, H., WANG, X., CHIQUET, J., BELCRAM, H., TONG, C., SAMANS, B., CORRÉA, M., DA SILVA, C., JUST, J., FALENTIN, C., KOH, C. S., LE CLAINCHE, I., BERNARD, M., BENTO, P., NOEL, B.,

LABADIE, K., ALBERTI, A., CHARLES, M., ARNAUD, D., GUO, H., DAVIAUD, C., ALAMERY, S., JABBARI, K., ZHAO, M., EDGER, P. P., CHELAIFA, H., TACK, D., LASSALLE, G., MESTIRI, I., SCHNEL, N., LE PASLIER, M.-C., FAN, G., RENAULT, V., BAYER, P. E., GOLICZ, A. A., MANOLI, S., LEE, T.-H., THI, V. H. D., CHALABI, S., HU, Q., FAN, C., TOLLENAERE, R., LU, Y., BATTAIL, C., SHEN, J., SIDEBOTTOM, C. H. D., WANG, X., CANAGUIER, A., CHAUVEAU, A., BÉRARD, A., DENIOT, G., GUAN, M., LIU, Z., SUN, F., LIM, Y. P., LYONS, E., TOWN, C. D., BANCROFT, I., WANG, X., MENG, J., MA, J., PIRES, J. C., KING, G. J., BRUNEL, D., DELOURME, R., RENARD, M., AURY, J.-M., ADAMS, K. L., BATLEY, J., SNOWDON, R. J., TOST, J., EDWARDS, D., ZHOU, Y., HUA, W., SHARPE, A. G., PATERSON, A. H., GUAN, C. & WINCKER, P. 2014. Early allopolyploid evolution in the post-Neolithic Brassica napus oilseed genome. *Science,* 345**,** 950-953.

CORDOVEZ, V., MOMMER, L., MOISAN, K., LUCAS-BARBOSA, D., PIERIK, R., MUMM, R., CARRION, V. J. & RAAIJMAKERS, J. M. 2017. Plant Phenotypic and Transcriptional Changes Induced by Volatiles from the Fungal Root Pathogen *Rhizoctonia solani*. *Frontiers in Plant Science,* 8.

DE VOS, M., VAN OOSTEN, V. R., VAN POECKE, R. M. P., VAN PELT, J. A., POZO, M. J., MUELLER, M. J., BUCHALA, A. J., METRAUX, J. P., VAN LOON, L. C., DICKE, M. & PIETERSE, C. M. J. 2005. Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Molecular Plant-Microbe Interactions,* 18**,** 923-937.

DICKE, M. & VAN POECKE, R. M. P. 2002. Signaling in plant-insect interactions: signal transduction in direct and indirect plant defence**.,** 289-316.

DICKE, M., VAN POECKE, R. M. P. & DE BOER, J. G. 2003. Inducible indirect defence of plants: from mechanisms to ecological functions. *Basic and Applied Ecology,* 4**,** 27-42.

DOMBRECHT, B., XUE, G. P., SPRAGUE, S. J., KIRKEGAARD, J. A., ROSS, J. J., REID, J. B., FITT, G. P., SEWELAM, N., SCHENK, P. M., MANNERS, J. M. & KAZAN, K. 2007. MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*. *Plant Cell,* 19**,** 2225-2245.

DRAKULIC, J., CAULFIELD, J., WOODCOCK, C., JONES, S. P. T., LINFORTH, R., BRUCE, T. J. A. & RAY, R. V. 2015. Sharing a Host Plant (Wheat *Triticum aestivum*) Increases the Fitness of *Fusarium graminearum* and the Severity of *Fusarium* Head Blight but Reduces the Fitness of Grain Aphids (*Sitobion avenae*). *Applied and Environmental Microbiology,* 81**,** 3492-3501.

DRIZOU, F., GRAHAM, N. S., BRUCE, T. J. A. & RAY, R. V. 2017. Development of high-throughput methods to screen disease caused by *Rhizoctonia solani* AG 2-1 in oilseed rape. *Plant Methods,* 13**,** 45.

ELZINGA, D. A., DE VOS, M. & JANDER, G. 2014. Suppression of Plant Defenses by a *Myzus persicae* (Green Peach Aphid) Salivary Effector Protein. *Molecular Plant-Microbe Interactions,* 27**,** 747-756.

FAOSTAT Food and Agriculture Organisation Statistical Database FAOSTAT. [http://faostat.fao.org/].

FOLEY, R. C., GLEASON, C. A., ANDERSON, J. P., HAMANN, T. & SINGH, K. B. 2013. Genetic and Genomic Analysis of *Rhizoctonia solani* Interactions with *Arabidopsis*; Evidence of Resistance Mediated through NADPH Oxidases. *Plos One,* 8.

GLAZEBROOK, J. 2005. Contrasting Mechanisms of Defense Against Biotrophic and Necrotrophic Pathogens. *Annual Review of Phytopathology,* 43**,** 205-227.

JAOUANNET, M., RODRIGUEZ, P. A., THORPE, P., LENOIR, C. J. G., MACLEOD, R., ESCUDERO-MARTINEZ, C. & BOS, J. I. B. 2014. Plant immunity in plant–aphid interactions. *Frontiers in Plant Science,* 5.

KATARIA, H. R. & VERMA, P. R. 1992. *Rhizoctonia solani* damping-off and root rot in oilseed rape and canola. *Crop Protection,* 11**,** 8-13.

KHANGURA, R. K., BARBETTI, M. J. & SWEETINGHAM, M. W. 1999. Characterization and pathogenicity of *Rhizoctonia* species on canola. *Plant Disease,* 83**,** 714-721.

KIM, K. C., LAI, Z. B., FAN, B. F. & CHEN, Z. X. 2008. Arabidopsis WRKY38 and WRKY62 Transcription Factors Interact with Histone Deacetylase 19 in Basal Defense. *Plant Cell,* 20**,** 2357-2371.

KÖRBER, N., BUS, A., LI, J., HIGGINS, J., BANCROFT, I., HIGGINS, E. E., PAPWORTH PARKIN, I. A., SALAZAR-COLQUI, B., SNOWDON, R. J. & STICH, B. 2015. Seedling development traits in Brassica napus examined by gene expression analysis and association mapping. *BMC Plant Biology,* 15**,** 136.

LAZEBNIK, J., FRAGO, E., DICKE, M. & VAN LOON, J. J. A. 2014. Phytohormone Mediation of Interactions Between Herbivores and Plant Pathogens. *Journal of Chemical Ecology,* 40**,** 730-741.

LE GUIGO, P., MAINGENEAU, A. & LE CORFF, J. 2011. Performance of an aphid *Myzus persicae* and its parasitoid *Diaeretiella rapae* on wild and cultivated Brassicaceae. *Journal of Plant Interactions,* 7**,** 326-332.

LEATHER, S. R. & DIXON, A. F. G. 1984. Aphid growth and reproductive rates. *Entomologia Experimentalis et Applicata,* 35**,** 137-140.

LI, J., BRADER, G., KARIOLA, T. & PALVA, E. T. 2006. WRKY70 modulates the selection of signaling pathways in plant defense. *Plant Journal,* 46**,** 477-491.

LIU, L. J., SONBOL, F. M., HUOT, B., GU, Y. N., WITHERS, J., MWIMBA, M., YAO, J., HE, S. Y. & DONG, X. N. 2016. Salicylic acid receptors activate jasmonic acid signalling through a non-canonical pathway to promote effector-triggered immunity. *Nature Communications,* 7**,** 10.

LORENZO, O., CHICO, J. M., SÁNCHEZ-SERRANO, J. J. & SOLANO, R. 2004. JASMONATE-INSENSITIVE1 Encodes a MYC Transcription Factor Essential to Discriminate between Different Jasmonate-Regulated Defense Responses in Arabidopsis. *The Plant Cell,* 16**,** 1938-1950.

MAZUMDER, M., DAS, S., SAHA, U., CHATTERJEE, M., BANNERJEE, K. & BASU, D. 2013. Salicylic acid-mediated establishment of the compatibility between *Alternaria brassicicola* and *Brassica juncea* is

mitigated by abscisic acid in Sinapis alba. *Plant Physiology and Biochemistry,* 70**,** 43-51.

MORAN, P. J. & THOMPSON, G. A. 2001. Molecular responses to aphid feeding in Arabidopsis in relation to plant defense pathways. *Plant Physiology,* 125**,** 1074-1085.

NOVAKOVA, M., SASEK, V., DOBREV, P. I., VALENTOVA, O. & BURKETOVA, L. 2014. Plant hormones in defense response of *Brassica napus* to *Sclerotinia sclerotiorum* - Reassessing the role of salicylic acid in the interaction with a necrotroph. *Plant Physiology and Biochemistry,* 80**,** 308-317.

OCCHIPINTI, A. 2013. Plant coevolution: evidences and new challenges. *Journal of Plant Interactions,* 8**,** 188-196.

OGOSHI, A. 1987. Ecology and pathogenicity of anastomosis groups of *Rhizoctonia solani* Kühn *Annual Review of Phytopathology,* 25**,** 125-143.

PARAMETER, J. R. J. 1970. *Rhizoctonia solani: Biology and Pathology,* University of California Press.

PERL-TREVES, R., FOLEY, R. C., CHEN, W. Q. & SINGH, K. B. 2004. Early induction of the Arabidopsis GSTF8 promoter by specific strains of the fungal pathogen *Rhizoctonia solani*. *Molecular Plant-Microbe Interactions,* 17**,** 70-80.

PIETERSE, C. M. J. & DICKE, M. 2007. Plant interactions with microbes and insects: from molecular mechanisms to ecology. *Trends in Plant Science,* 12**,** 564-569.

PUINEAN, A. M., FOSTER, S. P., OLIPHANT, L., DENHOLM, I., FIELD, L. M., MILLAR, N. S., WILLIAMSON, M. S. & BASS, C. 2010. Amplification of a Cytochrome P450 Gene Is Associated with Resistance to Neonicotinoid Insecticides in the Aphid *Myzus persicae*. *Plos Genetics,* 6.

RADFORD, P. J. 1967. Growth Analysis Formulae - Their Use and Abuse1. *Crop Science,* 7**,** 171-175.

RAY, R. V., JENKINSON, P. & EDWARDS, S. G. 2004. Effects of fungicides on eyespot, caused predominantly by *Oculimacula*

acuformis, and yield of early-drilled winter wheat. *Crop Protection,* 23**,** 1199-1207.

SCHOONHOVEN, L. M., VAN LOON, J. J. A. & DICKE, M. 2005. *Insect-plant biology*, Oxford University Press, USA.

SCHULTZ, J. C., APPEL, H. M., FERRIERI, A. P. & ARNOLD, T. M. 2013. Flexible resource allocation during plant defense responses. *Frontiers in Plant Science,* 4**,** 11.

SIMON-DELSO, N., AMARAL-ROGERS, V., BELZUNCES, L. P., BONMATIN, J. M., CHAGNON, M., DOWNS, C., FURLAN, L., GIBBONS, D. W., GIORIO, C., GIROLAMI, V., GOULSON, D., KREUTZWEISER, D. P., KRUPKE, C. H., LIESS, M., LONG, E., MCFIELD, M., MINEAU, P., MITCHELL, E. A. D., MORRISSEY, C. A., NOOME, D. A., PISA, L., SETTELE, J., STARK, J. D., TAPPARO, A., VAN DYCK, H., VAN PRAAGH, J., VAN DER SLUIJS, J. P., WHITEHORN, P. R. & WIEMERS, M. 2015. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. *Environmental Science and Pollution Research,* 22**,** 5-34.

STURROCK, C. J., WOODHALL, J., BROWN, M., WALKER, C., MOONEY, S. J. & RAY, R. V. 2015. Effects of damping-off caused by *Rhizoctonia solani* anastomosis group 2-1 on roots of wheat and oil seed rape quantified using X-ray Computed Tomography and real-time PCR. *Frontiers in Plant Science,* 6.

TEO, B. K., YITBAREK, S. M., VERMA, P. R. & MORRALL, R. A. A. 1988. Influence of soil moisture, seeding date, and *Rhizoctonia solani* isolates (AG 2-1 and AG 4) on disease incidence and yield in canola. *Canadian Journal of Plant Pathology,* 10**,** 151-158.

THOMPSON, G. A. & GOGGIN, F. L. 2006. Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects. *Journal of Experimental Botany,* 57**,** 755-766.

VAN DAM, N. M., TYTGAT, T. O. G. & KIRKEGAARD, J. A. 2009. Root and shoot glucosinolates: a comparison of their diversity, function and interactions in natural and managed ecosystems. *Phytochemistry Reviews,* 8**,** 171-186.

VAN LOON, L. C., REP, M. & PIETERSE, C. M. J. 2006. Significance of inducible defense-related proteins in infected plants. *Annual Review of Phytopathology*.

VOS, I. A., PIETERSE, C. M. J. & VAN WEES, S. C. M. 2013. Costs and benefits of hormone-regulated plant defences. *Plant Pathology,* 62**,** 43-55.

WANG, X. L., JIANG, N., LIU, J. L., LIU, W. D. & WANG, G. L. 2014. The role of effectors and host immunity in plant-necrotrophic fungal interactions. *Virulence,* 5**,** 722-732.

WHITE, T. J., BRUNS, T., LEE, S., TAYLOR, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. . *Innis, M.A., et al. (Ed.), PCR Protocols: A Guide to Methods and Applications.* San Diego, California,: Academic Press.

WOODHALL, J. W., WEBB, K. M., GILTRAP, P. M., ADAMS, I. P., PETERS, J. C., BUDGE, G. E. & BOONHAM, N. 2012. A new large scale soil DNA extraction procedure and real-time PCR assay for the detection of Sclerotium cepivorum in soil. *European Journal of Plant Pathology,* 134**,** 467-473.

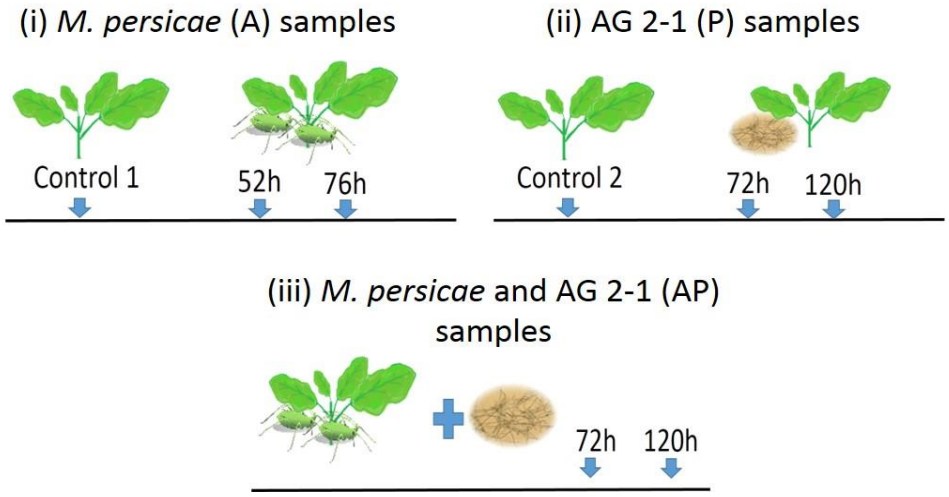
WU, Y., ZHANG, D., CHU, JEE Y., BOYLE, P., WANG, Y., BRINDLE, IAN D., DE LUCA, V. & DESPRÉS, C. 2012. The Arabidopsis NPR1 Protein Is a Receptor for the Plant Defense Hormone Salicylic Acid. *Cell Reports,* 1**,** 639-647.

WYATT, I. J. & WHITE, P. F. 1977. Simple estimation of intrinsic increase rates for aphids and tetranychid mites. *Journal of Applied Ecology,* 14**,** 757-766.

YITBAREK, S. M., VERMA, P. R., GUGEL, R. K. & MORRALL, R. A. A. 1988. Effect of soil-temperature and inoculum density on pre-emergence damping-off of canola caused by *Rhizoctonia solani Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie,* 10**,** 93-98.

ZHENG, S. J., VAN DIJK, J. P., BRUINSMA, M. & DICKE, M. 2007. Sensitivity and speed of induced defense of cabbage (*Brassica oleracea* L.): Dynamics of BoLOX expression patterns during insect and pathogen attack. *Molecular Plant-Microbe Interactions,* 20**,** 1332-1345.

4.10 Supplementary data



Supplementary Figure 4.1 Sampling method for different treatments for gene expression: (i) Control 1 samples collected from plants prior to aphid infestation, *M. persicae* (A) samples 52 h and 76 h after infestation with aphids (ii) Control 2 samples collected prior to infection with the pathogen, AG 2-1 (P) samples collected 72 h and 120 h after infection with the fungi. (iii) From plants that had been infested with *M. persicae* for 72h and then with *R. solani* AG 2-1 (AP), collected samples at 72 h and 120h after the infection with the later.

Supplementary Table 4.1 Sequence of forward and reverse primers for the ribosomal ITS1 region of the *R. solani* used in ITS and RT-PCR in the compost extractions, the target genes and *ACTIN* (reference gene)

Gene	Forward primer	Reverse primer
ITS1	5'-CTTCCTCTTTCATCCCACACA-3'	5'-TGAGTAGACAGAGGGTCCAATAACCTA-3'
LOX3	5'-GGCCTTACCCTAGACGGTGT-3'	5'-TTCAAATTGCTCGTCTCGTG-3'
MYC2	5'-TGCGTGAGCTCAATTCTTTG-3'	5'-GCTCTGTGTCATCGAAACCA-3'
NPR1	5'-AGGGGATATACGGTGCTTCA-3'	5'-GAGAGCCGTTCTACCTTCCA-3'
PR1	5'-ATGTCAACGCTCACAACCAA-3'	5'-TCTTAGTCGGTCGGCGTAGT-3'
WRKY38	5'-GGACCAGTACCGTGGGATAA-3'	5'-GGGATAACCGGTGACGATAA-3'
ACTIN	5'- TCAGGCCGTTCTTTCTCTTTAC-3'	5'-GAGCATAACCCTCGTAGATTGG-3'

Supplementary Table 4.2 Fungal DNA (log10 (DNA pg ng^{-1} of total DNA)) extracted from compost and Fresh weight (F.W.) of Canard and Temple under treatment with aphids and pathogen (AP) and pathogen only (P). For the comparison between treatments and varieties *P* value and LSD (for treatment-variety interaction) were used (ANOVA).

		DNA (pg ng^{-1})	F.W (g)
Canard	AP	2.16	11.98
	P	2.00	11.30
Temple	AP	2.06	11.45
	P	2.16	11.20
$P_{(t*v)}$		0.669	0.693
$LSD_{(t*v)}$		0.446	1.537

Chapter 5. **General Discussion**

Disease management has been an important part of agriculture through the centuries and one of the vital aspects ensuring food security. The increasing global food demand as a result of the global population increase in combination with the climate change and the negative effects of intensive agriculture highlights the need of new sustainable strategies for disease management (Savary, 2014, Tilman et al., 2011). This approach requires good understanding of the fundamental interactions occurring between pathogens and crops within the environment, as well as the impact of other organisms within the ecosystem to their interaction.

Globally there is a high demand for the cultivation of *Brassica napus*, however it has been addressed that in UK the achieved yields are behind the estimated potential yields (HGCA, 2014). Although the effect of individual agronomical factors alone is unclear, it is stated that one important restricting factor is the intensive cultivation of OSR, which is linked with higher disease pressure (HGCA, 2014). Oilseed rape is under pressure of many pathogens such as *Leptosphaeria maculans* and *L. biglobsa* (phoma leaf spot and stem canker respectively), *Sclerotinia sclerotiorum* (stem rot), *Plasmidiophora brassicae* (clubroot) and *Verticillium longiosporum* (verticillium wilt). Nonetheless, as I mentioned before the high frequency of OSR cultivation in combination with alterations in agricultural practises (cultivated varieties, crop rotations, pesticide application) could result in the emergence of new diseases that previously were not considered a major problem (Hannukkala et al., 2016). For example it has been recently shown that this was the case for *R. solani* AG 2-1 in OSR fields in Finland (Hannukkala et al., 2016). *Rhizoctonia solani* AG 2-1 is known to be present in UK fields (Brown et al., 2014). This in combination with the lack of genetic resistance of OSR germplasm to this pathogen (Babiker et al., 2013) makes it a potential threat for OSR cultivation.

The present study aimed to provide a better understanding of the interaction between *R. solani* AG 2-1 and OSR. While confirming that *R. solani* AG 2-1 is a highly virulent pathogen to a wide range of OSR germplasm (Chapter 2 and

3), improvements to disease measurement protocols were made. Insights were obtained into plant defence responses with preliminary evidence suggesting that AG 2-1 is capable of inducing both JA and SA related plant defences and overcome them through an unknown mechanism (Chapter 4). Also, for the first time, it is shown that *M. persicae* infestation indirectly increases susceptibility to AG 2-1, through the suppression of JA related plant defences (Chapter 4).

The current lack of genetic resistance in OSR is a great drawback for the development of sustainable control methods against this pathogen (Babiker et al., 2013). Identifying resistance traits could be a particularly challenging task in the absence of a suitable high-throughput screening method. Hence, the initial objective of this study was to develop and compare different high-throughput phenotyping methods to screen disease caused by AG 2-1 in OSR (Chapter 2). Considering that AG 2-1 infects seeds and the early seedling stage of OSR causing seed decay, root rot and damping off disease (Kataria and Verma, 1992, Khangura et al., 1999), the aim was to develop a method that would enable the screening of the early stages of disease progression. Apart from the identification of robust genetic resistance, which is the main target in breeding programs, other traits linked with the rapid growth of the plant which enable faster establishment of a strong root system and disease escape are also important (Sturrock et al., 2015). For example it has been shown that there is a correlation between root morphology, nutrient concentration and OSR type (winter or spring) (Thomas et al., 2016a), with winter OSR and fodder types to have larger roots and lower concentration of micronutrients in their leaves compared to spring OSR (Thomas et al., 2016a). The four methods (1- nutrient media plates, 2- hydroponic growth in pouch and wick system, and growth in multi-well trays with 3- compost or 4- LECA) developed here, were assessed for their ability to screen for disease resistance and tolerance. Out of the four methods, the most suitable appeared to be trays with LECA: this method embraced the majority of the benefits of screening in compost but also enabled disease assessments of the root system. It thus provided a high-throughput and low-cost method to assess disease in roots and hypocotyls of OSR seedlings during the early infection stages.

Here it needs to be mentioned that although screening under controlled environmental conditions tries to simulate field conditions, infection in the field it is expected to be different. This is related not only with the amount and the source of inoculum but also with the interactions within the ecosystem and environment that affect the epidemiology of the pathogen and disease progression. Therefore, identification of resistant and/or tolerant germplasm should be also tested under field conditions.

All genotypes tested during the development of these methods, were susceptible to the pathogen; although differences among them were observed, overall disease severity was elevated even within 5 dpi, ranking from approximately 30-85 % (disease on hypocotyl; Chapter 2). Among the set of 8 genotypes tested were 'Temple' and 'Canard', parental lines of the 'Temple' x 'Canard' mapping population (TCDH). In both tray methods with compost and LECA, those genotypes had contrasting responses, with 'Canard' to performing better in terms of disease resistance.

For the identification of *R. solani* resistance in *Brassica* germplasm, studies so far have tested a wide range of *B. napus*, *B. rapa*, *B. oleracea* and other *Brassica* species (Acharya et al., 1984, Babiker et al., 2013, Yang and Verma, 1992). Despite the diverse screening that has been performed, no resistant genotype has been identified yet (Babiker et al., 2013). The present study aimed to screen a diversity of *B. napus* genotypes (Chapter 3) starting with a selection of commercial cultivars and genotypes from two diversity sets (group 1), then a selection of genotypes from the ASSYST population (group 2) and the TCDH mapping population (group 3; based on the results from Chapter 2). The screening was performed in trays with compost. That was because the LECA material, and its suitability for the screening method, was only found after the completion of screening (Chapter 3) and towards the end of the third year of the project. Nonetheless, as discussed previously, screening in compost is the second most suitable screening method developed in this study. Additionally, the results from Chapter 3 show a correlation between the responses of the genotypes between trays with compost and LECA; confirming that compost trays do provide valid data.

Overall, screening of a range of different germplasm showed that there was no genetic resistance to AG 2-1 infection, confirming our current knowledge (Babiker et al., 2013, Yang and Verma, 1992). However, an important aspect that needs to be taken into account is that 10 dpi was a very long exposure period to the pathogen, considering the increased susceptibility of the germplasm and the high virulence of AG 2-1. Perhaps screening again with a selection of this germplasm in trays with LECA under 5 dpi might enable the detection of more pronounced differences with respect to disease severity.

Differences between genotypes regarding their response to AG 2-1 were observed (Chapter 3): For example in group 1, ASSYST 224 had lower disease levels compared to the other genotypes at 10 dpi. Also, variation in emergence and survival was observed in group 2, where genotypes ASSYST 269, -194 and -279 had equally good emergence with compared to non-inoculated controls. Similarly, during the screening of the TCDH population, emergence and survival was significantly different within 10 dpi and when the experimental protocol altered from 10 dpi to 5 dpi, differences on disease within the group of selected genotypes were also observed. Therefore, potentially tolerance traits related to plant growth could be identified. In order to evaluate this it would be useful to screen these genotypes on nutrient media plates (Chapter 2). That method would provide information regarding their root length and lateral root density, data that could be correlated with emergence and survival and identify if these genotypes had better emergence and/or survival because of the early establishment of their root system which promote their growth and disease escape (Sturrock et al., 2015). Considering the increased susceptibility and the lack of resistance in *B. napus* germplasm to AG 2-1, breeding programs should also focus on the screening of a wide range of wild relative species. Wild *Brassica* germplasm is likely to obtain resistant traits that have been lost under domestication. Furthermore, probably a phylogenetic approach, taking into account the polyploid events that resulted in the current genetic variation of *Brassicas* (Renny-Byfield and Wendel, 2014, Soltis et al., 2009) it is also important for the identification of resistant traits. Studies so far have screened a range of *B. napus*, *B. oleracea* and *B. rapa* (genomes C and A respectively), as well as other *Brassica* species

(Babiker et al., 2013, Yang and Verma, 1992). However, as no resistance to AG 2-1 has identified within these close related species probably a wider selection of germplasm needs to be screened, including different genera and species from Brassicales. Potential differences of germplasm, to their response to AG 2-1, could be further analysed with transcriptomic approaches for the identification of genetic traits of resistance.

Another aspect that was examined was to test the hypothesis that induced defence could be a transgenerational trait. It has been established that exposure to biotic stresses or compounds can induce plant resistance, which could be also expressed in future generations (Bruce et al., 2007, Holeski et al., 2012). For example exogenous treatment with JA as well as herbivory by the small cabbage butterfly *Pieris rapae* on *Raphanus raphanistrum* induced transgenerational resistance to their progeny compared to control plants (Agrawal, 2002). Additionally, infection of tobacco seedlings (*Nicotiana tabacum*) with the tobacco mosaic virus resulted in resistance of progeny plants to infection by the same virus and also *Pseudomonas syringae* bacterium and to the oomycete *Phytophthora nicotiannae* (Kathiria et al., 2010). Regarding *R. solani* AG 2-1 and OSR, it has been suggested that resistance could be achieved via selection (Yang and Verma, 1992) and therefore it could be a result of transgenerational induction. However, the results in the present work from both OSR germplasm and *A. thaliana* do not support this hypothesis (Chapter 3). Conflicting with our hypothesis, the origin of the seeds had a negative effect on plant's susceptibility to the pathogen with progeny of infected plants emerging and surviving worse than their parents. It might be that inoculation of parental plants compromised seeds' health and this affected progeny performance or simply that lack of any induced defence in the parental germplasm did not enable the identification of such a transgenerational effect. Probably testing more generations will enable to validate this finding. Nonetheless, the present results do not support that resistance, could be passed to the next generation or even that it exists in the first generation.

When no resistance exists, understanding plant defence mechanisms could be a useful tool for the development of alternative methods of disease management.

In addition, when studying plant defence mechanisms it is essential to take into account that in natural ecosystems, plant are exposed to an array of attackers (Dicke et al., 2003, Pieterse and Dicke, 2007). This is of great importance when considering that different organisms sharing the same host indirectly affect one another (Lazebnik et al., 2014). The present work aimed to gain a better insight into *B. napus* plant defences against AG 2-1 and illustrate if and how these are altered when the plant is also attacked by *M. persicae* aphids (Chapter 4). Experiments were firstly performed to establish if there was an indirect interaction between AG 2-1 and *M. persicae*, where it was shown that 'Canard' plants exposed to herbivory (AP) had increased disease on their stems, whilst in 'Temple' the disease was similar between the two treatments. Additionally, the amount of fungal DNA was not different between aphid and pathogen (AP) combined treatments compared to treatment with pathogen (P) alone in both cultivars, indicating that the observed increase in disease in 'Canard' plants with AP treatment was possibly due to alterations in plant defence status induced by *M. persicae*. For a different study system, it is known that during simultaneous attack, aphid infestation can alter plant response and benefit the pathogen but not the aphids (Drakulic et al., 2015). In our study *M. persicae* aphids were not affected by AG 2-1 infection and aphid infestation affected only the response of one cultivar, 'Canard', to AG 2-1, which highlights not only the interspecific variability of plant defences that is already known (Sarwar and Sattar, 2013), but also the variability within the same plant species. As it is discussed before (Chapter 4), the different response of the two cultivars could be due to their potentially different GSL profile. So it would be interesting for future work to establish if there are differences in the GSL profiles of those cultivars and if these are linked with the observed differences.

For a better understanding of the interaction and the alterations that take place, the expression of different marker genes related with JA and SA signalling and plant defences was examined. Infection by *R. solani* alone upregulated the expression of *LOX3*, *MYC2* and *PR1* indicating the involvement of both JA and SA signalling. As a necrotrophic fungus *R. solani* is expected to induce JA related plant defences (Glazebrook, 2005) but considering that JA signalling

pathway is separated into two branches, with the MYC branch negatively affecting defence against necrotrophic fungi, it is surprising that there was an upregulation of *MYC2.* This could potentially indicate that AG 2-1 suppresses plant defences for its own benefit. Regarding the upregulation of *PR1* by AG 2-1, it has been shown previously in Arabidopsis (Foley et al., 2016) and also more evidence states that there is a coordination of signalling pathways and hormones (Liu et al., 2016), thus SA signalling is likely to be also activated for necrotrophic pathogens. Overall, this study showed that *M. persicae* induced changes in the JA signalling pathway, expressed as downregulation of *MYC2* and *LOX3* under AP treatment. The upregulation of *NPR1* in AP treatment implies a role of SA signalling during dual attack. Negative crosstalk between JA- and SA-signalling pathways is well known (Bruce et al., 2007) and therefore it is expected that suppressing of JA-signalling will increase SA-signalling. It is well established that these interactions are characterised by great complexity with other plant hormones to interfere with JA and SA and affect the final outcome (Shigenaga and Argueso, 2016, Vos et al., 2015). Therefore future transcriptomic work will enable the better understanding of the observed plant responses. Following to this, transcriptomic analysis on 'Temple' could also help us to understand why this cultivar had a different response compared to 'Canard', under aphid infestation. Nonetheless, this study provided for the first time an indication of how *B. napus*, the primary host of AG 2-1 responds to infection by this elegant pathogen and how this response changes in the presence of *M. persicae*. This knowledge is particularly useful considering the increase pressure of pest such as *M. persicae* in OSR fields, after the changes on pesticides legislation and the need to develop integrated methods for disease control. Additionally, further research on this field could also benefit breeding programs aiming to develop resistant genotypes for multiple attackers.

5.1 Summary of Conclusions

- Multi-well trays with LECA provide a new low cost, high-throughput screening method for the identification resistant OSR germplasm to *R. solani* AG 2- 1. This method can be used as an early step for the evaluation of germplasm prior to testing under field conditions.

- AG 2-1 is very pathogenic to OSR germplasm and results in high disease level and reduced seedling performance.
- Differences in emergence, survival and disease severity of OSR germplasm, could potentially indicate tolerance.
- Resistance of OSR to AG 2-1 is unlikely to involve transgenerational induction of resistance, inherited as result of epigenetic stress responses.
- AG 2-1 infection induces the upregulation of marker genes from both JA and SA signalling pathways.
- AG 2-1 infection does not indirectly affect *M. persicae* performance but *M. persicae* infestation increases susceptibility to AG 2-1 in a cultivar specific manner.
- Induced changes and suppression of the JA signalling pathway by *M. persicae* is probably the reason for increased susceptibility to AG 2-1.

5.2 Future work

- Important aspect that needs to be considered in future screening for resistance to AG 2-1, should be the phylogenetic relationship between *B. napus* and other *Brassica* species. Future work will be highly benefited from the screening of a collection with accessions of both *B. rapa* (genome A) and *B. oleracea* (genome C). In addition to this, further screening of diverse populations of *B. napus* and wild *Brassica* species is another essential step to elucidate if there is any resistance against this destructive pathogen.
- Based on the finding of this PhD, screening of germplasm should be conducted with the LECA method and under 5 dpi, for the detection of differences in disease severity even with highly susceptible germplasm.
- Alternative method to elucidate the interaction between OSR and AG 2-1, could be the screening of different fungal isolates within AG 2-1. It could be very interesting to obtain and test a global collection of isolates. In this way it could be identified if there is variation within AG 2-1 and understand the phylogenetic link in their global distribution.

- Following the previous point, development of impaired mutants and their ability to infect or/and cause disease in OSR, could also illustrate the role of key genes in plant-pathogen communication and plant defences and potentially enable the development of novel control strategies for AG 2-1.
- Examination of additional genes from JA and SA signalling pathway, as well as genes related with ET and ABA will provide a better understanding on the *M. persicae*-AG 2-1 indirect interaction. A better approach would be to perform transcriptomic analysis, as it will unravel the role of multiple signalling pathways providing the profile of an array of genes.
- Elucidation of plant defences in cultivar 'Temple', regarding its response to *M .persicae* and AG 2-1, will potentially reveal intraspecific differences between 'Canard' and 'Temple'.
- Detection of alterations in the expression of virulent genes in AG 2-1, is also an interesting aspect, as it would enable us to understand how the pathogen overcome/ manipulates plant defence mechanisms.

5.3 References

ACHARYA, S. N., VERMA, P. R., DUECK, J. & DOWNEY, R. K. 1984. Screening rapeseed/canola for resistance to damping-off and seedling root rot caused by *Rhizoctonia solani*. *Canadian Journal of Plant Pathology,* 6**,** 325-328.

AGRAWAL, A. A. 2002. Herbivory and maternal effects: Mechanisms and consequences of transgenerational induced plant resistance. *Ecology,* 83**,** 3408-3415.

AGRIOS, G. N. 2005. *Plant Pathology,* London, UK, Elsevier Academic Press.

AJAYI-OYETUNDE, O. O., BUTTS-WILMSMEYER, C. J. & BRADLEY, C. A. 2017. Sensitivity of *Rhizoctonia solani* to succinate dehydrogenase inhibitor and demethylation inhibitor fungicides. *Plant Disease,* 101**,** 487-495.

AL-NAEMI, F. & HATCHER, P. E. 2013. Contrasting effects of necrotrophic and biotrophic plant pathogens on the aphid *Aphis fabae*. *Entomologia Experimentalis Et Applicata,* 148**,** 234-245.

ALLENDER, C. J. & KING, G. J. 2010. Origins of the amphiploid species *Brassica napus* L. investigated by chloroplast and nuclear molecular markers. *BMC Plant Biology,* 10**,** 1-9.

ANDERSON, N. A. 1982. The genetics and pathology of *Rhizoctonia solani*. *Annual Review of Phytopathology,* 20**,** 329-347.

ATANASOVA, L., LE CROM, S., GRUBER, S., COULPIER, F., SEIDL-SEIBOTH, V., KUBICEK, C. P. & DRUZHININA, I. S. 2013. Comparative transcriptomics reveals different strategies of *Trichoderma* mycoparasitism. *Bmc Genomics,* 14**,** 15.

ATKINSON, J. A., WINGEN, L. U., GRIFFITHS, M., POUND, M. P., GAJU, O., FOULKES, M. J., LE GOUIS, J., GRIFFITHS, S., BENNETT, M. J., KING, J. & WELLS, D. M. 2015. Phenotyping pipeline reveals major seedling root growth QTL in hexaploid wheat. *Journal of Experimental Botany*.

BABIKER, E., HULBERT, S., SCHROEDER, K. & PAULITZ, T. 2013. Evaluation of *Brassica* species for resistance to *Rhizoctonia solani* and

binucleate *Rhizoctonia* (*Ceratobasidum spp*.) under controlled environment conditions. *European Journal of Plant Pathology,* 136**,** 763-772.

BARDGETT, R. D., WARDLE, D. A. & YEATES, G. W. 1998. Linking above-ground and below-ground interactions: How plant responses to foliar herbivory influence soil organisms. *Soil Biology & Biochemistry,* 30**,** 1867-1878.

BARI, R. & JONES, J. 2009. Role of plant hormones in plant defence responses. *Plant Molecular Biology,* 69**,** 473-488.

BASS, C., PUINEAN, A. M., ANDREWS, M., CUTLER, P., DANIELS, M., ELIAS, J., PAUL, V. L., CROSSTHWAITE, A. J., DENHOLM, I., FIELD, L. M., FOSTER, S. P., LIND, R., WILLIAMSON, M. S. & SLATER, R. 2011. Mutation of a nicotinic acetylcholine receptor β subunit is associated with resistance to neonicotinoid insecticides in the aphid *Myzus persicae*. *BMC Neuroscience,* 12**,** 51.

BENITEZ, T., RINCON, A. M., LIMON, M. C. & CODON, A. C. 2004. Biocontrol mechanisms of *Trichoderma* strains. *International Microbiology,* 7**,** 249-260.

BLACKMAN, R. L. & EASTOP, V. F. 2000. *Aphids on the world's crops An identification and information guide*, John Willey & sins Ltd.

BORGEN, B. H., THANGSTAD, O. P., AHUJA, I., ROSSITER, J. T. & BONES, A. M. 2010. Removing the mustard oil bomb from seeds: transgenic ablation of myrosin cells in oilseed rape (*Brassica napus*) produces MINELESS seeds. *Journal of Experimental Botany,* 61**,** 1683-1697.

BROWN, M., WOODHALL, J., MOONEY, S. & RAY, R. The occurrence and population dynamics of *Rhizoctonia solani* in soil of winter wheat. The Dundee Conference. Crop Protection in Northern Britain 2014, Dundee, UK, 25-26 February 2014, 2014. The Association for Crop Protection in Northern Britain, 107-112.

BRUCE, T. J. A. 2014. Glucosinolates in oilseed rape: secondary metabolites that influence interactions with herbivores and their natural enemies. *Annals of Applied Biology,* 164**,** 348-353.

BRUCE, T. J. A. 2015. Interplay between insects and plants: dynamic and complex interactions that have coevolved over millions of years but act in milliseconds. *Journal of Experimental Botany,* 66**,** 455-465.

BRUCE, T. J. A., MATTHES, M. C., NAPIER, J. A. & PICKETT, J. A. 2007. Stressful "memories" of plants: Evidence and possible mechanisms. *Plant Science,* 173**,** 603-608.

BUDGE, G. E., SHAW, M. W., COLYER, A., PIETRAVALLE, S. & BOONHAM, N. 2009. Molecular tools to investigate *Rhizoctonia solani* distribution in soil. *Plant Pathology,* 58**,** 1071-1080.

BUS, A., KÖRBER, N., PARKIN, I. A. P., SAMANS, B., SNOWDON, R. J., LI, J. & STICH, B. 2014. Species- and genome-wide dissection of the shoot ionome in *Brassica napus* and its relationship to seedling development. *Frontiers in Plant Science,* 5**,** 485.

BUS, A., KÖRBER, N., SNOWDON, R. J. & STICH, B. 2011. Patterns of molecular variation in a species-wide germplasm set of *Brassica napus*. *Theor Appl Genet,* 123.

BUTTNER, G., PFAHLER, B. & MARLANDER, B. 2004. Greenhouse and field techniques for testing sugar beet for resistance to *Rhizoctonia* root and crown rot. *Plant Breeding,* 123**,** 158-166.

CARLING, D. E., BAIRD, R. E., GITAITIS, R. D., BRAINARD, K. A. & KUNINAGA, S. 2002. Characterization of AG-13, a newly reported anastomosis group of *Rhizoctonia solani*. *Phytopathology,* 92**,** 893-899.

CARRÉ, P. & POUZET, A. 2014. Rapeseed market, worldwide and in Europe. *OCL,* 21**,** D102.

CHALHOUB, B., DENOEUD, F., LIU, S., PARKIN, I. A. P., TANG, H., WANG, X., CHIQUET, J., BELCRAM, H., TONG, C., SAMANS, B., CORRÉA, M., DA SILVA, C., JUST, J., FALENTIN, C., KOH, C. S., LE CLAINCHE, I., BERNARD, M., BENTO, P., NOEL, B., LABADIE, K., ALBERTI, A., CHARLES, M., ARNAUD, D., GUO, H., DAVIAUD, C., ALAMERY, S., JABBARI, K., ZHAO, M., EDGER, P. P., CHELAIFA, H., TACK, D., LASSALLE, G., MESTIRI, I., SCHNEL, N., LE PASLIER, M.-C., FAN, G., RENAULT, V., BAYER, P. E., GOLICZ, A. A., MANOLI, S., LEE, T.-H., THI, V. H. D., CHALABI, S., HU, Q., FAN, C.,

TOLLENAERE, R., LU, Y., BATTAIL, C., SHEN, J., SIDEBOTTOM, C. H. D., WANG, X., CANAGUIER, A., CHAUVEAU, A., BÉRARD, A., DENIOT, G., GUAN, M., LIU, Z., SUN, F., LIM, Y. P., LYONS, E., TOWN, C. D., BANCROFT, I., WANG, X., MENG, J., MA, J., PIRES, J. C., KING, G. J., BRUNEL, D., DELOURME, R., RENARD, M., AURY, J.-M., ADAMS, K. L., BATLEY, J., SNOWDON, R. J., TOST, J., EDWARDS, D., ZHOU, Y., HUA, W., SHARPE, A. G., PATERSON, A. H., GUAN, C. & WINCKER, P. 2014. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. *Science,* 345**,** 950-953.

CHUNG, W. C., HUANG, J. W., HUANG, H. C. & JEN, J. F. 2002. Effect of ground *Brassica* seed meal on control of *Rhizoctonia* damping-off of cabbage. *Canadian Journal of Plant Pathology,* 24**,** 211-218.

COHEN, M. F., YAMASAKI, H. & MAZZOLA, M. 2005. *Brassica napus* seed meal soil amendment modifies microbial community structure, nitric oxide production and incidence of *Rhizoctonia* root rot. *Soil Biology & Biochemistry,* 37**,** 1215-1227.

COOK, R. J. 2001. Management of wheat and barley root diseases in modern farming systems. *Australasian Plant Pathology,* 30**,** 119-126.

CORDOVEZ, V., MOMMER, L., MOISAN, K., LUCAS-BARBOSA, D., PIERIK, R., MUMM, R., CARRION, V. J. & RAAIJMAKERS, J. M. 2017. Plant Phenotypic and Transcriptional Changes Induced by Volatiles from the Fungal Root Pathogen *Rhizoctonia solani*. *Frontiers in Plant Science,* 8.

CUBETA, M. A. & VILGALYS, R. 1997. Population biology of the *Rhizoctonia solani* complex. *Phytopathology,* 87**,** 480-484.

DE VOS, M., VAN OOSTEN, V. R., VAN POECKE, R. M. P., VAN PELT, J. A., POZO, M. J., MUELLER, M. J., BUCHALA, A. J., METRAUX, J. P., VAN LOON, L. C., DICKE, M. & PIETERSE, C. M. J. 2005. Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Molecular Plant-Microbe Interactions,* 18**,** 923-937.

DICKE, M. & VAN POECKE, R. M. P. 2002. Signaling in plant-insect interactions: signal transduction in direct and indirect plant defence**.**, 289-316.

DICKE, M., VAN POECKE, R. M. P. & DE BOER, J. G. 2003. Inducible indirect defence of plants: from mechanisms to ecological functions. *Basic and Applied Ecology,* 4**,** 27-42.

DOMBRECHT, B., XUE, G. P., SPRAGUE, S. J., KIRKEGAARD, J. A., ROSS, J. J., REID, J. B., FITT, G. P., SEWELAM, N., SCHENK, P. M., MANNERS, J. M. & KAZAN, K. 2007. MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*. *Plant Cell,* 19**,** 2225-2245.

DOUDS, D. D., LEE, J., UKNALIS, J., BOATENG, A. A. & ZIEGLER-ULSH, C. 2014. Pelletized biochar as a carrier for AM fungi in the on-farm system of inoculum production in compost and vermiculite mixtures. *Compost Science & Utilization,* 22**,** 253-262.

DRAKULIC, J., CAULFIELD, J., WOODCOCK, C., JONES, S. P. T., LINFORTH, R., BRUCE, T. J. A. & RAY, R. V. 2015. Sharing a host plant (Wheat *Triticum aestivum*) increases the fitness of *Fusarium graminearum* and the severity of fusarium head blight but reduces the fitness of grain aphids (*Sitobion avenae*). *Applied and Environmental Microbiology,* 81**,** 3492-3501.

DRIZOU, F., GRAHAM, N. S., BRUCE, T. J. A. & RAY, R. V. 2017. Development of high-throughput methods to screen disease caused by *Rhizoctonia solani* AG 2-1 in oilseed rape. *Plant Methods,* 13**,** 45.

ELZINGA, D. A., DE VOS, M. & JANDER, G. 2014. Suppression of plant defenses by a *Myzus persicae* (green peach aphid) salivary effector protein. *Molecular Plant-Microbe Interactions,* 27**,** 747-756.

FAOSTAT Food and Agriculture Organisation Statistical Database FAOSTAT. [http://faostat.fao.org/].

FOLEY, R. C., GLEASON, C. A., ANDERSON, J. P., HAMANN, T. & SINGH, K. B. 2013. Genetic and genomic analysis of *Rhizoctonia solani* interactions with *Arabidopsis*; Evidence of resistance mediated through NADPH oxidases. *Plos One,* 8.

FOLEY, R. C., KIDD, B. N., HANE, J. K., ANDERSON, J. P. & SINGH, K. B. 2016. Reactive oxygen species play a role in the infection of the necrotrophic fungi, *Rhizoctonia solani* in wheat. *Plos One,* 11.

GHORBANI, R., WILCOCKSON, S., KOOCHEKI, A. & LEIFERT, C. 2009. *Soil management for sustainable crop disease control: A Review,* New York, Springer.

GLAZEBROOK, J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology,* 43**,** 205-227.

GOLL, M. B., SCHADE-SCHUTZE, A., SWART, G., OOSTENDORP, M., SCHOTT, J. J., JASER, B. & FELSENSTEIN, F. G. 2014. Survey on the prevalence of *Rhizoctonia* spp. in European soils and determination of the baseline sensitivity towards sedaxane. *Plant Pathology,* 63**,** 148-154.

GRABER, A. & JUNGE, R. 2009. Aquaponic systems: nutrient recycling from fish wastewater by vegetable production. *Desalination,* 246**,** 147-156.

GUILLEMAUT, C., EDEL-HERMANN, V., CAMPOROTA, P., ALABOUVETTE, C., RICHARD-MOLARD, M. & STEINBERG, C. 2003. Typing of anastomosis groups of *Rhizoctonia solani* by restriction analysis of ribosomal DNA. *Canadian Journal of Microbiology,* 49**,** 556-568.

GUPTA, S. K. 2012. *Brassicas,* New York, Springer.

HANDISENI, M., BROWN, J., ZEMETRA, R. & MAZZOLA, M. 2013. Effect of Brassicaceae seed meals with different glucosinolate profiles on Rhizoctonia root rot in wheat. *Crop Protection,* 48**,** 1-5.

HANNUKKALA, A. O., RASTAS, M., LAITINEN, P. & LATVALA, S. 2016. *Rhizoctonia solani* injuries in oilseed crops in Finland and impacts of different crop management practices on disease incidence and severity. *Annals of Applied Biology,* 169**,** 257-273.

HÉMATY, K., CHERK, C. & SOMERVILLE, S. 2009. Host–pathogen warfare at the plant cell wall. *Current Opinion in Plant Biology,* 12**,** 406-413.

HGCA 2014. Oilseed rape guide. second ed. Warwickshire.

HOLESKI, L. M., JANDER, G. & AGRAWAL, A. A. 2012. Transgenerational defense induction and epigenetic inheritance in plants. *Trends in Ecology & Evolution,* 27**,** 618-626.

HU, Q., HUA, W., YIN, Y., ZHANG, X., LIU, L., SHI, J., ZHAO, Y., QIN, L., CHEN, C. & WANG, H. 2017. Rapeseed research and production in China. *The Crop Journal,* 5**,** 127-135.

HUND, A., TRACHSEL, S. & STAMP, P. 2009. Growth of axile and lateral roots of maize: I development of a phenotying platform. *Plant and Soil,* 325**,** 335-349.

JAAFFAR, A. K. M., PAULITZ, T. C., SCHROEDER, K. L., THOMASHOW, L. S. & WELLER, D. M. 2016. Molecular characterization, morphological characteristics, virulence, and geographic distribution of *Rhizoctonia* spp. in Washington State. *Phytopathology,* 106**,** 459-473.

JAOUANNET, M., RODRIGUEZ, P. A., THORPE, P., LENOIR, C. J. G., MACLEOD, R., ESCUDERO-MARTINEZ, C. & BOS, J. I. B. 2014. Plant immunity in plant–aphid interactions. *Frontiers in Plant Science,* 5.

JESTIN, C., BARDOL, N., LODE, M., DUFFE, P., DOMIN, C., VALLEE, P., MANGIN, B., MANZANARES-DAULEUX, M. J. & DELOURME, R. 2015. Connected populations for detecting quantitative resistance factors to phoma stem canker in oilseed rape (*Brassica napus* L.). *Molecular Breeding,* 35**,** 16.

KARLSSON, M., ATANASOVA, L., JENSEN, D. F. & ZEILINGER, S. 2017. Necrotrophic mycoparasites and their genomes. *Microbiology Spectrum,* 5**,** 21.

KATARIA, H. R. & VERMA, P. R. 1992. *Rhizoctonia solani* damping-off and root rot in oilseed rape and canola. *Crop Protection,* 11**,** 8-13.

KATHIRIA, P., SIDLER, C., GOLUBOV, A., KALISCHUK, M., KAWCHUK, L. M. & KOVALCHUK, I. 2010. Tobacco mosaic virus infection results in an increase in recombination frequency and resistance to viral, bacterial, and fungal pathogens in the progeny of infected tobacco plants. *Plant Physiology,* 153**,** 1859-1870.

KHANGURA, R. K., BARBETTI, M. J. & SWEETINGHAM, M. W. 1999. Characterization and pathogenicity of *Rhizoctonia* species on canola. *Plant Disease,* 83**,** 714-721.

KHARBANDA, P. D. & TEWARI, J. P. 1996. Integrated management of canola diseases using cultural methods. *Canadian Journal of Plant Pathology,* 18**,** 168-175.

KIM, K. C., LAI, Z. B., FAN, B. F. & CHEN, Z. X. 2008. *Arabidopsis* WRKY38 and WRKY62 transcription factors interact with histone Deacetylase 19 in basal defense. *Plant Cell,* 20**,** 2357-2371.

KIRKEGAARD, J. A., SARWAR, M., WONG, P. T. W., MEAD, A., HOWE, G. & NEWELL, M. 2000. Field studies on the biofumigation of take-all by *Brassica* break crops. *Australian Journal of Agricultural Research,* 51**,** 445-456.

KIRKEGAARD, J. A., WONG, P. T. W. & DESMARCHELIER, J. M. 1996. In vitro suppression of fungal root pathogens of cereals by *Brassica* tissues. *Plant Pathology,* 45**,** 593-603.

KLEIN-GEBBINCK, H. W. & WOODS, D. L. 2002. Yield Loss Assessment in Canola: Effects of brown girdling root rot and Maggot damage on single plant yield. *Plant Disease,* 86**,** 1005-1010.

KNECHT, K., SEYFFARTH, M., DESEL, C., THURAU, T., SHERAMETI, I., LOU, B. G., OELMULLER, R. & CAI, D. G. 2010. Expression of BvGLP-1 encoding a germin-Like protein from sugar beet in *Arabidopsis thaliana* leads to resistance against phytopathogenic fungi. *Molecular Plant-Microbe Interactions,* 23**,** 446-457.

KÖRBER, N., BUS, A., LI, J., HIGGINS, J., BANCROFT, I., HIGGINS, E. E., PAPWORTH PARKIN, I. A., SALAZAR-COLQUI, B., SNOWDON, R. J. & STICH, B. 2015. Seedling development traits in *Brassica napus* examined by gene expression analysis and association mapping. *BMC Plant Biology,* 15**,** 136.

KÖRBER, N., WITTKOP, B., BUS, A., FRIEDT, W., SNOWDON, R. J. & STICH, B. 2012. Seedling development in a *Brassica napus* diversity set and its relationship to agronomic performance. *Theoretical and Applied Genetics,* 125**,** 1275-1287.

KREMEN, C. & MILES, A. 2012. Ecosystem Services in Biologically Diversified versus Conventional Farming Systems: Benefits, Externalities, and Trade-Offs. *Ecology and Society,* 17**,** 25.

LAMICHHANE, J. R., DURR, C., SCHWANCK, A. A., ROBIN, M. H., SARTHOU, J. P., CELLIER, V., MESSEAN, A. & AUBERTOT, J. N. 2017. Integrated management of damping-off diseases. A review. *Agronomy for Sustainable Development,* 37**,** 25.

LAMPRECHT, S. C., TEWOLDEMEDHIN, Y. T., CALITZ, F. J. & MAZZOLA, M. 2011. Evaluation of strategies for the control of canola and lupin seedling diseases caused by *Rhizoctonia* anastomosis groups. *European Journal of Plant Pathology,* 130**,** 427-439.

LAZEBNIK, J., FRAGO, E., DICKE, M. & VAN LOON, J. J. A. 2014. Phytohormone mediation of interactions between herbivores and plant pathogens. *Journal of Chemical Ecology,* 40**,** 730-741.

LAZNIK, Z., ZNIDARCIC, D. & TRDAN, S. 2011. Control of *Trialeurodes vaporariorum* (Westwood) adults on glasshouse-grown cucumbers in four different growth substrates: an efficacy comparison of foliar application of *Steinernema feltiae* (Filipjev) and spraying with thiamethoxamn. *Turkish Journal of Agriculture and Forestry,* 35**,** 631-640.

LE COINTE, R., SIMON, T. E., DELARUE, P., HERVE, M., LECLERC, M. & POGGI, S. 2016. Reducing the use of pesticides with site-specific application: The chemical control of *Rhizoctonia solani* as a case of study for the management of soil- borne diseases. *Plos One,* 11**,** 18.

LE GUIGO, P., MAINGENEAU, A. & LE CORFF, J. 2011. Performance of an aphid *Myzus persicae* and its parasitoid *Diaeretiella rapa*e on wild and cultivated *Brassicaceae*. *Journal of Plant Interactions,* 7**,** 326-332.

LEATHER, S. R. & DIXON, A. F. G. 1984. Aphid growth and reproductive rates. *Entomologia Experimentalis et Applicata,* 35**,** 137-140.

LI, J., BRADER, G., KARIOLA, T. & PALVA, E. T. 2006. WRKY70 modulates the selection of signaling pathways in plant defense. *Plant Journal,* 46**,** 477-491.

LI, L. X., LUO, Y. J., CHEN, B. Y., XU, K., ZHANG, F. G., LI, H., HUANG, Q., XIAO, X., ZHANG, T. Y., HU, J. H., LI, F. & WU, X. M. 2016. A

genome-wide association study reveals new loci for resistance to clubroot disease in *Brassica napus*. *Frontiers in Plant Science,* 7**,** 12.

LIU, L. J., SONBOL, F. M., HUOT, B., GU, Y. N., WITHERS, J., MWIMBA, M., YAO, J., HE, S. Y. & DONG, X. N. 2016. Salicylic acid receptors activate jasmonic acid signalling through a non-canonical pathway to promote effector-triggered immunity. *Nature Communications,* 7**,** 10.

LORENZO, O., CHICO, J. M., SÁNCHEZ-SERRANO, J. J. & SOLANO, R. 2004. JASMONATE-INSENSITIVE1 Encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in *Arabidopsis*. *The Plant Cell,* 16**,** 1938-1950.

LUNA, E., BRUCE, T. J. A., ROBERTS, M. R., FLORS, V. & TON, J. 2012. Next-generation systemic acquired resistance. *Plant Physiology,* 158**,** 844-853.

MASON, A. S. & SNOWDON, R. J. 2016. Oilseed rape: learning about ancient and recent polyploid evolution from a recent crop species. *Plant Biology,* 18**,** 883-892.

MAZUMDER, M., DAS, S., SAHA, U., CHATTERJEE, M., BANNERJEE, K. & BASU, D. 2013. Salicylic acid-mediated establishment of the compatibility between *Alternaria brassicicola* and *Brassica juncea* is mitigated by abscisic acid in *Sinapis alba*. *Plant Physiology and Biochemistry,* 70**,** 43-51.

MELZER, M. S., YU, H., LABUN, T., DICKSON, A. & BOLAND, G. J. 2016. Characterization and pathogenicity of *Rhizoctonia* spp. from field crops in Canada. *Canadian Journal of Plant Pathology,* 38**,** 367-374.

MORAN, P. J. & THOMPSON, G. A. 2001. Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. *Plant Physiology,* 125**,** 1074-1085.

NOVAKOVA, M., SASEK, V., DOBREV, P. I., VALENTOVA, O. & BURKETOVA, L. 2014. Plant hormones in defense response of *Brassica napus* to *Sclerotinia sclerotiorum* - Reassessing the role of salicylic acid in the interaction with a necrotroph. *Plant Physiology and Biochemistry,* 80**,** 308-317.

O'BRIEN, P. A. 2017. Biological control of plant diseases. *Australasian Plant Pathology,* 46**,** 293-304.

OCCHIPINTI, A. 2013. Plant coevolution: evidences and new challenges. *Journal of Plant Interactions,* 8**,** 188-196.

OGOSHI, A. 1987. Ecology and pathogenicity of anastomosis groups of *Rhizoctonia solani* Kühn *Annual Review of Phytopathology,* 25**,** 125-143.

OGOSHI, A., COOK, R. J. & BASSETT, E. N. 1990. *Rhizoctonia* species and anastomosis groups causing root-rot of wheat and barley in Pacific-Northwest *Phytopathology,* 80**,** 784-788.

OKUBARA, P. A., DICKMAN, M. B. & BLECHL, A. E. 2014. Molecular and genetic aspects of controlling the soilborne necrotrophic pathogens *Rhizoctonia* and *Pythium*. *Plant Science,* 228**,** 61-70.

PANNECOUCQUE, J. & HOFTE, M. 2009. Interactions between cauliflower and *Rhizoctonia* anastomosis groups with different levels of aggressiveness. *Bmc Plant Biology,* 9**,** 12.

PAPAVIZAS, G. C., ADAMS, P. B., LUMSDEN, R. D., LEWIS, J. A., DOW, R. L., AYERS, W. A. & KANTZES, J. G. 1975. Ecology and epidemiology of *Rhizoctonia solani* in field soil. *Phytopathology,* 65**,** 871-877.

PARAMETER, J. R. J. 1970. *Rhizoctonia solani: Biology and pathology,* University of California Press.

PERL-TREVES, R., FOLEY, R. C., CHEN, W. Q. & SINGH, K. B. 2004. Early induction of the *Arabidopsis* GSTF8 promoter by specific strains of the fungal pathogen *Rhizoctonia solani*. *Molecular Plant-Microbe Interactions,* 17**,** 70-80.

PIETERSE, C. M. J. & DICKE, M. 2007. Plant interactions with microbes and insects: from molecular mechanisms to ecology. *Trends in Plant Science,* 12**,** 564-569.

PINK, D., BAILEY, L., MCCLEMENT, S., HAND, P., MATHAS, E., BUCHANAN-WOLLASTON, V., ASTLEY, D., KING, G. & TEAKLE, G. 2008. Double haploids, markers and QTL analysis in vegetable brassicas. *Euphytica,* 164**,** 509-514.

PUINEAN, A. M., FOSTER, S. P., OLIPHANT, L., DENHOLM, I., FIELD, L. M., MILLAR, N. S., WILLIAMSON, M. S. & BASS, C. 2010. Amplification of a cytochrome P450 gene is associated with resistance

to neonicotinoid insecticides in the aphid *Myzus persicae*. *Plos Genetics*, 6.

RADFORD, P. J. 1967. Growth Analysis Formulae - Their Use and Abuse1. *Crop Science,* 7**,** 171-175.

RAY, R. V., JENKINSON, P. & EDWARDS, S. G. 2004. Effects of fungicides on eyespot, caused predominantly by *Oculimacula acuformis*, and yield of early-drilled winter wheat. *Crop Protection,* 23**,** 1199-1207.

RENNY-BYFIELD, S. & WENDEL, J. F. 2014. Doubling down on genomes: polypoidy and crop plants. *American Journal of Botany,* 101**,** 1711-1725.

SARWAR, M., KIRKEGAARD, J. A., WONG, P. T. W. & DESMARCHELIER, J. M. 1998. Biofumigation potential of brassicas - III. In vitro toxicity of isothiocyanates to soil-borne fungal pathogens. *Plant and Soil,* 201**,** 103-112.

SARWAR, M. & SATTAR, M. 2013. Varietals variability of winter rapes (*Brassica napus* L.) for their susceptibility to green aphid, *Myzus persicae* (Sulzer) (Homoptera: Aphididae). *Pakistan Journal of Zoology,* 45**,** 883-888.

SAVARY, S. 2014. The roots of crop health: cropping practices and disease management. *Food Security,* 6**,** 819-831.

SCHNEIDER, C. A., RASBAND, W. S. & ELICEIRI, K. W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat Meth,* 9**,** 671-675.

SCHOONHOVEN, L. M., VAN LOON, J. J. A. & DICKE, M. 2005. *Insect-plant biology*, Oxford University Press, USA.

SCHULTZ, J. C., APPEL, H. M., FERRIERI, A. P. & ARNOLD, T. M. 2013. Flexible resource allocation during plant defense responses. *Frontiers in Plant Science,* 4**,** 11.

SHARMA-POUDYAL, D., PAULITZ, T. C. & DU TOIT, L. J. 2015. Stunted patches in onion bulb crops in Oregon and Washington: Etiology and yield loss. *Plant Disease,* 99**,** 648-658.

SHARON, M., FREEMAN, S. & SNEH, B. 2011. Assessment of resistance pathways induced in *Arabidopsis thaliana* by hypovirulent *Rhizoctonia* spp. isolates. *Phytopathology,* 101**,** 828-38.

SHIGENAGA, A. M. & ARGUESO, C. T. 2016. No hormone to rule them all: Interactions of plant hormones during the responses of plants to pathogens. *Seminars in Cell & Developmental Biology,* 56**,** 174-189.

SIMON-DELSO, N., AMARAL-ROGERS, V., BELZUNCES, L. P., BONMATIN, J. M., CHAGNON, M., DOWNS, C., FURLAN, L., GIBBONS, D. W., GIORIO, C., GIROLAMI, V., GOULSON, D., KREUTZWEISER, D. P., KRUPKE, C. H., LIESS, M., LONG, E., MCFIELD, M., MINEAU, P., MITCHELL, E. A. D., MORRISSEY, C. A., NOOME, D. A., PISA, L., SETTELE, J., STARK, J. D., TAPPARO, A., VAN DYCK, H., VAN PRAAGH, J., VAN DER SLUIJS, J. P., WHITEHORN, P. R. & WIEMERS, M. 2015. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. *Environmental Science and Pollution Research,* 22**,** 5-34.

SMITH, B. J. & KIRKEGAARD, J. A. 2002. In vitro inhibition of soil microorganisms by 2-phenylethyl isothiocyanate. *Plant Pathology,* 51**,** 585-593.

SOLTIS, D. E., ALBERT, V. A., LEEBENS-MACK, J., BELL, C. D., PATERSON, A. H., ZHENG, C. F., SANKOFF, D., DEPAMPHILIS, C. W., WALL, P. K. & SOLTIS, P. S. 2009. Polyploidy and angiosperm diversification. *American Journal of Botany,* 96**,** 336-348.

STAVI, I., BEL, G. & ZAADY, E. 2016. Soil functions and ecosystem services in conventional, conservation, and integrated agricultural systems. A review. *Agronomy for Sustainable Development,* 36**,** 12.

STODART, B. J., HARVEY, P. R., NEATE, S. M., MELANSON, D. L. & SCOTT, E. S. 2007. Genetic variation and pathogenicity of anastomosis group 2 isolates of *Rhizoctonia solani* in Australia. *Mycological Research,* 111**,** 891-900.

STURROCK, C. J., WOODHALL, J., BROWN, M., WALKER, C., MOONEY, S. J. & RAY, R. V. 2015. Effects of damping-off caused by *Rhizoctonia solani* anastomosis group 2-1 on roots of wheat and oil seed rape quantified using X-ray Computed Tomography and real-time PCR. *Frontiers in Plant Science,* 6.

TEO, B. K., YITBAREK, S. M., VERMA, P. R. & MORRALL, R. A. A. 1988. Influence of soil moisture, seeding date, and *Rhizoctonia solani* isolates (AG 2-1 and AG 4) on disease incidence and yield in canola. *Canadian Journal of Plant Pathology,* 10**,** 151-158.

TEWOLDEMEDHIN, Y. T., LAMPRECHT, S. C., MCLEOD, A. & MAZZOLA, M. 2006. Characterization of *Rhizoctonia* spp. Recovered from Crop Plants Used in Rotational Cropping Systems in the Western Cape Province of South Africa. *Plant Disease,* 90**,** 1399-1406.

THOMAS, C. L., ALCOCK, T. D., GRAHAM, N. S., HAYDEN, R., MATTERSON, S., WILSON, L., YOUNG, S. D., DUPUY, L. X., WHITE, P. J., HAMMOND, J. P., DANKU, J. M. C., SALT, D. E., SWEENEY, A., BANCROFT, I. & BROADLEY, M. R. 2016a. Root morphology and seed and leaf ionomic traits in a *Brassica napus* L. diversity panel show wide phenotypic variation and are characteristic of crop habit. *BMC Plant Biology,* 16**,** 214.

THOMAS, C. L., GRAHAM, N. S., HAYDEN, R., MEACHAM, M. C., NEUGEBAUER, K., NIGHTINGALE, M., DUPUY, L. X., HAMMOND, J. P., WHITE, P. J. & BROADLEY, M. R. 2016b. High-throughput phenotyping (HTP) identifies seedling root traits linked to variation in seed yield and nutrient capture in field-grown oilseed rape (*Brassica napus* L.). *Annals of Botany*.

THOMPSON, G. A. & GOGGIN, F. L. 2006. Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects. *Journal of Experimental Botany,* 57**,** 755-766.

TILMAN, D., BALZER, C., HILL, J. & BEFORT, B. L. 2011. Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences,* 108**,** 20260-20264.

TOMASO-PETERSON, M. & TREVATHAN, L. E. 2007. Characterization of *Rhizoctonia*-like fungi isolated from agronomic crops and turfgrasses in Mississippi. *Plant Disease,* 91**,** 260-265.

TRDAN, S., ZNIDARCIC, D. & VIDRIH, M. 2007. Control of *Frankliniella occidentalis* on glasshouse-grown cucumbers: an efficacy comparison of foliar application of *Steinernema feltiae* and spraying with abamectin. *Russian Journal of Nematology,* 15**,** 25-34.

USDA, N. A. S. S. 2017. *Crop production historical track records* [Online]. United States Department of Agriculture. Available: http://usda.mannlib.cornell.edu/usda/current/htrcp/htrcp-04-13-2017.pdf [Accessed].

VAN DAM, N. M., TYTGAT, T. O. G. & KIRKEGAARD, J. A. 2009. Root and shoot glucosinolates: a comparison of their diversity, function and interactions in natural and managed ecosystems. *Phytochemistry Reviews,* 8**,** 171-186.

VAN LOON, L. C., REP, M. & PIETERSE, C. M. J. 2006. Significance of inducible defense-related proteins in infected plants. *Annual Review of Phytopathology.*

VERMA, P. R. 1996. Biology and control of *Rhizoctonia solani* on rapeseed: A review. *Phytoprotection,* 77**,** 99-111.

VILGALYS, R. & CUBETA, M. A. 1994. Molecular Systematics and Population Biology of *Rhizoctonia*. *Annual Review of Phytopathology,* 32**,** 135-155.

VINALE, F., SIVASITHAMPARAM, K., GHISALBERTI, E. L., MARRA, R., WOO, S. L. & LORITO, M. 2008. *Trichoderma*–plant–pathogen interactions. *Soil Biology and Biochemistry,* 40**,** 1-10.

VOS, I. A., MORITZ, L., PIETERSE, C. M. J. & VAN WEES, S. C. M. 2015. Impact of hormonal crosstalk on plant resistance and fitness under multi-attacker conditions. *Frontiers in Plant Science,* 6.

VOS, I. A., PIETERSE, C. M. J. & VAN WEES, S. C. M. 2013. Costs and benefits of hormone-regulated plant defences. *Plant Pathology,* 62**,** 43-55.

WANG, X. L., JIANG, N., LIU, J. L., LIU, W. D. & WANG, G. L. 2014. The role of effectors and host immunity in plant-necrotrophic fungal interactions. *Virulence,* 5**,** 722-732.

WEI, W. & DARMENCY, H. 2008. Gene flow hampered by low seed size of hybrids between oilseed rape and five wild relatives. *Seed Science Research,* 18**,** 115-123.

WHITE, T. J., BRUNS, T., LEE, S., TAYLOR, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. .

Innis, M.A., et al. (Ed.), PCR Protocols: A Guide to Methods and Applications. San Diego, California,: Academic Press.

WOODHALL, J. W., WEBB, K. M., GILTRAP, P. M., ADAMS, I. P., PETERS, J. C., BUDGE, G. E. & BOONHAM, N. 2012. A new large scale soil DNA extraction procedure and real-time PCR assay for the detection of Sclerotium cepivorum in soil. *European Journal of Plant Pathology,* 134**,** 467-473.

WU, Y., ZHANG, D., CHU, JEE Y., BOYLE, P., WANG, Y., BRINDLE, IAN D., DE LUCA, V. & DESPRÉS, C. 2012. The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Reports,* 1**,** 639-647.

WYATT, I. J. & WHITE, P. F. 1977. Simple estimation of intrinsic increase rates for aphids and tetranychid mites. *Journal of Applied Ecology,* 14**,** 757-766.

XUE, A. G., COBER, E., MORRISON, M. J., VOLDENG, H. D. & MA, B. L. 2007. Effect of seed treatments on emergence, yield, and root rot severity of soybean under *Rhizoctonia solani* inoculated field conditions in Ontario. *Canadian Journal of Plant Science,* 87**,** 167-174.

YANG, J. & VERMA, P. R. 1992. Screening genotypes for resistance to pre-emergence damping-off and postemergence seedling root rot of oilseed rape and canola caused by *Rhizoctonia solani* AG-2-1. *Crop Protection,* 11**,** 443-448.

YITBAREK, S. M., VERMA, P. R., GUGEL, R. K. & MORRALL, R. A. A. 1988. Effect of soil-temperature and inoculum density on pre-emergence damping-off of canola caused by *Rhizoctonia solani Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie,* 10**,** 93-98.

YITBAREK, S. M., VERMA, P. R. & MORRALL, R. A. A. 1987. Anastomosis groups, pathogenicity, and specificity of *Rhizoctonia solani* isolates from seedling and adult rapeseed/canola plants and soils in Saskatchewan. *Canadian Journal of Plant Pathology,* 9**,** 6-13.

ZEUN, R., SCALLIET, G. & OOSTENDORP, M. 2013. Biological activity of sedaxane a novel broad-spectrum fungicide for seed treatment. *Pest Management Science,* 69**,** 527-534.

ZHANG, X. Y., HUO, H. L., WANG, W., ZHOU, H. Y., DONG, B. Z. & ZHAO, G. Q. 2016. First report of damping-off and seedling blight on oat caused by *Rhizoctonia solani* AG 2-1 in China. *Plant Disease,* 100**,** 653-654.

ZHENG, S. J., VAN DIJK, J. P., BRUINSMA, M. & DICKE, M. 2007. Sensitivity and speed of induced defense of cabbage (*Brassica oleracea* L.): Dynamics of BoLOX expression patterns during insect and pathogen attack. *Molecular Plant-Microbe Interactions,* 20**,** 1332-1345.

Professional Internships for PhD Students Reflection Form

Name of Organization

Benaki Phytopathological Institute (BPI), 8 Stefanou Delta Street, Kifissia, Athens, Greece

Details of Placement

Please describe your main activities during the placement (150-200 words)

The main aim of the placement was to work in the field of chemical ecology of herbivorous insects and learn how to perform electroantennography (EAG). My PIP supervisor and I designed the project and the main research questions were 1.How *Tuta absoluta* feeding on tomato plant changes the emission of plant volatile organic compounds (VOCs) and 2. How these affect the oviposition choice of conspecific females.

The first period of the PIP and in order to get familiar with the study system, I helped the technical team with the rearing of insects: *T. absoluta*, *Trichogramma* species, *Lobesia botrana* and *Ephestia kuehniella.* I was also responsible for the sowing and growth of the experimental tomato plants. During this period, the research team demonstrated to me the lab equipment related to my work (air entrainments, gas chromatography –GC) and taught me how to perform the EAG technique.

Additionally, I was able to practice with the EAG, develop my skills on handling insect antennae (different species) and test their activity on collected VOC blends and synthetic compounds. Finally, I designed and performed the experimental work, which included choice-experiments (bioassays), the collection and analysis of VOCs (air entrainment collection and GC analysis) and performance of EAD-GC.

Placement Achievements

Please detail all outcomes from the placement, including any publications, presentations given and reports written etc. (150-200 words)

The scientific outcome of this study was the demonstration that female moths understand through their antennae, conspecifics' herbivory/presence and choose to oviposit on un-infested plants. The findings support the theory 'Mother knows the best', stating that females choose to lay eggs on plants that will be more suitable hosts for their progenies. Additionally, in natural ecosystems, this behaviour of ovipositing females seems to protect the progenies from natural enemies that are recruited from plants under herbivory. Future work with identification and synthetic production of key compounds will enable us to develop more suitable control methods for this pest.

After the completion of the PIP, the group identified with Mass Spectrometry (MS), which compounds were altered in the emitted blend. The combined results from bioassays, EAD-GC and MS were used to produce the following manuscript submitted to the Journal of Chemical Ecology: Anastasaki E., Drizou F., Milonas PG., Electrophysiological and oviposition responses of *Tuta absoluta* females on herbivore induced volatiles in tomato plants.

Skill development

Has this Placement helped you developed any new skills or enhanced your previous skill set? (100-150 words)

The PIP mainly helped me to improve my knowledge on techniques used in chemical ecology. Having not used gas-chromatography for three years, this PIP gave me the opportunity to gain a better technical knowledge on the functions of the GC machine and I understood how these could affect the output of the EAG. Additionally, the most important aspect was that learned

how to perform GC-EAD with two different methods (micropipettes with saline solution and metal electrodes with electrically conductive gel). Furthermore, although I was familiar with the headspace volatile collection, samples concertation and their analysis, through the placement I learned to perform them with different protocols and alternative methods. Another important outcome is that I developed my professional network in Greece (my home country) and that I also experienced the working environment of an institutional organisation.

Future Work

Has this Placement influenced your future career aspirations? If so, in what way? (150-200 words)

I believe that the placement overall had a very positive impact by enabling me not only to work in an interesting research field but also to consider the direct application of knowledge for the development of pest control methods.

Since my MSc I was convinced that my scientific interest is on plant defences and plant-insect interactions. With my PhD I added another aspect (that of plant-pathogen interaction) but I felt that I would like to discover more in the field of chemical ecology.
Additionally as my work so far was based mainly in the understanding of the fundamental aspects, I wanted to search the potential application of this knowledge for the development of more sustainable integrated control methods. BPI, through its role as the primary governmental institute for plant health and plant protection was an ideal place to explore applied science. The last impact of the PIP is that through the working experience of the institute, I can also see myself being outside academia.

Therefore, the aim of my future career, would be to combine the different fields of insect chemical ecology with plant pathology and plant defences for the understanding of multi-interactions between host-plants and different enemies.