Understanding the genetic basis of Ramularia disease resistance in barley

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Declaration

I hereby declare that this thesis has been composed by me and that the work is my own, except as acknowledged by means of references. This thesis has not been submitted for any other degree or professional qualification except as specified.

Laura RÖHRIG

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List of abbreviations

$^1O_2$	Singlet oxygen
6-BAP	6-benzylaminopurine, cytokinin derivate
ABA	Abscisic acid
ABC	ATP-binding cassette transporters
ACC	1-Aminocyclopropane-1-carboxylate
ACO	ACC-oxidase
AFLPs	Amplified Fragment Length Polymorphism
ANOVA	Analysis of variance
AP2	Apetala2
APX	Ascorbate peroxidase
ARCK	Aromatic core, N6 position of cytokinins
AUDPC	Area Under the Disease Progress Curve
Avr	Pathogen avirulence effectors
BC	Backcross
bHLH	basic helix-loop-helix-protein
BIC	Bayesian Information Criterion
BLAST	Basic Alignment Search Tool
BOPA	Barley oligonucleotide pool assay
BR	Brassinosteroids
bZIP	basic leucine zipper domain
Ca^{2+}	Calcium
CaM	Calmodulin
CAT	Catalase
CBL	Calcineurin-B-like protein
CDPK	Calcium-dependent protein kinase
CK	Cytokinins
CKX	Cytokinin oxidases/dehydrogenases
CML	CaM-like proteins

CN	Carbon and nitrogen analysis
CTR	Constitutive Triple Response
CWINV	Cell wall invertase
CYP	Cytochrome P450 monooxygenases
cZ	Cis-zeatin
DAMPs	Danger-associated molecular patterns
DECO	Barley cultivars Decanter X Cocktail population
DEGs	Differentially regulated genes
DH	double haploid
DK05	Danish *Ramularia collo-cygni* isolate
DMI	sterol demethylation inhibitor
dpi	Days post inoculation
dpt	Days post treatment
DRE	Dehydration-Responsive Element
DREB	dehydration-responsive element-binding proteins
DRR	Dry root rot
ECGC	Environmental Covariate Search Affecting Genetic Correlations
EF-1α	Elongation factor 1 α
EIN	Ethylene Insensitive
EMMA	Efficient mixed model association
EMR	*Enhanced Magnaporthe Resistance*
ERF	AP2/ethylene response factor
ERS	Ethylene Response Sensor
ET	Ethylene
ETI	Effector triggered immunity
ETR	Ethylene-Responsive
F leave	Flag leave
F-1	Flag leave -1
FDR	False discovery rate

G x E	gene by environment interaction
GA	Gibberellin
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GPC	Protein grain content
GS	Growth Stage
GSH	Glutathione
GWAS	Genome-wide association scan
H_2O_2	Hydrogen peroxide
HK	Histidine kinase
HR	Hypersensitive response
iP	Isopentenyladenine
IPM	Integrated pest management
IPT	Isopentenyl transferase
ISR	Induced systemic resistance
ISRK	Isoprene side chain, N6 position of cytokinins
ITS	Internal transcribed spacer
JA	Jasmonates
LD	Linkage disequilibrium
Leaf N	Leaf nitrogen
LMEM	Linear mixed-effect model
LMM	Lesion mimic mutants
LOG	LONELY GUY family of enzymes
LRR	Leucine-rich-repeat
LysM	Lysine motif
MAMPs	Microbial associated molecular patterns
MAPK	Mitogen-activated protein kinase
MAS	Marker assisted selection
MeSA	Methyl salicylate
MLO/*mlo*	Mildew resistance locus O wildtype/Mildew resistance locus O mutant

Mock	non-inoculated
N	Nitrogen
NBS	Nucleotide binding site
NFNB	Net form net blotch
NIL	Near isogenic lines
NLR	Nucleotide-binding domains and leucine-rich repeat receptors
NPI1	Necrosis inducing protein 1
NRP	Non-ribosomal peptide
NRPS	NRP synthetases
$O_2^{.-}$	Superoxide
PAMPs	Pathogen associated molecular patterns
PCA	Principal component analysis
PCD	Programmed cell death
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PLS	Physiological leaf spots
POD	Peroxidase
PR	Pathogenesis related gene
PRR	Pattern recognition receptors
PTI	Pattern triggered immunity
QDR	Quantitative disease resistance
QoI	quinone outside inhibitor
Q-Q	Quantile-Quantile
QTL	Quantitative trait loci
r	Pearson correlation coefficient
R proteins	Resistance proteins
RAV	Related to Abscisic Acid Insensitive 3/Viviparous 1
REML	Restricted maximum likelihood algorithm
RIL	Recombinant inbred line

RLK	Receptor like kinase
RLP	Receptor-like protein
RLS	Ramularia leaf spot
ROR	Required for *mlo* Resistance
ROS	Reactive oxygen species
SA	Salicylic acid
SAG	Senescence-associated gene
SAM	S-Adenosylmethionine
SAR	Systemic acquired resistance
SC19 B5	*Ramularia collo-cygni* isolate Scotland 2019
SDHI	succinate dehydrogenase inhibitor
SDW	Sterile distilled water
SM	Secondary metabolite
SNP	Single Nucleotide Polymorphism DNA marker
SOD	Superoxide dismutase
SPAD	Soil Plant Analysis Development
SSR	Simple Sequence Repeat
STB	Septoria tritici blotch
SW	Specific weight
TF	Transcription factor
TMV	Tobacco mosaic virus
tZ	Trans-zeatin
Ug99	Puccinia graminis f. sp. tritici, strain Uganda 1999
UK	United Kingdom
WAK	Wall associated kinase

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“Ever tried, ever failed. No matter. Try again. Fail again. Fail better.”

Samuel Beckett (Worst Ho)

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Abstract

Since the 1980's, barley (*Hordeum vulgare*) production across many regions in the world, has been facing challenges posed by the fungus *Ramularia collo-cygni,* which causes Ramularia leaf spot (RLS). The fungus causes substantial yield losses ranging from 20% to 70% and reduces grain quality. The appearance of fungicide resistance in *R. collo-cygni* populations to a number of fungicide actives, together with the lack of known genetic resistance in widely grown barley varieties, indicates limited options to control this disease in the medium to long-term. This highlights the importance of investigating the potential for genetic control of RLS and gain an improved understanding of the host/pathogen interaction.

In this study, one major quantitative trait loci (QTL) involved in RLS resistance was identified, by using a genome-wide association scan (GWAS) on 238 spring barley varieties. Based on the GWAS results, a subset of the identified spring barley cultivars was tested in field trials in both Scotland and Germany between 2021 to 2022. The results on disease development during both seasons, support the conclusion that resistant QTL carrying barley lines exhibit an increased resistance to RLS. This was supported by results from controlled inoculation experiments in a subset of spring barley seedling experiments. Results at both the adult and seedling stage of barley found that *R. collo-cygni*-DNA did not correlate with symptom expression, suggesting that endophytic colonisation by the fungus may not always lead to the appearance of symptoms.

The observations from previous studies and the field trial in 2021 of this study, have linked the appearance of RLS symptoms late in the season to monocarpic senescence. Therefore, this study examined the role of senescence during RLS development and fungal colonisation under controlled *R.collo-cygni*-inoculation studies. This study found that host senescence is prematurely induced by *R. collo-cygni* in various spring barley genotypes differing in suceptibility to the fungus. Moreover, results have indicated that senescence is induced both in partially resistant and susceptible barley genotypes, suggesting that other factors contribute to the fungus transitioning to the necrotrophic lifestyle. Indeed, delayed leaf senescence experiments in this study showed that RLS symptoms and fungal DNA were increased, whereas during early senescence RLS symptoms were reduced and fungal colonisation was increased. Contrasting the hypothesis that extending the biotrophic phase of *R. collo-cygni* will lead to increased resistance to RLS and highlighting that other factors contribute to disease development.

Increased resistance to *R. collo-cygni* in barley plants in controlled inoculation experiments was also found post foliar treatment with the ethylene precursor 1-Aminocyclopropane-1-carboxylic acid (ACC). Furthermore, gene expression analysis of barley ethylene response factors in this study, indicated that one ethylene response factor was upregulated in response to foliar ACC treatment. Altogether, these results suggest a putative ET-mediated disease resistance to RLS and represent potential targets for barley breeding programmes.

Lay summary

Ramularia leaf spot (RLS) is a fungal disease of barley, which represents after wheat, maize and rice, the fourth most important crop in the world. *Ramularia collo-cygni*, the causative agent of RLS, can lead to substantial yield losses and a reduction in barley quality worldwide. In the United Kingdom these have been estimated to range between 0.5 to 1 tonne per hectare based on average spring barley yields of 6 tonnes per hectare. Currently, the control of RLS mainly relies on the use of fungicides, however; the fungus has evolved resistance to the most common classes of fungicides over the past twenty years and therefore, poses a threat to barley production worldwide. At present, there are no barley varieties resistant to RLS for farmers to grow, which highlights the importance to investigate the genetic control for RLS.

The symptoms of RLS typically occur late in the barley growing season as the disease remains symptomless for the majority of it. Usually, symptoms become apparent after the crop has flowered and makes it difficult to predict RLS and thus, control the disease in a timely manner. In some growing seasons, the fungus remains symptomless, despite having grown within the barley leaves. These observations have led to many studies over the past 20 years, investigating the factors that trigger the fungus to produce symptoms. Environmental stresses, the yellowing of the crop late in the season after flowering and the barley genetics have been attributed to play major roles in the transition. However, the interaction between the host and fungus under these factors have been largely unexplored. The aim of this study was to investigate the potential to develop barley lines with increased resistance to the disease and to examine the link between the yellowing of the crop and RLS disease development.

This study used information on RLS disease levels and genetic make-up of varieties from a previous study to explore the association between disease levels and host genetics. Moreover, field trials performed in different countries with varying environments were conducted to confirm the findings at the adult plant stage over two barley seasons. The findings of this study confirmed the previous hypothesis that RLS is controlled by quantitative trait loci, which represent variations in the barley genome that contribute to quantitative differences in disease levels. The field trials in Scotland and Germany showed that barley varieties carrying the resistant locus obtained reduced RLS disease levels compared to the ones carrying the susceptible locus. However, this study has also shown that this effect varies highly with the environment, which supports previous hypothesis that disease symptoms are governed by interaction of the barley genes and the local environment.

Environmental factors such as drought and flooding, and the genetics of barley can lead to an early yellowing of the barley leaves before flowering is completed. Therefore, the interaction between the yellowing of barley leaves and RLS disease symptom development was investigated under controlled fungal-inoculation experiments by using different methods to extend the greenness of barley leaves and induce early yellowing in barley seedlings. The results contrasted with the hypothesis that delaying the yellowing of the leaves would extend the symptomless stage and thus, reduce RLS disease development. Results showed that extending the greenness led to enhanced symptom formation and increased fungal growth in the host plant, whereas early yellowing resulted in increased resistance to RLS. However, increased colonisation was observed in early yellowing leaves and the results also showed that *R. collo-cygni* induces premature leaf yellowing, indicating that other factors trigger the production of symptoms.

Chapter 1:

General Introduction

1.1 Current and past agricultural challenges

The Green Revolution of the mid-20th century (1960-2000) focused primarily on agricultural research into increasing crop productivity and plant protection, before focussing on yield losses caused by biological and non-biological causes (Evans, 1998). The hallmarks of the Green Revolution were genetically improved cultivars, enhanced soil fertility by chemical fertilisation, pest control through synthetic pesticides, and improved irrigation techniques. Since the 1960's, the world food production has doubled. Along with increased pesticide use, the average yield productivity in the major sources for human nutrition (maize, wheat and rice) has more than doubled (Oerke et al., 2004).

The conflict between efficient production and use of inputs remains still challenging at the beginning of the 21st century, as further complexity has been reached. The growing demand for food, which is projected to increase by 60% by 2050 (FAO, 2016), highlights the challenges of sustaining food security, diminishing environmental damage, and managing water and resource under shifting climate conditions (FAO, 2009). Future crop production is predicted to be affected the most by climate change due to increased extreme weather events, leading to droughts and flooding, which subsequently, may affect plant pathogens and related plant diseases (Juroszek et al., 2020; Pereira, 2016). In Europe, climate change projections indicate an increase of the mean annual air temperature ranging from 1.0 to 4.5°C before the end of the century (Miedaner and Juroszek, 2021). Disease prediction models forecast a latitudinal shift of the two most threating pathogens *Magnaporthe oryzae,* causing rice blast, and *Zymoseptoria tritici,* causing Septoria blotch in wheat, as temperatures rise (Chaloner et al., 2021). In the major crops such as wheat (*Triticum aestivum*), maize (*Zea mays*), and soybean (*Glycine max*), model predictions further estimate 6-16% yield decrease for each degree the world temperature increases by (Asseng et al., 2015; Kucharik and Serbin, 2008). Juroszek et al. (2020) recently reviewed the direct and indirect effects of climate change on pathogens. The changes related to pathogens have been concluded to affect 1) the geographical distribution; 2) seasonal phenology and 3) population dynamics of pathogens (Miedaner and Juroszek, 2021). Those direct or indirect effects of climate change can ultimately lead to altered disease prevalence, incidence and severity (Juroszek et al., 2020). Currently, crop protection against fungal diseases relies primarily on single-site antifungals and cultivars carrying major disease resistance R genes (Fones et al., 2020). However, the approaches of the current agroecosystems are prone to be volatile due to monocultures provoking the emergence of new fungal strains, including fungicide resistant populations and the ability to overcome host resistance (Fisher et al., 2012). Most wheat cultivars conferred resistance to *Puccinia graminis f. sp. tritici* causing stem rust via a single gene, *Secale cereale 31* (*Sr31*), between 1970 to the early 2000, however, in 1999 the resistance broke due to a newly emerging strain in Uganda, Ug99 (Singh et al., 2011). Moreover, fungicide efficacy was indicated to be temperature dependent and that temperature-mediated pathogen adaption to non-specific fungicides appears to be possible (Greiner et al., 2019; He et al., 2018; Matzrafi, 2019). He et al. (2018) found a correlation between fungicide tolerance of different temperature adapted *Alternaria alternata* strains and local temperatures. Additionally, fungicide resistance has emerged against major classes of single-site fungicides in several crop pathogens (Fones et al., 2020). The barley pathogen, *Ramularia collo-cygni,* causing Ramularia leaf spot (RLS), has quickly evolved resistance against the single-site class of quinone outside inhibitors (QoI) and has recently also shown a shift in sensitivity to two major fungicide classes, the succinate dehydrogenase inhibitors (SDHIs) and sterol demethylation inhibitors (DMIs; Erreguerena et al., 2022; Fountaine and Fraaije, 2009; Piotrowska et al., 2017). While chlorothalonil carries currently a market value of US$205 million per annum and has been used for wheat and barley fungicide programmes, it has been banned from the European market in May 2020 (Fones et al., 2020). Although it provided the only remaining, reliable control against RLS in Europe before 2020 (Dussart et al. 2020). Altogether, it highlights the challenges the 21st century faces and the importance of breeding for genetic control for RLS. Cultivars with durable multi-disease resistance are not only needed for wheat (Miedaner and Juroszek, 2021), but also barley, rice and maize which comprise the four most important crops in the world (FAOSTAT, 2023). This project investigates the potential for a quantitative genetic control against Ramularia leaf spot (RLS) that can help breeding programmes to incorporate Ramularia resistant barley to their cultivars.

1.2 Barley – a resilient cereal crop

1.2.1 The history of domestication

Barley (*Hordeum vulgare*) is one of the oldest cultivated cereal crops, which was domesticated around 10,000 years ago in the Fertile Crescent (Nevo, 1992). This estimation was confirmed by archaeological data which indicated the fixation of non-brittle mutations and the subsequent emergence of the six-rowed, hulled, and naked barley types (Badr et al., 2000). It is thought that naturally occurring mutants with a non-brittle rachis, the central axis of the inflorescence, were preferentially harvested during the initial domestication stages (Newton et al., 2011). The brittleness of rachis in barley along with the rough awn, promotes the spreading of seeds. Therefore, a selection of non-brittleness ensures a more efficient harvest as it limits the loss of grains (Bothmer et al., 2003). A molecular analysis of Amplified Fragment Length Polymorphism loci (AFLPs) frequencies applied to wild and cultivated barley, narrowed down the area of origin to the Israel-Jordan area in the southern part of the Fertile Crescent (Badr et al., 2000). The wild relative of modern barley is known as *Hordeum vulgare ssp. spontaneum* C. Koch (Zohary and Hopf, 2000). Phenotypically similar, the cultivated barley shows broader leaves, shorter stems and awns, tough ear rachis, a shorter and thicker spike, and larger grains compared to the wild barley (Badr et al., 2000).

Barley, being one of the first domesticated crops, was submitted to a wide range of diverse environmental conditions because of human expansion over several millennia. This contributed to the development and distribution of a myriad of locally adapted varieties (Bothmer et al., 2003). These form a valuable genepool source of variation which is represented in these local varieties known as landraces, that were created by intentional selection of desired traits such as a non-brittle rachis at an early stage of domestication. These landraces shaped the basic material for modern plant breeding which has started some 150 years ago (Bothmer et al., 2003).

Taxonomically, cultivated barley belongs to the tribe of *Triticeae*, which evolved 12 million years ago within the family of grasses, *Poaceae* (Gaut, 2002) and includes crop genera *Triticum* (wheat), *Hordeum* as well as *Secale* (rye; Kilian et al. 2010). Barley is an annual, diploid (2n=14 chromosomes) inbreeding species with a genome size of 5.1 gigabases (Gb; Bennett and Smith 1976). The relatively simple genetic system of barley compared to hexaploid bread wheat (*T. aestivum*) cultivars, its genetic diversity and the availability of whole genome sequencing, makes it suitable for genetic studies and breeding efforts (Dawson et al., 2015). Progress in molecular studies on barley genes and traits have not only resulted in an improved understanding of *Poaceae*, and plant biology in general, but also discovered barley specific trait expression controlled by highly conserved genes in related grass species (Schulte et al., 2009).

1.2.2 Morphological and physiological crop diversity

Florets, known as the flowers of grasses, are produced on a characteristic short branch called spikelet (Koppolu and Schnurbusch, 2019). The morphological structure in the arrangement of the inflorescence branches, spikelets and florets vary among grass species (Clifford, 1987). Each spikelet of barley has a single floret. The spike is composed of triplets (each with one central and two lateral spikelets), alternating at rachis nodes. The feature of the triplet spikelet gives barley spikes a characteristic called “row-type” (Koppolu and Schnurbusch, 2019). Morphologically barley can be thus divided into two- and six-rowed barley based on ear types (Figure 1.1).

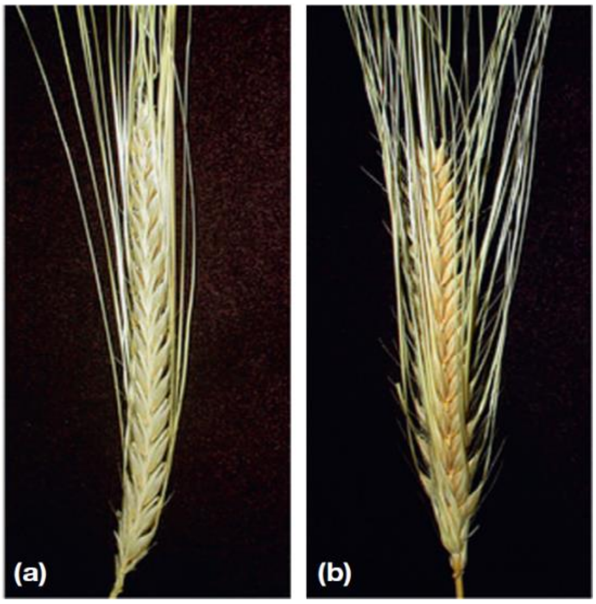

Figure 1.1: Morphological diversity of barley based on ear types.
Heads of (a) two-row barley and (b) six row barley. (Taken from Cuesta-Marcos et al. (2015)).

The wild progenitor of barley is two-rowed and is composed of arrow-like spikelets (Zohary and Hopf, 2000). In the modern six-rowed barley cultivars, all the three spikelets are fertile and able to develop into grains. In contrast, the lateral spikelets of two-rowed barley are reduced in size and are sterile (Komatsuda *et al.*, 2007). Cultivated barley can be two- or six-rowed. Two morphological loci are known to differentiate between these row-type traits. The development of a six-rowed spike is controlled by a single allele, *vrs1* which is recessive to the dominant allele leading to a two-rowed spike *Vrs1*. A second allele, *intermedium spike-c.h* (*Int-c.* or *vrs5*) occurs in six-rowed barley and is involved in the broadening of lateral spikelets (Koppolu et al., 2013). The allele constellation of the majority of six-rowed barley varieties is represented by *vrs1.a* and *Int,c,a*, a missense allele of *VRS5,* which encodes for a *TEOSINTE BRANCHED1*/*CYCLOIDEA*/*PCF1* (*TCP*) transcription factor orthologous to the *TEOSINTE BRANCHED 1 (TB1)* gene of maize (Ramsay et al., 2011). However, the presence of *vrs1* itself is sufficient to initiate two-rowed barley to become six-rowed barley (Franckowiak *et al.*, 1997; Komatsuda et al., 2007). The less functional allele of barley *HvTB1* together with *vrs1.a* contributes to increased lateral grain size in commercial six-rowed cultivars (Ramsay et al., 2011). While two-rowed barley varieties exhibit higher tiller number as well as larger and heavier seeds compared to six-rowed barley varieties (Hayes et al., 2003).

1.2.3 Seasonal diversity in barley production

Agronomic systems used for barley production throughout the world, reflect a large diversity which is adapted to both climatic and soil conditions in a given region as well as the requirements of the end user for the crop (Newton et al., 2011). Barley cultivars can broadly be distinguished between winter and spring barley depending on whether they require vernalisation or not. Winter barley has a growing period of around 10 months and is usually sown during the autumn (Jeanty et al., 2023). Winter-sown varieties require a certain exposure to colder temperatures known as vernalisation to promote flowering. Photosensitivity usually prevents these varieties from flowering in winter and reduces the chance of cold damage of the sensitive floral meristem (Ellis and Russell, 1984). Facultative varieties do not require vernalisation but do have some cold tolerance and therefore are suitable for sowing also in spring (Cuesta-Marcos et al., 2015; Jeanty et al., 2023).

The requirement for vernalisation is driven by the allelic diversity regulating growth habit (*VRN genes*) and divides temperate cereals like barley and wheat into winter and spring classes. Allelic differences in the *VRN1*, *VRN2* and *VRN3* vernalisation genes are mainly associated with the natural variation in vernalisation requirement. Whilst photoperiod response *PPD* genes, divide temperate cereals into photoperiod-sensitive and photoperiod-insensitive classes (Distelfeld et al., 2009). The response to photoperiod appears to be determined by allelic differences in the *PPD1* gene, which belongs to the family of pseudo-response regulators (Turner et al., 2005).

1.2.4 Barley production

Barley is by production the fourth world's most important cereal crop after maize, rice, and wheat (Newton et al., 2011). The geographic range of barley is wider than that of almost every other cereal crop species due to its high adaptability. Barley can be cultivated under extreme environments that are encountered for instance at altitudes of 4,500m in the Himalayan mountains, or in monsoon paddies in south east Asia, or in the arid regions of the Mediterranean (Bothmer et al., 2003; Hayes et al., 2003). The ability of barley to consistently maintain high yields in challenging conditions compared to other cereal crops such as wheat, resulted in barley being used widely amongst farmers with limited resources (Newton et al., 2011).

Worldwide, the total area of barley production is approximately 50 million hectares based on data collected in 2021 (Figure 1.2.A; FAOSTAT, 2023). In the period between 2011 to 2021, Europe was the biggest producer contributing 61% of the global barley production, Asia contributed 15%, America 13%, Oceania 7% and Africa 5% (Figure1.2.A). Globally, the main producers were the Russian Federation with 18.1 million metric tonnes (mt), France (11.3 mt), Germany (10.6 mt) and Australia (9.8 mt) (Figure 1.2.B; FAOSTAT, 2023).

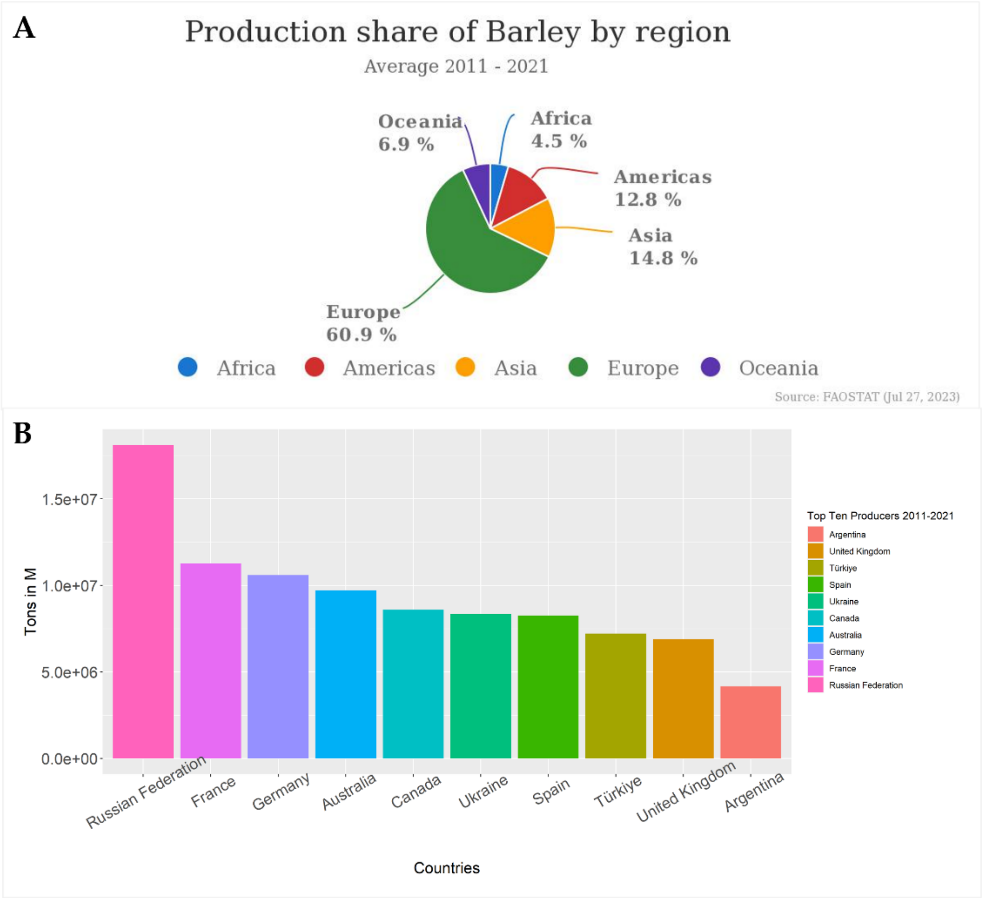

Figure 1.2: Barley production from 2011-2021.
(A) Global annual barley production by region. Comprising both winter and spring barley. (B) Average barley production in the top 10 producing countries. Bar plot was generated in R using the ggplot2 package with data accessed from FAOSTAT (2023).

Although barley is grown in highly productive areas, the rising threat from changes in climate on more marginal and stressed areas where barley plays a crucial role in sustaining food security, should not be underestimated. Barley production has substantial socio-economic and political importance followed by considerable high food security issues (Newton et al., 2011).

1.2.5 The main uses of barley

In ancient cultures of Asia, Africa and Europe, barley was the most important food grain and literature refers to barley as being used in alcoholic beverages of different types as well as for fermented food production (Newman and Newman, 2006). Archaeological evidence indicated the consumption of wild emmer (*T. dicoccoides*) and wild barley taking place 17,000 BC (Kislev et al., 1992). However, its importance as a major food crop has decreased over time, especially in highly developed countries where barley is now mainly used for animal feed and alcoholic beverages production (Newton et al., 2011). In Ethiopia, India, China, and Morocco the proportion of barley production used for human consumption still amounts to 79%, 73% and 62% and 61% respectively. However, the situation in western countries like the USA is drastically different, with 65% of the barley production used for animal feed, 30% for malting and alcohol production and only 1.5% for human consumption (Newman and Newman, 2006). Globally, the main drivers for using barley as food source are 1) the greater environmental diversity barley can be grown in compared to other cereals such as wheat, and 2) with the ability of barley to maintain high yields despite unfavourable growing conditions (Newton et al., 2011). The cholesterol lowering effect of β-glucan, a cell-wall polysaccharide found in both oats and barley grains, has increased the interest in using barley as a food product, especially as high cholesterol is a major health issue in developed countries (Newman et al., 1989). The beneficial and nutritious properties of barley were already known in ancient Egypt when barley was used in therapeutic and medicinal applications. Ground barley mixed with oil was used as purgatives and applied to wounds to decrease healing time (Newman and Newman, 2006). Similarly, in Japan a green-coloured drink made from powder of young barley grass known as "Aojiru" containing 29.5% dietary fibre, 27.3% protein and 4.5% fat is consumed regularly (Takano et al., 2013, Ikeguchi et al., 2014). The promising health benefits of barley grass include controlling blood sugar levels due to a high content in insoluble fibre (Takano et al., 2013). Further highlighting the importance of barley usage in human diet, young barley grass powder is rich in β-glucan, phenolic acids and flavonoids making it a good food supplement to support human health by reducing cholesterol, preventing diabetes and decreasing gastric cancer risk respectively (Zeng et al., 2018) .

Barley is also used to produce malt and due to the presence of the husk, and its enzymatic activities, barley is preferred over other cereals (Kumar et al., 2023). During malting, barley seeds are germinated to start the hydrolysis of starch into simple sugars which are used in the fermentation process (Greenwood and Thomson, 1959; Hayes et al., 2003). While a minor proportion of malt is used in a variety of food products to enhance flavour such as malty or roasted flavours (Newman and Newman, 2006), malt is primarily used in the production of alcoholic beverages in the brewing and distilling industries. On a global scale, beer production represents 94%, distillation 4% and 2% are used for other food stuff of malt production (Newton et al., 2011). The primary product gained from distilling barley, is whisky, which has been traditionally produced in Scotland, Ireland, the USA and Canada. For distilling purposes malt is required to drive alcohol production whereas in the brewing industry malt contributes mainly to head retention, colour and flavour of the beer (Newton et al., 2011).

The quality requirements needed for malting differ from that of feed barley. Malting barley requires a germination rate above 95%, a protein content of 11-12.5% (dry weight), moisture content of maximum 13.5%, grain uniformity, specific weight (SW), quantity of screenings as well as the levels of damaged grains below 5% (Hoyle et al., 2020; BMRI, 2015).

The varied applications of barley either as livestock feed, direct human consumption or alcohol production, highlight the importance of protecting the crop from both biotic and abiotic threats.

1.2.6 Threats to barley production

1.2.6.1 Abiotic stresses

Abiotic stress contributes to up to 50% of all major crop yield losses worldwide and thus, represents an important threat to food security (Wang et al., 2003). Abiotic stresses, such as frost, drought, extreme temperatures, salinity, alkaline soil, flooding or nutrient deficiency limit agricultural production (Atkinson and Urwin, 2012). Abiotic stresses affect the duration of the growing season therefore having major impacts on barley development, and ultimately contribute to barley production volatility (Newton et al., 2011). The frequency of these extreme weather patterns is predicted to increase as a result of climate change and will increase food insecurity (Saijo and Loo, 2020).

Although barley can adapt to a large range of growing conditions, its tolerance to environmental stresses has decreased as a consequence of the narrowing of its genetic diversity (Nevo, 2009). Crop breeding programmes are mostly responsible for this loss of genetic diversity as only around 40% of the alleles found in wild barley are present in cultivated varieties. However, the genetic diversity present in the genome of wild barley could be a useful source for alleles conferring resistance to abiotic stresses (Ellis et al., 2000). Nevo and Chen (2010) showed that the considerable genetic diversity present in the genome of wild barley can be directly used to improve drought and salt tolerance in cultivated barley (Nevo and Chen, 2010).

Physiological leaf spots (PLS) are an abiotic condition commonly observed in barley under temperate climate. PLS has been described in winter and spring barley since the 1980s in Germany and its occurrence depends on varieties, regions, and weather conditions (Wu and Von Tiedemann, 2002). Necrotic symptoms caused by PLS closely resemble those caused by pathogens such as net blotch, spot blotch, leaf scald and RLS caused by *Pyrenophora teres f. teres* (*P. teres f. teres*), *Bipolaris sorokiniana, Rynchosporium commune* and *R. collo-cygni* respectively (Wu and Von Tiedemann, 2002). Wu and Von Tiedemann (2004) also suggested that spring barley cultivars carrying the loss of function mutation *mlo* which confers total resistance to the powdery mildew fungus *Blumeria graminis f. sp. hordei* were more susceptible to form PLS. Congruently, Piffanelli et al. (2002) also indicated that the MLO protein acts as a negative regulator of programmed cell death, plant defence and stress response; and that *mlo*-enhanced cell death was associated with hydrogen peroxide accumulation in the mesophyll of plant cells. Wu and Von Tiedemann (2004) studied PLS symptoms induction in the field and under controlled glasshouse conditions and found a link to oxidative stress due to a diminished antioxidant system. Moreover, PLS formation was related to elevated levels of reactive oxygen species (ROS) in the plant (Wu and Von Tiedemann, 2002).

The plant response to abiotic stress involves complex signalling cascades, including kinases cascades, ROS, phytohormones and transcription factors that can induce stress-responsive genes (Fraire-Velazquez et al. 2011). The function of transcription factors that control the expression of abiotic stress-related genes have been characterised intensively in barley (Gürel et al., 2016). These include transcription factor families such as Apetala2 (AP2), basic leucine zipper domain (bZIP), dehydration-responsive element-binding proteins (DREBs), NAC (NAM, ATAF and CUC), myelocytomatosis (MYC) oncogene, myeloblastosis (MYB) oncogene, and WRKY (X. Li et al., 2013).

Plant-specific NACs represent one of the largest families of stress-responsive transcription factors both to abiotic and biotic stresses (Nakashima et al., 2012; Puranik et al. 2012). In barley, overexpression of *stress-responsive NAC* (*HvSNAC1*) leads to increased drought tolerance (Al Abdallat et al.,2014). A recent gene expression study on barley seedlings under controlled conditions showed that several subgroup II c *WRKY* genes were upregulated under simulated drought, Cadmium toxicity, and high salt treatments (Zheng et al., 2021).

1.2.6.2 Biotic stresses

Barley production can be threatened by several biotic organisms including insects, nematodes, bacteria, and viruses as well as fungi. This report will only focus on the main fungal pathogens threating barley production globally.

The most important fungal diseases in barley are (1) leaf and stem rust caused by *Puccinia hordei* and *Puccinia gramininis f.sp. tritici* respectively, (2) powdery mildew caused by *B. graminis f.sp. hordei*, (3) leaf scald caused by *Rynchosporium commune*, (4) net-type and spot-type net blotch caused by *P. teres f. teres* and *P. teres f. maculata* respectively, (5) spot blotch caused by *Bipolaris sorokiniana*, (6) Fusarium head blight caused by *Fusarium* species, (7) leaf blast caused by *M. oryzae*, a rice and barley pathogen, and (8) RLS caused by *R. collo-cygni* (Schmid and Thorwarth, 2014). These pathogens can all be placed along a gradient of different lifestyles ranging from obligate biotrophic (*B. graminis* and *Puccinia sp.*) over hemi-biotrophic (*B. sorokiniana, P. teres, M. oryzae, R. commune*) to necrotrophic pathogens (*Fusarium sp.*; Schmid and Thorwarth, 2014). However, a clear distinction in the lifestyle of a plant pathogen is not always feasible. A good example is represented by *R. collo-cygni,* the causative agent of RLS of barley. The long asymptomatic development of the fungus, the ability of *R. collo-cygni* to be seed transmitted and association with symptom development under abiotic stress, suggest that this fungus is an endophyte that becomes necrotrophic under adverse conditions (McGrann and Havis, 2017).

The obligate biotrophic stem rust fungus, *Puccinia graminis f.sp. tritici* belongs to the family of *Pucciniaceae* within the order of *Uredinales* (Kirk et al., 2008). The primary host of the stem rust fungus are common hexaploid bread wheat (*T. aestivum*), tetraploid durum wheat (*Triticum turgidum* var. *durum*), barley (*H. vulgare*), triticale (X *Triticosecale*) and wheat progenitors (Singh et al., 2011). Between 1970 to the early 2000 resistance against the pathogen was conferred due to genetic resistance (Leonard and Szabo, 2005; Singh et al., 2011). The emergence of a new super virulent wheat stem rust isolate *Ug99* in Africa, also known as black rust, *P. graminis f.sp. tritici*, poses a serious threat as 80% of wheat and 97% of barley varieties currently cultivated are highly susceptible to this strain (Dawson et al., 2015; Lewis et al., 2018; Singh et al., 2011). Over the past decade, wheat and barley stem rust re-occurred in isolated outbreaks after being almost eradicated in Western Europe (Lewis et al., 2018). Its first infrequent outbreak as wheat stem rust reported from Germany, Sweden and Denmark in 2013 and its first report in the UK in 2018 after nearly 60 years of eradication, has become a growing concern (Lewis et al., 2018). In 2019, stem rust was also reported on late-sown spring barley varieties in the UK (Orton et al., 2019). Orton et al., (2019) suggested that early maturating cultivars of UK wheat and barley varieties may be used an effective control as the shift of summer temperatures could contribute to the rust re-emergence. This highlights, the impacts climate change has on abiotic factors influencing crop productivity of barley, as well as on changes in disease and pest prevalence (Dawson et al., 2015).

Rynchosporium leaf scald caused by the hemi-biotrophic pathogen *R. commune*, is one of the most destructive and economically important diseases of barley in the world (Avrova and Knogge, 2012). The disease not only reduces yields by as much as 40%, but also affects the grain quality (Xi et al., 2000). The use of cultivars resistant to *R. commune* alongside fungicide application and cultural practices are currently used to control the disease (Avrova and Knogge, 2012; Xi et al., 2000). Fungicide programmes consist of a mixture of azoles with quinone outside inhibitors (QoI) or a succinate dehydrogenase inhibitor (SDHI; Phelan et al., 2017). To date, nine major resistance genes against *R. commune* have been mapped (Zhan et al., 2008). The first mapped resistance locus was *Rrs1* on chromosome 3H (Thomas et al., 1995) and 11 alleles at this locus have been described so far (Bjørnstad et al., 2002). *Rrs1* is the only resistance gene against leaf scald having a corresponding virulence factor, the necrosis inducing protein 1 (NIP1) (Rohe et al., 1995). NIP1 which belongs to a family of necrosis-inducing small, secreted proteins induces the expression of several plant defence genes such as *pathogenesis-related* (*PR*) genes *PR1, PR5, PR9* and *PR10* (Avrova and Knogge, 2012). However, modern marker-assisted breeding calls for the identification of closely linked markers along with new sources of resistance. Diagnostic markers for *R. commune* resistance loci have been developed for *Rrs2* (Hanemann et al., 2009) and *Rrs1*$_{Rh4}$ (Looseley et al., 2020).

M. oryzae, the hemi-biotrophic fungus responsible for rice blast disease has recently emerged as a potential threat to barley production in South America and south east Asia as the disease has also been reported on barley, wheat and triticale (Newton et al., 2011). The observation that barley varieties carrying the *mlo* mutation exhibit increased susceptibility to *M.*

oryzae further highlight the devastative potential of this disease as 70% of spring barley grown in Europe carry this mutation (Jarosch et al., 1999).

1.2.7 Basic plant responses to stress

The exposure to abiotic and/or biotic stress leads to the activation of calcium (Ca^{2+}) signalling and kinases cascades (Fraire-Velazquez et al., 2011), metabolite sensing (Zhang and Sonnewald, 2017), as well as ROS and phytohormone accumulation. Moreover, it leads to a reprogramming of transcriptional responses resulting ultimately in defence reactions to increase plant tolerance and minimise tissue damage (Atkinson and Urwin, 2012; Ben Rejeb et al., 2014).

It is well accepted that Ca^{2+} acts as an universal secondary messenger for primary stress signals, which transduces the stress signals from membrane receptors located at the cell surface to cytosolic effector proteins (Gong et al., 2020; Sandrini et al., 2022). This in turn initiates the activation of other calcium binding proteins, such as calmodulin (CaM), CaM-like proteins (CMLs), and calcineurin-B-like proteins (CBLs; Yang and Poovaiah 2003). In addition, the perception of molecules such as ROS, Ca^{2+} or phytohormones is perceived by receptors such as receptor like kinases (RLKs), and messengers such as calcium-dependent protein kinases (CDPKs), histidine kinases (HKs) and mitogen-activated protein kinases (MAPKs; Cheng et al. 2002; Liang and Zhou 2018). All of which are key components in the transduction of stress signalling to alleviate stress by transcriptional reprogramming (Fichman and Mittler, 2020).

Perturbations in the ROS homeostasis, which lead to over accumulation of ROS form another key cellular response to biotic and abiotic stress (Nephali et al., 2020; Sandrini et al., 2022). ROS represent reduced or activated forms of oxygen and include hydrogen peroxide, superoxide, hydroxyl radical and singlet oxygen (Halliwell and Gutteridge, 2015). Two major sources of ROS that are produced primarily in the chloroplasts, mitochondria, peroxisomes and the apoplast during abiotic stress derive from; 1) metabolic ROS that is produced from disruptions in metabolic activity and 2) signalling ROS that results from signal transduction (Choudhury et al. 2017; Miller et al. 2010). ROS accumulation, in turn results in the oxidation of those cell components and interferes with metabolic activities including photorespiration and negatively affect organelle integrity (Suzuki et al., 2012). To minimise the damage to DNA, lipids and proteins caused by ROS and maintain redox balance, plants have evolved enzymes such as catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX) as well as non-enzymatic antioxidants such as glutathione (GSH ; Foyer and Noctor 2005; Choudhury et al. 2017).

During biotic or abiotic stress, the production of phytohormones as one of the primary signalling molecules in response to the stress, induces the expression of stress-related genes via complex signalling cascades (Nephali et al., 2020; Sandrini et al., 2022). These phytohormones include auxins, gibberellins (GA), cytokinins (CYT), ABA, ethylene (ET), salicylic acid (SA), jasmonates (JA), brassinosteroids (BR), and strigolactones (Verma et al., 2016). For example, ABA accumulation is induced in response to abiotic stress such as drought, salinity and heat (Zhang et al., 2006), whereas, SA, JA and ET play major roles in response to biotic stresses by stimulating the expression of *PR* genes (Bari and Jones, 2009). The signalling pathways of these hormones are known to interact in an antagonistic-, synergistic-, as well as in an additive manner in hormonal crosstalk's (Aerts et al., 2021).

1.3 Ramularia collo-cygni

1.3.1 Taxonomy

R. collo-cygni is the causative agent of RLS on barley, which has been identified as a major threat to barley production in the temperate region of the world over the past 40 years (Dussart et al. 2020). This ascomycete fungus has been known for over 100 years, with a first report in Italy, where it was identified as *Ophiocladium hordei* (Cavara, 1893). Later, Sprague, (1946) reclassified the fungus to *Ovularia hordei* based on thorough morphological studies between *Ovularia* and *Ophiocladium* species. Based on further investigations of conidiophore morphology between *Ovularia* and *Ramularia* species, Sutton and Waller, (1988), proposed a reclassification of the genus into *Ramularia* within the family *Mycosphaerellaceae* in the class *Dothideomycetes*. The species name *R. collo-cygni* derives from the morphological structure of the conidiophores resembling a curved swan neck (from the Latin: *collum* meaning neck and *cygnus*- swan; Figure 1.3).

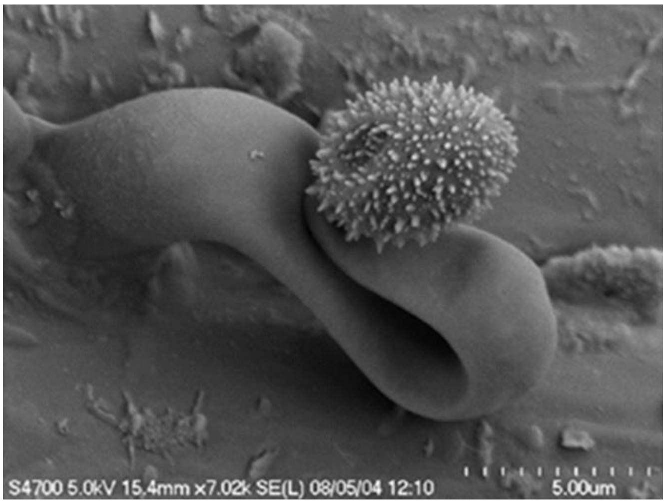

Figure 1.3: Swan-neck shaped conidiophore of *Ramularia collo-cygni*.
Taken from Walters et al. (2008).

The taxonomy of *R. collo-cygni* was later confirmed based on a phylogenetic analysis of internal transcribed spacer (ITS) regions ITS-1, ITS-2 and the 5.8S ribosomal RNA of 46 *Mycosphaerella* species including *Ramularia* anamorphs (Crous et al., 2000). A phylogenetic analysis based on the full genome of *R. collo-cygni* confirmed the classification of the fungus within the *Mycosphaerellaceae* and identified the wheat pathogen *Z. tritici* as the sister species of *R. collo-cygni* (McGrann et al., 2016). Moreover, *R. collo-cygni* is closely related to the banana pathogen *Pseudocercospora fijiensis* (syn. *Mycosphaerella fijiensis*) , the pine pathogen *Dothistroma septosporum* and *Cladosporium fulvum* a pathogen of tomatoes (McGrann et al., 2016).

1.3.2 Distribution of *R. collo-cygni*

Following, the first detection of the fungus in Italy (Cavara, 1893) *R.collo-cygni* was identified in Norway in 1925 (Jorstad, 1930). However, it was not until the 1980s that *R. collo-cygni* was attracting increasing interest as a threat to barley production (Walters et al., 2008). The main reason for this lack of interest was the fact that RLS was often confused with PLS or other diseases such as *P. teres* (Sachs et al., 1998).

From the late 19th century the disease was identified throughout Northern Europe, in countries like Austria (Huss et al., 1987), Germany (Sachs et al., 1998), Scotland (Oxley et al., 2002), Norway (Salamati et al., 2002), Denmark (Pinnschmidt et al., 2006), and the Czech Republic (Minarikova et al., 2002). Furthermore, the identification of RLS seed samples and crops from Slovakia, Estonia, Russia, Poland and Spain was reported more recently (Havis et al., 2015). Outside of Europe, RLS had been reported in Argentina, Chile, Uruguay, Columbia, Mexico, the USA and New Zealand (Walters et al., 2008).

Despite being usually diagnosed upon recognition of disease symptoms and the morphology of sporulating structures of the fungus, the use of molecular diagnostic tools showed that *R. collo-cygni* DNA is present in seed samples without causing disease symptoms (Havis et al., 2015). Moreover, the findings of a seed-borne stage in the lifecycle of *R. collo-cygni* (P. Matusinsky et al., 2011) has not only contributed to a better understanding of the life cycle, but is also thought to be the cause of the sporadic global distribution of pathogen (Dussart et al., 2020).

1.3.3 Ramularia leaf spot symptoms

The typical symptoms of RLS in spring and winter barley are identified by the 5R's (Havis et al., 2018) which are: (1) rectangular, (2) reddish-brown in colour, (3) restricted by the leaf veins, (4) going right through the leaf and (5) ringed with a halo of chlorosis (Figure 1.4). Identification of RLS symptoms can be difficult, especially in early stages of their development. Initial RLS symptoms which are often referred to as "pepper spots" as they form small brown spots on the leaf, can be easily confused with PLS (Sachs et al., 1998). With the progression of the fungus, those "pepper spots" turn into typical RLS lesions. Symptoms generally appear post flowering around growth stage (GS) 70 (Zadoks et al., 1974) which led to the classification of *R. collo-cygni* as a late season pathogen (Walters et al., 2008). However, under field conditions in crops grown under waterlogging stress, RLS symptoms and sporulation was observed during vegetative growth stages on senescent leaves (Havis et al., 2015). Fungal sporulation emerges typically on the abaxial side of leaves in necrotic tissue, as conidiophores which can be seen as whitish clusters appear through stomata (Sutton and Waller, 1988).

Figure 1.4: Ramularia leaf spot symptoms.
Picture shows typical RLS leaf spot symptoms: ringed with yellow margin of chlorosis, rectangular shape, restricted by the leaf veins, and reddish-brown coloration. Picture taken at Boghall Farm in August 2021 at the end of the growing season.

1.3.4 Plant-fungus interactions in the cause of symptom formation

The exact cause for symptom formation is yet to be determined (Dussart et al. 2020). At the cell level, it was long thought that the release of secondary metabolites (SMs) by *R. collo-cygni* in barley contributed to the formation of RLS symptoms (Sutton and Waller, 1988). This hypothesis was supported by the discovery of non-host specific toxins, called rubellins by Miethbauer et al., (2003) who identified rubellin D from mycelium and culture filtrate of *R. collo-cygni* isolated from infected barley leaves. This anthraquinone derivative can induce cell death in a light-and concentration-dependent way (Heiser et al., 2003). Furthermore, an *in vitro* study with incubated barley leaves demonstrated the production of ROS in response to light-activated rubellins (Heiser et al., 2003). This has not yet been shown *in planta*, however, some light activated fungal phytotoxins of other *Dothideomycetes* were already shown to induce the formation of ROS in plants as seen by several *Cercospora* species. These fungi produce cercosporin, a perylenequinone that induces singlet oxygen ($^1O_2$) and superoxide ($O_2.^-$) production in a photodynamic reaction (Daub et al., 1983). The peroxidation of membrane lipids can lead to loss in membrane fluidity, leakage of nutrients, and rapid death of the plant cell (Daub, 1987). Later, McGrann and Brown, (2018) supported also the link between RLS and ROS by detecting the ROS hydrogen peroxide (H_2O_2) in infected barley leaves. Dussart et al, (2018a) investigated the role of rubellin D in five barley landraces in a preliminary study and indicated that rubellin D does not reproduce typical RLS symptoms that are linked to rubellin sensitivity as highlighted in several other studies (Heiser et al., 2003; Miethbauer et al., 2003). Hence, rising doubts of the direct link between rubellin release and RLS symptom development. Interestingly, Dussart et al. 2018a showed that the genome of *R. collo-cygni* appears to have several clusters involved in the biosynthesis of other SMs and highlights the importance of further studies to uncover putative compounds potentially involved in the formation of RLS symptoms. Moreover, the genes present in these clusters were also shown to be highly expressed during the asymptomatic growth stage in barley. Recently, Dussart and Jakubczyk (2022) have demonstrated in controlled inoculation barley seedling assays that all of the genes in the rubellin candidate biosynthetic gene cluster are mainly expressed during the asymptomatic and to a lesser extend during the early formation of RLS disease symptoms, suggesting that rubellins may play a role in mediating the host-pathogen interactions rather than being directly involved in the formation of symptoms.

1.3.4.1 Factors influencing Ramularia leaf spot development

The ability of *R. collo-cygni* to develop asymptomatically within its host, sometimes without causing disease epidemics has not only contributed to a difficult prediction of RLS in the past, but also raised the question of knowing which factors determine disease severity. Similarly, the conditions leading to the fungus changing from endophytic to necrotrophic stage is still unknown despite several studies linking RLS development to the host genetics (McGrann et al., 2015; McGrann et al., 2015, 2014). Changes in environmental conditions seem to play an important role in the transition from asymptomatic to necrotrophic fungal development.

Abiotic and biotic stresses share common signalling and responsive genes (Chan, 2012; Ma and Bohnert, 2007), and thus, the combination of both as often observed in the field, can cause a negative, neutral or positive effect on plants (Sewelam et al., 2021). Király et al. (2008) showed that high temperature suppressed the hypersensitive-response (HR)-mediated resistance of tobacco (*Nicotiana tabacum*) against Tobacco Mosaic Virus (TMV). Similarly, elevated temperatures partially suppressed SA signalling in *A. thaliana* resulting in increased susceptibility to *Pseudomonas syringae* (Huot et al., 2017). Recently, Röhrig and Dussart (2022) extensively reviewed the impact of abiotic stresses on the host in the development of diseases caused by *Dothideomycete* fungi. It was highlighted that abiotic stress can alter the host-pathogen interaction between barley and *R. colly-cygni*, belonging to the family of *Mycosphaerellaceae* in the class of *Dothideomycetes* (McGrann et al., 2016). A field trial study by Hoheneder et al. (2021) showed that extended drought periods leads to increased resistance to RLS and reduced fungal DNA in the plant compared to plants grown in irrigated plots. The authors speculated that drought-induced leaf senescence could potentially inhibit the development of RLS symptoms (Hoheneder et al., 2021). In Ireland, Mulhare et al. (2021) observed similar trends with prolonged drought periods post stem extension resulting in no disease development. Congruently, previous studies showed that the overexpression of salt and drought-responsive *HvSNAC1* in transgenic barley lines led to increased resistance to RLS and lower fungal DNA in the absence of stress under controlled conditions (McGrann et al., 2015a).

Abiotic stresses such as high light intensity and waterlogging have also been linked to the development of RLS (Röhrig and Dussart, 2022). Barley plants subjected to waterlogging prior to inoculations with *R. collo-cygni* exhibited significantly higher diseases levels compared to plants grown under normal conditions (McGrann and Brown, 2018). Havis et al. (2018) highlighted a correlation between high rainfall and temperatures during the growing season and elevated RLS levels in spring barley crops. This correlation is also supported by the observation that RLS symptom expression was promoted by rainy days and lower average daily temperature in the three weeks post heading (Mařík et al., 2011). A recent study by Havis et al. (2023) suggested that extended leaf wetness in barley crops might have resulted in the initiation of spore release from barley in the UK sites. Light and humidity are also known to play an important role in disease development as Huss et al., (2003) showed that dew was crucial for symptom development and Makepeace et al., (2008) showed that plants gown under high light intensity displayed increased RLS severity.

1.3.5 The epidemiology of *R. collo-cygni*

Infection by *R. collo-cygni* eventually results in a loss of green leaf area in infected plants which can lead to yield losses ranging between 10- to 70% in barley, with an average loss in Scotland to be 15- to 20% (Walters et al. 2008; Havis et al. 2015; Dussart et al. 2020; N. Havis personal communication). The transmission of the fungus can occur either from spore dispersal or from infected seeds (Havis et al., 2014, 2006; Mäe et al., 2018). While developing within an infected seed, *R. collo-cygni* is transmitted vertically into developing plant tissue. Fungal colonisation can be followed developing asymptomatically within the host from seeds to seedling stage (Havis et al., 2014b). As a late season pathogen, RLS symptoms can be seen emerging on all aerial parts of the plant including stems, awns and grains (Walters et al., 2008).

The infection process from airborne spores results in spore germination on the leaf surface and is followed by the hyphae penetrating the leaf stomata (Stabentheiner et al., 2009). In addition to airborne spores, Salamati and Reitan, (2006) observed the development of a secondary spore type of *R. collo-cygni*, described as *asteromella*. The role of these structures has yet to be fully established, although it has been proposed to function as a form of sexual reproduction (Kaczmarek, 2013). However, the teleomorph form of *R. collo-cygni* has not been identified to date (Havis et al., 2015).

The understanding of the fungal life cycle has become clearer over the past 15 years. The confirmation of a seedborne stage (Havis et al., 2014a), was suggested to be responsible for the global distribution of *R. collo-cygni* (Dussart et al. 2020). The role the different stages of the fungal life cycle play on the disease development are still unknown, especially that of airborne spores in the biology of the fungus (Dussart et al., 2020; Havis et al., 2015; McGrann and Havis, 2017). At present, the role of airborne spores relies on studies performed with spore traps in the field, and thus on natural occurrences of airborne spores. Controlled inoculation experiments in glasshouses are problematic to achieve as the isolation of spores poses difficulties. A method providing an artificial inoculation with a spore suspension of *R. collo-cygni,* rather than the current use of a mycelial suspension (Makepeace et al., 2008b), would help to better understand the role of airborne spores in the lifecycle. The importance of this is highlighted due to the conflicting findings in literature investigating the spore movement within the crop canopy. Field studies from infected seeds in the absence of an external inoculum source performed by Havis et al. (2014b) indicated that epidemics in the United Kingdom emerge from a seedborne infection and that late season spore movement does not impact occurring disease epidemics. While trials completed in Germany and Estonia have found a closer relationship between spore movement and disease epidemics (Schützendübel et al., 2008) which supported the original life cycle of the fungus, proposing a role for spore movement in disease epidemiology (Mäe et al., 2018; Sachs, 2006). This was supported by findings of Zamani-Noor, (2011), who indicated that spore release from winter crops contributed to disease levels in spring crops. Recently, Havis et al. (2023) observed an association between *R. collo-cygni* spore levels around GS 49 (first awns visible) and final RLS disease levels in winter barley. Moreover, the study indicates a single peak of spore in the UK, Germany, and Poland, however, the timing of the peak differed among countries. The data from the two UK sites, in which sporulation is low early in the season, support the theory that the initial inoculum comes from the seed and is vertically transmitted through the host plant (Havis et al., 2023).

Although primarily being a pathogen of barley, *R. collo-cygni* can also infect other cereals such as wheat and oats (Huss et al., 2005) as well as the model grass *Brachypodium distachyon* (Peraldi et al., 2014). The role of alternative hosts in RLS epidemiology remains to be determined, but studies from Estonia, where the pathogen has recently emerged have suggested that wild grasses such as ryegrass and blackgrass may act an important source of inoculum (Mäe et al., 2018).

1.3.6 Plant-fungus interaction

A study of transcriptional changes associated with *R. collo-cygni* infection of barley based on genome wide transcriptional profiling of both organism was performed to identify disease-associated molecular components (Sjokvist et al., 2019). The study found that during the asymptomatic colonisation of mesophyll tissue, the expression of barley transmembrane sugar transporters was increased. Likewise, *R. collo-cygni* upregulated its own sugar and nutrient transporters suggesting that the fungus derives its nutrients from the apoplastic medium (Sjokvist et al., 2019). During the asymptomatic stage of RLS development, genes involved in lignification and cell wall reinforcement were upregulated whereas photosynthesis-related genes were downregulated (Lemcke et al., 2021; Sjokvist et al., 2019).

Like several related fungal pathogens, *R. collo-cygni* also appears to avoid recognition by the host immune system as genes encoding for molecules containing a chitin-binding Lysine motif (LysM)-domain were upregulated during the initial stages of infection (Sjokvist et al., 2019). LysM-domain–containing proteins act as scavengers of chitin, a well-known pathogen-associated molecular pattern (PAMP), to avoid recognition by the host plant pattern recognition receptors (PRRs), therefore circumventing pattern-triggered immunity (PTI; Dodds and Rathjen, 2010; Sánchez-Vallet et al., 2015). In turn, the expression of RLKs, along with several genes encoding for proteins involved in the plant immune response was induced in barley, indicating a recognition of *R. collo-cygni*-PAMPS (Sjokvist et al., 2019).

Like many other Dothideomycete pathogens, *R. collo-cygni* may also use SMs to control their host (Muria-Gonzalez et al., 2015). SMs also exhibit a vast structural diversity and can be grouped into four main groups based on their biosynthetic origin. Polyketides such as aflatoxins are synthesised by polyketide synthases, non-ribosomal peptides (NRPs) such as penicillin are produced by NRP synthetases (NRPSs), terpenoids which includes gibberellins result from the terpene pathway, and tryptophan-derived metabolites such as ergot and indole alkaloids are produced by dimethylallyl tryptophan synthases (Wiemann and Keller, 2014). A study by Dussart et al. (2018) identified several genes involved in SM biosynthesis, including PKSs and NRPSs in the genome of *R. collo-cygni*. Based on the findings by Dussart et al., (2018), Sjokvist et al. (2019) performed RNA-seq on the predicted gene clusters and revealed a similar situation, with higher expression rates of genes encoding enzymes to be involved in SM metabolism at both 3 days post inoculation (dpi) and 7 dpi.

1.3.7 Crop disease management of Ramularia leaf spot in barley

Grain yield loss in barley results from necrotic RLS disease symptoms causing reduction in green leaf area (Dussart et al. 2020). Currently the impetus to adapt integrated pest management (IPM) strategies to offer a viable solution to control RLS, should include the continuous development of pest control strategies to minimise the need for pesticides (Lamichhane et al., 2016; Mulhare et al., 2021). The principles of IPM as defined by the European Commission in 2009, include the prevention and suppression of the pest, monitoring, decision-making, non-chemical methods, pesticide selection, reduced pesticide use and anti-resistance strategies (Marchand and Robin 2019; Dussart et al. 2020). At present limited information is available on varietal resistance against RLS, even though quantitative resistance has been observed in many countries in Europe and appears a promising solution (Havis et al., 2015). Difficulties of utilising quantitative resistance against RLS are presented by observations of strong genotype x environment interactions and the link to *mlo* alleles that provide broad-spectrum resistance to powdery mildew, but come with the trade-off of susceptibility to necrotrophic pathogens such as *R. collo-cygni* (McGrann et al., 2014).

Currently, the impact of agronomic practices in controlling RLS is still debated and appears to be rather limited. Practices that might lead to a reduction of RLS include removing crop residues by ploughing and increasing the rotation length of barley crops, which may be able to eradicate secondary spore structures, and in turn RLS development in the following crop season (Dussart et al. 2020).

In the absence of effective genetic resistance, seed treatment control and IPM strategies, RLS management currently relies on foliar application of fungicides (Mulhare et al., 2021). Initially, the QoI fungicides provided a good control of RLS but resistance to this class of fungicide has quickly developed and is now widespread in *R. collo-cygni* populations (Fountaine and Fraaije, 2009; Pavel Matusinsky et al., 2011). Therefore, fungicide programmes relied in the past on a combination of single site active ingredients including fungicides from the SDHI and DMI families as well as multisite fungicides such as chlorothalonil (Havis et al., 2015). However, controlling RLS has recently become challenging as resistance to SDHI and DMI have been reported in Western Europe by mutations present in the target genes of both fungicide classes (Rehfus et al., 2019). Furthermore, the ban of chlorothalonil in Europe will further the pressure on barley growers as RLS control will become more difficult (Dussart et al. 2020; Fones et al. 2020). The absence of varietal resistance, the lack effective seed treatments and the ability to quickly evolve resistance to single-site actives, demonstrates the urgency to better understand the genetic basis of RLS disease resistance in barley.

1.4 Project Aims

This project investigates the potential genetic control of RLS in spring barley genotypes and examine genotype by environment interaction in RLS epidemiology. A genome-wide association scan (GWAS) was performed to identify quantitative trait loci (QTL) associated with increased resistance to RLS and candidate genes underlying the QTL. Field trials were conducted both in Scotland and Germany to study the effect of genotype by environment interactions on RLS development in a subset of spring barley genotypes carrying the resistant/susceptible QTL identified by the GWAS. To better understand the link between senescence and RLS symptom development, this project studied the response of RLS symptom development and fungal colonisation in response to delayed- and early senescence. Following the results from the GWAS of identifying an ethylene-responsive gene which is known to be involved both in the regulation of senescence in plants and plant immune responses, the response to ethylene application on RLS symptom development was tested. Moreover, the fungal colonisation and impact on host senescence was monitored under controlled *R. collo-cygni* inoculation experiments. Based on these results, the expression of ethylene-responsive genes was investigated in different barley genotypes to associate changes in transcript levels with the expression of disease symptoms observed in the field and under controlled conditions.

Chapter 2:

Investigating the interaction between host physiology and Ramularia leaf spot in barley

2.1 Introduction

The endophytic characteristic of *Ramularia collo-cygni* allows the fungus to develop asymptomatically during the growing season, sometimes without causing disease epidemics (Havis et al., 2014). Ramularia leaf spot (RLS) symptoms become typically apparent late in the growing season on older senescing leaves, when plants undergo reproductive development (Figure 2.1; McGrann and Havis 2017). Due to its prolonged asymptomatic growth, predicting the exact moment of symptoms appearance has challenged growers and scientist alike. Identifying factors that promote RLS outbreaks remains one of the main barrier to effective disease control (Dussart et al. 2020). The environmental conditions as well as the role of the host plant genetic makeup resulting in the fungus transiting from endophytic to necrotrophic growth is at present not fully understood. Several studies have linked abiotic stresses such as waterlogging and high light intensity with increased RLS symptoms (Huss et al., 2003; McGrann and Brown, 2018). Similarly, McGrann et al. (2014) showed that the host genetics mediated the interaction between barley and *R. collo-cygni* as barley lines carrying the *mildew locus O* (*mlo*) mutation, a mutation conferring full resistance to powdery mildew, exhibited increased RLS symptoms resulted in increased susceptibility of barley to RLS. Whether there is a causal relationship between senescence and RLS remains to be ascertained, since most elite barley varieties senesce early due to high levels of disease (McGrann and Brown, 2018; Oxley et al., 2008).

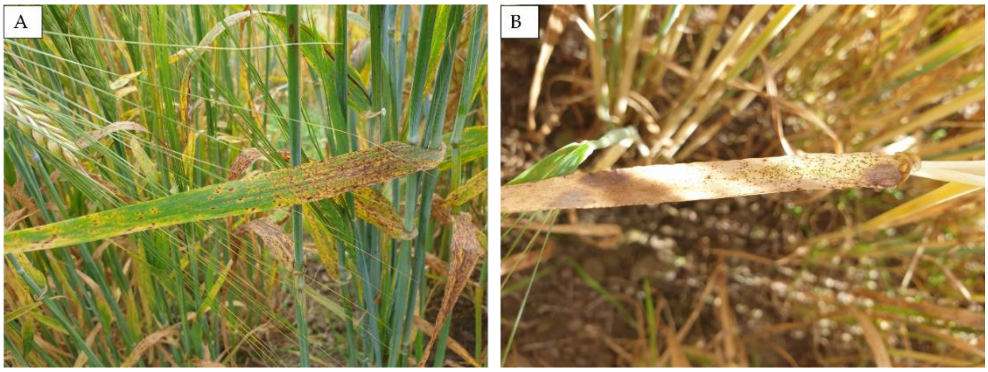

Figure 2.1: Spring barley cultivars Barke (left) and Scarlett (right) showing different rates of senescence and Ramularia leaf spot (RLS) symptom expression at growth stage (GS) 79 in field trials in Scotland (2021).
Barke is a moderately susceptible elite variety exhibiting around 10% RLS disease symptoms. Scarlet is a highly resistant elite variety exhibiting 5% RLS disease symptoms.

2.1.1 The interaction between Ramularia leaf spot and senescence

In 2005 and 2006 the host antioxidative system was studied under field conditions in relation to RLS symptom development in winter barley undergoing monocarpic senescence of the flag (F) and F-1 leaves (Schützendübel et al., 2008). Monocarpic senescence marks the physiological processes accompanied with the loss of antioxidative capacity and as a result, leads to an increase of reactive oxygen species (ROS; Zimmermann and Zentgraf 2004). ROS are activated forms of atmospheric oxygen and include hydrogen peroxide or superoxide (Halliwell and Gutteridge, 2015). ROS are primarily produced in the chloroplasts, mitochondria, peroxisomes and the apoplast of plants during stress responses (Choudhury et al., 2017). ROS accumulation leads to oxidative damage to DNA, lipids and proteins. To minimise ROS-associated damage, plants produce antioxidants, such as ascorbic acid and glutathione (GSH), as well as ROS-scavenging enzymes such as ascorbate peroxidase (APX), catalase (CAT) and GSH peroxidase (Mittler et al., 2004). Schützendübel et al. (2008), found that first visible symptoms of RLS corresponded to the breakdown of the GSH pool, supporting the hypothesis that barley susceptibility to RLS might be influenced by the decline of the antioxidative systems in the host. The alteration of increased ROS levels in the host caused by the antioxidative breakdown during monocarpic senescence could lead to an imbalance between the asymptomatic development of *R. collo-cygni* and the plant immune system and may lead to late season outbreaks (Heiser et al., 2003; Schützendübel et al., 2008). Authors also speculated that senescence-induced nutrient remobilisation leading to changes in the leaf nitrogen status might trigger the transition from asymptomatic to symptomatic growth of *R. collo-cygni* (Schützendübel et al., 2008). Transcriptome analysis by Sjokvist et al., (2019) of spring barley cv. Fairytale during controlled inoculation experiments with *R. collo-cygni* has explored gene transcription profiles of genes that have been previously reported to be upregulated during senescence in barley (Kucharewicz et al., 2017). Those genes belonged to five functional categories: transcription factor (TF), photo-synthesis-, stress-, remobilisation-, and degradation-related genes (Kucharewicz et al., 2017). Results by Sjokvist et al. (2019) have shown that upon *R. collo-cygni* infection those senescence-associated genes (*SAGs*) were upregulated and resembled patterns monitored in naturally senescing barley leaves, indicating that the fungus induces premature senescence (Kucharewicz et al., 2017). Moreover, control plants appeared green, and no senescence related genes were upregulated between 3 to 12 days post inoculation (dpi; Sjokvist et al. (2019). Similar observations were made in *R. collo-cygni* sister species *Zymoseptoria tritici*, the agent responsible for Septoria tritici blotch in wheat. *Z. tritici* also appears to trigger host senescence as an important component of its colonisation strategy (Ma et al., 2018; McGrann et al., 2016). TF families associated with senescence such as NAC and WRKY were upregulated during *R. collo-cygni* colonisation of the apoplast at 7 and 12 dpi (Sjokvist et al., 2019). Interestingly, a study by McGrann et al. (2015), showed that the overexpression of the barley *stress-responsive NAC* (*HvSNAC1*) TF leads to reduced RLS symptom formation and fungal DNA. *SNAC1*s are associated with the regulation of diverse developmental processes including senescence as well as biotic and abiotic stress tolerance (Puranik et al., 2012). However, the study by McGrann et al. (2015) also indicated that the overexpression of *SNAC1* in *R. collo-cygni*-infected plants did not affect the regulation of ROS scavengers, suggesting that the observed reduced susceptibility to RLS cannot be attributed to an increased tolerance to ROS-induced damage. The authors hypothesised that the increased resistance to RLS may be mediated by mechanisms controlled by *HvSNAC1* that are involved in tolerance to abiotic stresses and inhibition of senescence (McGrann et al., 2015a). Taken together, the results of these studies on senescence and RLS development suggest that processes occurring during host senescence may play a role in the epidemiology of *R. collo-cygni*. McGrann and Brown (2018) observed differences in dark-induced senescence assays under controlled inoculation experiments among eight spring barley genotypes, however; no direct correlation existed between the rate of senescence and varietal susceptibility to RLS. Furthermore, they showed that a treatment with high light intensity during RLS infection lead to premature leaf senescence compared to control plants and found that disease severity was enhanced in the two susceptible varieties Braemar and Golden Promise but not in the partially resistant cv. Power (McGrann and Brown 2018). Interestingly, the fungus remained in the endophytic stage compared to the controls in experiments performed on white leafed *albostrians* mutants impaired in chloroplasts development and in the *Bipolaris sorokiniana tolerant 1-7 (bst1-7)* mutant that is resistant to spot blotch and in which chloroplast degradation occurs rapidly (McGrann and Brown 2018).

Similarly, *Z tritici* exhibited reduced virulence on wheat plants containing mutations of the *phytoene desaturase and Magnesium chelatase H subunit*, which resulted in non-functional chloroplasts (Lee et al., 2015). Lee et al., (2015) also showed accelerated *Z. tritici*-induced ROS production which has also been shown in *R. collo-cygni* (McGrann and Brown 2018). RLS development has been linked to an increase in either hydrogen peroxide (H_20_2) accumulation or superoxide (O_2^-) as shown in the 3`,3 diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) stained leaves during RLS development. Moreover, infiltration with either H_20_2 or catalase resulted in this study in enhanced symptom expression in the susceptible barley varieties (McGrann and Brown, 2018). However, this was not as consistent as the effect of abiotic stress on RLS symptom development, which indicates that barley varieties might respond differently to pathogen-induced changes in H_20_2 (McGrann and Brown, 2018). Altogether, this implies that chloroplast function and ROS may present important factors in the transition of *Mycosphaerellaceae* fungi to necrotrophic parasitism. Abiotic stress, elevated ROS levels and senescence might not be the decisive factors triggering the necrotrophic transition, however, ROS-mediated processes such as abiotic stresses resulting in senescence might initiate the pathogenic transition of *R. collo-cygni* (Lee et al., 2015; McGrann et al., 2015a; McGrann and Brown, 2018). Considering that senescence appears to play a role in this pathosystem, this chapter aims to better understand how senescence in barley influences RLS development in seedlings.

2.1.2 Senescence in monocarpic plants

Some of the most globally important crops are monocarpic plants including maize, rice, wheat, and barley. Monocarpic plants complete their life cycle within one growing season, which implies the termination of the plant life once reproduction is complete (Davies and Gan, 2012). Senescence is the last stage of the development of plant cells, and tissue organs. It can be divided into organ senescence which can occur throughout the life-cycle of the plant and post-anthesis senescence, also known as monocarpic or terminal senescence (Leopold, 1961). Senescing organs translocate nutrients during the vegetative growth to younger plant parts and is associated with leaf age and affected by environmental factors, such as limited water and nitrogen (N) availability (Gan and Amasino, 1997). Characteristic for a hallmark of monocarpic senescence is the rapid transition from green to yellow crops (Figure 2.2) and happens during the reproductive stage when all tissue and organs of the parental plant die (Davies and Gan, 2012).

Figure 2.2: Monocarpic senescence in barley.
Representative pictures of field trials at Boghall Farm, Scotland 2021. Right picture was taken on the 5th of July and the left picture was taken on the 5th of August 2021.

Monocarpic plants are characterised by remobilising all their resources from senescing organs to the grains after flowering (Distelfeld et al., 2014). Key agronomic traits including N-use efficiency, yield and quality parameters such as protein grain content (GPC) are influenced by the timing of senescence including the onset, initiation and rate of senescence (Distelfeld et al., 2014; Gregersen et al., 2013; Munier-Jolain and Salon, 2005; Zakari et al., 2020). N availability has a well-studied influence on post-anthesis senescence, as low N levels can induce early senescence (Martre et al., 2006). Whereas high N levels can lead to a delay in leaf senescence due to a higher extent of stored inorganic and organic nitrogen which, in turn, results in higher photosynthetic activity (Distelfeld et al., 2014; Martre et al., 2006).

2.1.2.1 Sugar signalling during senescence

Barley and wheat leaf senescence has been studied in the past using steam girdling which interrupts the phloem export by killing the tissue in a narrow zone from detached mature cereal leaves at the base of the leave (Feller and Fischer, 1994). Steam girdling results in a rapid accumulation of soluble carbohydrates, such as fructose, glucose and sucrose from photosynthetic active detached leaves and to rapid leaf senescence (Distelfeld et al., 2014). In *Arabidopsis thaliana* and tobacco (*Nicotiana tabacum*) plants, sugar signals were higher in senescing leaves compared to non-senescing leaves (Kumar et al., 2018). Girdling has been used by Parrott et al., (2007) in a transcriptomic study of two-week-old barley leaves which led to the identification of several *Senescence-Associated Genes* (*SAGs*). These genes included WRKY and MYB transcription factors involved in nitrogen metabolism and amino acid transporters, suggesting that these genes could be involved in nitrogen translocation (Distelfeld et al., 2014; Parrott et al., 2007). Alongside *SAGs*, genes encoding for transmembrane sugar transporters were also expressed in barley leaves following infection by *R. collo-cygni* (Sjokvist et al., 2019). Taken together, these results could explain the premature leaf senescence observed during RLS development.

2.1.2.2 The role of phytohormones during senescence

Post-anthesis senescence in monocarpic plants is under correlative regulation of reproduction (Davies and Gan, 2012) and therefore, dependent on the plant developmental processes and influenced by environmental factors (Jibran et al., 2013). A transcriptomic analysis of barley flag leaves at 21 days post-anthesis showed that several phytohormones related genes were upregulated in near isogenic germplasm differing in early and late senescence (Jukanti et al., 2008). These included genes encoding for abscisic acid (ABA)-responsive element binding proteins, cytokinin (CK) oxidases/dehydrogenases (CKX) as well as genes involved in ethylene (ET) biosynthesis. The transcriptome analysis results indicated that barley senescence might be favoured by low CK levels, however; senescence appears to be associated with enhanced ABA and ET levels (Jukanti et al., 2008). ABA levels positively correlated with the age of leaves in several species, including rice and maize, as older leaves exhibited higher levels of ABA compared to younger leaves. Similarly, the expression of genes associated with ABA biosynthesis and signalling also increased in older leaves (Jibran et al., 2013). Schlüter et al. (2011) observed that barley flag leaf senescence was associated with an increased activity of CKX in field grown barley plants. An even higher activity of CKX was observed in detached barley leaf segments incubated in a light/dark cycle that was associated with a decrease in chlorophyll levels (Schlüter et al., 2011). Distelfeld et al. (2014) indicated that cereal leaf senescence appears to be linked with lower cytokinin, but higher ABA levels. The decrease of CK content in senescencing leaves has been long proposed to act as a key signal to initiate senescence (Gan and Amasino, 1997). Studies of the transcriptome in *A. thaliana* leaves demonstrated that expression of CK biosynthetic genes decrease during senescence, while transcript levels of CK degrading enzymes increase (Breeze et al., 2011; Buchanan-Wollaston et al., 2005). Evidence supporting its role as an endogenous negative regulator of senescence is based on studies examining changes in cytokinin content, expression during senescence or manipulating senescence by exogenous application of CK and its derivates (Cortleven and Valcke, 2012; Gan and Amasino, 1997; Zwack and Rashotte, 2013). Strong evidence for the negative regulation of leaf senescence by CK, was provided by Gan and Amasino (1995)` design of a heterologous, auto-regulatory senescence-inhibition system in transgenic tobacco plants. The system is based on the regulation of the expression of an *Agrobacterium tumefaciens* gene isopentenyl transferase (IPT), an enzyme that catalyses the rate-limiting step in CK biosynthesis, under the control of a senescence-specific promotor, *SAG12*, which leads to a delay of leaf senescence. Due to the striking delay of leaf senescence by the pro*SAG:IPT* system, it has been implemented in various crop species, including tomato, cauliflower, and cotton (Y. D. Liu et al., 2012; Nguyen et al., 2008; Swartzberg et al., 2006). The exact mechanism of cytokinin delayed leaf senescence is not fully understood, however; it is widely accepted to involve the regulation of source-sink relations (Zwack and Rashotte, 2013). Lara et al. (2004) demonstrated that CK can upregulate expression of extracellular cell wall invertase (CWINV) by using transgenic tobacco plants expressing *SAG12:ipt.* The extracellular enzyme invertase plays a crucial role in source-sink regulation, and has been shown to be required for the delay of leaf senescence (Hönig et al., 2018). CKs have also been suggested to be involved in cellular oxidative stress by increasing the antioxidant activity in plants (Brizzolari et al., 2016). During dark-induced senescence of wheat leaves, the external application of 6-benzylaminopurine, a cytokinin derivate (6-BAP;

cytokinin) increased the activity of the antioxidant enzymes CAT and APX and led to a reduction of hydrogen peroxide as well as to a delay of senescence (Zavaleta-Mancera et al., 2007). Authors suggested that externally applied CKs may be involved in protecting cell membranes and the photosynthetic apparatus of plants from oxidative damage during the delay of senescence in the dark (Hönig et al., 2018; Zavaleta-Mancera et al., 2007).

2.1.3 Biosynthesis of cytokinin

CK plays a major role in the regulation of several developmental processes, including chloroplast biogenesis, seed development, growth, root-, shoot- and, inflorescence branching, nutrient balance, stress tolerance and leaf senescence (Gupta et al., 2020). Naturally occurring CKs are purine based molecules that can be substituted at the N-6-position of the adenine ring either by an isoprene side chain (ISRKs) or an aromatic core (ARCK; Bielach et al. 2017; Hönig et al. 2018). In plants, the most common group of ISRKs comprising an isoprenoid side chain, include isopentenyladenine (iP)-, trans-zeatin (tZ)-, and cis-zeatin (cZ; Mok and Mok 2001). ARCKs which contain an aromatic side chain, include 6-BAP, 6-furfurylaminopurine, also known as kinetin (Kin) and methoxylated derivates of 6-BAP, called topolins (Strnad, 1997). The cytokinin 6-BAP and Kin are not considered naturally occurring CKs, but due to their strong effect on delaying senescence in plants via exogenous applications in numerous studies, these molecules formed the basis of the first generation of synthetic ARCKs (Plíhalová et al., 2016). The biosynthesis of CKs depends on the precursor isopentenyladenine which is catalysed by ATP/ADP IPT. Followed by cytochrome P450 monooxygenases CYP735A1 and CYP735A2, identified from *Arabidopsis thaliana,* which catalyse the hydroxylation of isopentenyladenine-type CKs (Sakakibara, 2006; Takei et al., 2004). Enzymes from the LONLEY GUY (LOG) family convert N6-modified adenosine monophosphate derivates into active CKs. The degradation of CK in turn, is catalysed by CKX enzymes and present an irreversible degradation by oxidative side chain cleavage (Frébort et al., 2011). The regulation of the CK biosynthesis gene IPT is also used by plants to trigger a hypersensitive response (HR) during fungal attacks. HR is characterised by programmed cell death (PCD) occurring near infection sites to prevents biotrophic pathogens from accessing nutrient therefore limiting the spread of the infection (Novák et al., 2013).

2.1.4 The role of cytokinin in plant/pathogen interactions

Many plant pathogens have the ability to secrete CKs or to induce CK production in host plants (Gupta et al., 2020). The ability to secrete CKs was found in several cereal and grass fungal pathogens such as *Claviceps pupurea* (Hinsch et al., 2015), *Colletotrichum graminicola* (Behr et al., 2012), *Ustilago maydis* (Bruce et al., 2011), *Magnaporthe oryzae* (Chanclud et al., 2016) and *Fusarium pseudograminearum* (Sørensen et al., 2018). The detailed production and perception of cytokinin in fungi has been recently extensively reviewed by Anand et al. (2022). Findings to date suggest that fungal induced CK signalling favours biotrophic pathogens as it may help hijacking plant defence systems, appears to promote fungal virulence and because biotrophs derive their nutrients from living host cells (Anand et al., 2022). Van der Plank (1968) defined virulence as the capacity of a pathogen to infect a particular host genotype, while the American Society of Phytopathologist adapted the term virulence as the degree of damage caused to the host (Sacristán and García-Arenal, 2008). CKs can delay senescence by limiting the oxidative burst and sustaining photosynthetic activity and in accordance to this role, CKs appear to be accumulated in “green islands” which are photosynthetically active zones surrounding pathogenic lesions caused by biotrophic pathogens (Chanclud et al., 2016; Walters and McRoberts, 2006). Spores of powdery mildew and rust diseases contain high levels of cytokinins and were proposed to be the root cause of the green island formation associated with the development of these diseases (Walters and McRoberts, 2006). Moreover, CKs were also suggested to mobilise nutrients towards infection sites by increasing the host invertase activity which alongside of fungal invertase activity, would lead to a strong nutrient sink (Walters and McRoberts, 2006). Despite the presence of the genetic material required for CKs biosynthesis, most necrotrophic fungi do not produce CKs (Chanclud and Morel, 2016). However; an increase of CK content in detached host tissue was observed after infection by the necrotrophic pathogens *Pyrenophora teres* and *Drechslera maydis*, the agents responsible for tan spot and southern corn leaf blight in barley and maize, respectively (Angra-Sharma and Sharma, 2000). Interestingly, CK levels were lower in resistant barley and maize detached leaf experiments upon infection and steadily decreased with time of incubation (Angra-Sharma and Sharma, 2000). Gupta et al. (2020) showed that CKs applied via soil drench can also act systemically to induce immunity to foliar infection by *Botrytis cinera* in tomato plants. Both exogenously applied CK and endogenously manipulated CK by using mutants that exhibited increased CK levels, led to the induction of ET biosynthesis, ion leakage and induced the expression of defence genes such as the pathogenesis related 1 (PR1) PR1a and PR1b genes in tomato (Gupta et al., 2020). Similarly, the treatment of rice plants with high levels of CK led to increased defence responses against the biotrophic fungus *Magnaporthe oryzae*, the agent responsible for rice blast disease (Jiang et al., 2013). Work in *A. thaliana* is supporting these findings and furthermore, it appears that CK-induced immunity partially relies on content and signalling of salicylic acid

(SA; Albrecht and Argueso 2017). In the *A. thaliana* mutants *npr1* (*NON-EXPRESSOR OF PR-1*) which is impaired in the master regulator of SA signalling, a lack of CK-induced immunity was observed (Choi et al., 2010). It was further supported by the findings that CK-treated plants showed enhanced SA-dependent gene expression upon pathogen perception, suggesting a priming activity of CK (Albrecht and Argueso, 2017; Choi et al., 2010). Controlled inoculation experiments have shown that CK-treated spring barley plants exhibited lower RLS symptom development than control plants (Burrell, 2021; unpublished). Therefore, the impact of CK on RLS symptom development and fungal growth *in planta* was investigated in a more comprehensive study to understand whether delaying leaf senescence in barley plants would prolong the endophytic stage of *R. collo-cygni*. Furthermore, the contrary was investigated by using nitrogen-starvation to induce early senescence in barley plants. Taken together, this study will examine the relationship between senescence and RLS development in barley seedlings grown under controlled conditions.

2.1.5 Aims and Objectives

- Delaying leaf senescence via exogenous foliar application of cytokinin and assessing the impact on RLS symptoms development and *R. collo-cygni* growth.
- Manipulating the leaf N status prior to *R. collo-cygni* infection and assessing fungal colonisation and RLS symptoms development.
- Monitoring physiological traits during the entire *R. collo-cygni* infection process in both experiments.

2.2 Material and Methods

2.2.1 Plant material used in this study

Throughout this chapter RLS susceptible spring barley cultivar (cv.) Fairing was used. Seeds were placed in two rows on dampened, absorbent paper with the root primordia pointing downwards to ensure rectilinear growth of the roots and shoots. The dampened absorbent paper was folded and stacked upright in a 1L beaker containing 100 mL distilled water and wrapped with a transparent zip bag to ensure high humidity and to prevent the papers from drying out. A total of thirty seed stacks were prepared, each stack containing 60 seeds which were kept in the dark at 4°C for 36 hours to induce an even germination rate. Subsequently, seeds were kept in the dark for four days at 20°C.

2.2.2 Growing Conditions

After four days in the dark, plants were subsequently transferred into 20.5 cm x 15.4 cm x 5.2 cm trays containing vermiculite. Vermiculite was watered with 500 ml of a modified Hoagland solution to provide all seedlings in this experiment with homogenous nutrient supply. The modified nutrient solution was made up as according to the micronutrient- and macronutrient solution found in Table 2.1 and Table 2.2 Table respectively. Two trays were placed in a big tray watered with additional 500 ml distilled water. Seedlings were grown for five weeks in MC1000 high specification climate chambers (Snijders, Tilbourg, Netherlands) with a photoperiod of 16h light (220 μmol m^{-2} s^{-1})/8h dark and a temperature of 18°C/12°C day/night, respectively. Relative humidity was kept at 90% throughout the day/night cycle. Barley seedlings were watered with 10ml/plant nutrient solution every second day.

Table 2.1: Composition of the modified Hoagland micronutrient solution used to grow plants.

Micronutrients	Chemical Name	Concentration
H_3BO_3	Boric acid	30 µM
$MnSO_4 \bullet 4H_2O$	Manganese (II) sulphate	5 µM
EDTA Na-Fe	Iron (III)-EDTA-sodium salt	5 µM
$CuSO_4 \bullet 5H_2O$	Copper (II) sulphate	0.3 µM
$ZnSO_4 \bullet 7H_2O$	Zinc sulphate	0.7 µM
$Na_2MoO_4 \bullet 2H_2O$	Sodium molybdate dihydrate	0.1 µM

Table 2.2: Composition of the modified Hoagland macronutrient solution used to grow the plants.

Macronutrients	Chemical Name	Concentration
KH_2PO_4	Monopotassium phosphate	1 mM
K_2SO_4	Potassium sulphate	1.5 mM
$MgSO_4\,7H_2O$	Magnesium Sulphate, heptahydrate	2 mM
$CaCl_2\,2H_2O$	Calcium chloride, dihydrate	3.5 mM
NH_4NO_3	Ammonium nitrate	4 mM

2.2.3 Fungal material used in this study

The isolate of *R. collo-cygni* used in this study was isolated from infected spring barley leaves collected from field trials performed in 2019 at the SRUC trial site at Drumalbin farm, Lanarkshire, using the method described by Frei, 2012. The isolate *Rcc*-SC19-B5 was used for all inoculation experiments.

2.2.4 Barley seedling inoculation with *Ramularia collo-cygni*

Isolate *Rcc*-SC19-B5 was grown for two weeks in the dark at 18°C on potato dextrose agar (PDA; Sigma, Dorset, UK) containing 5 µg mL^{-1} streptomycin. Liquid cultures were prepared by adding two ground 1 cm^2

mycelial plug to 250 mL of potato dextrose broth (PDB, Sigma, Dorset, UK). Cultures were incubated for ten days in the dark at 18°C under constant agitation at 125 rpm. Inoculum was prepared by homogenising the culture in a disperser (IKA, Munich, Germany) for 2.5 minutes and then adding one drop of Tween20 (Sigma, Dorset, UK) per 50 ml of inoculum. Negative controls were achieved by mock-inoculating plants using PDB as inoculum instead of fungal mycelial suspension. Two-week old seedlings were inoculated using an artist's air brush (Clarke Wiz Air®, Clarke International, Essex, UK) at a rate of 0.5 mL of inoculum per seedling. To ensure an even leaf coverage, seedlings were sprayed from every direction. Inoculated seedlings were then placed in plant propagator (50cm×40cm×5cm) covered with a clear plastic lid. Prior to closing the lid, each tray of inoculated plants was given an additional 1 L of nutrient solution. To ensure maximum humidity, lids were sealed and incubated in constant dark with a temperature regime of 18°C for 16 hours and 12°C for 8 hours. The light regime was restored after three days post-inoculation (dpi) and after five dpi the lids were removed.

2.2.5 Treatments to manipulate host senescence

2.2.5.1 Delaying physiological leaf senescence by using exogenously applied cytokinin

Host cytokinin levels were manipulated by exogenous application of cytokinin to delay age related leaf senescence in barley cv. Fairing plants and determine the effect on RLS development as well as fungal growth. Plants were grown as described in Section 2.2.2 and inoculated with *R. collo-cygni* as described in Section 2.2.4. Seven days post inoculation 0.5 mL/plant of 0.1 mM exogenous foliar 6-BAP (cytokinin) and sterile distilled water (SDW) as a control respectively were applied using an artist's air brush (Clarke Wiz

Air®, Clarke International, Essex, UK). Treatments were applied both to *R. collo-cygni*-inoculated and non-inoculated plants to assess the impact of treatments to delay leaf senescence relative to untreated plants on RLS development. The development of RLS was assessed visually as described in 2.2.7.1 between 10 and 28 dpi. *In planta* colonisation by *R. collo-cygni* (2.2.7.2*)* during RLS development was assessed between 5 and 28 dpi. To assess the relationship between symptom development and physiological-related senescence, the relative chlorophyll content as described in 2.2.7.3 was monitored between 7 and 28 dpi every second day. Additionally, the leaf nitrogen content was monitored at 5, 7, 10, 14, 21 and 28 dpi, as described in 2.2.7.4.

2.2.5.2 Nitrogen starvation to induce leaf senescence

Plants were grown as described in 2.2.3 except in this experiment two nutrient solutions differing in their nitrogen concentration were prepared as described in Table 2.1 and Table 2.2 each containing the same micronutrients. Ammonium nitrate was the only nitrogen source used in the macronutrient nutrient solution which permitted the manipulation of nitrogen concentration without changing any other nutrient sources. For a moderate nitrogen (+N) supply 4 M ammonium nitrate was used and for no nitrogen (-N) no ammonium nitrate was added to the macronutrient solution to ensure total nitrogen starvation. After 14 days (Figure 2.3) barley seedlings were inoculated as described previously (Section 2.2.4). After restoring the light regime, plants were watered every two days with 200 mL of the respective (+N, -N) nutrient solution. RLS symptoms development was assessed visually between 5, 7, 10, 12, 15, 18 and 21 dpi and is described in 2.2.7.1. Additionally, *in planta* colonisation by *R. collo-cygni (*Section 2.2.7.2*)* during RLS development and the relative chlorophyll content (Section 2.2.7.3) as well as the leaf nitrogen content (Section 2.2.7.4) was monitored at 5, 7, 10, 12, 15, 18 and 21 dpi.

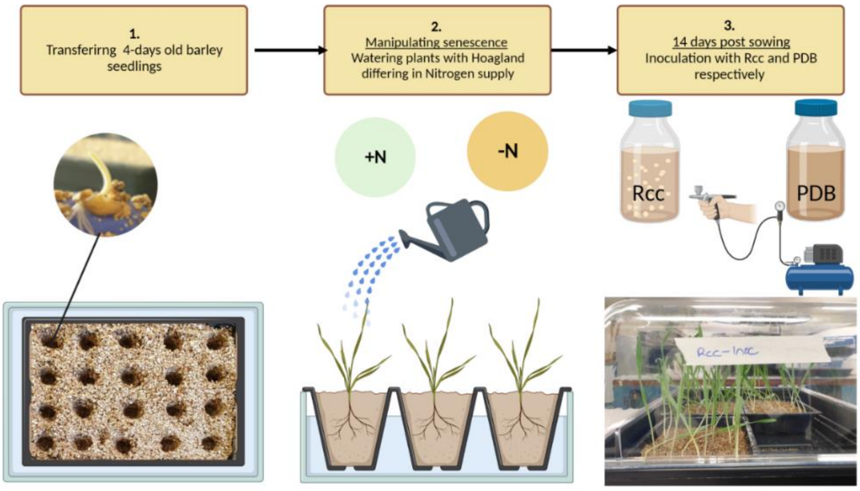

Figure 2.3: Examining the link between leaf nitrogen content and Ramularia leaf spot development.
Figure highlights the experimental flow from 1.: Transferring four-days old barley cv. Fairing seedlings; 2.: Manipulating host senescence by using two different nitrogen regimes (N0=0 ammonium nitrate; N1=4 M ammonium nitrate); to 3.: Seedling inoculation of +*Rcc*/+Mock with the aim to assess the impact on Ramularia leaf spot development, both independently and in combination.

2.2.6 Cytokinin assay on Ramularia collo-cygni growth in vitro

An *in vitro* assay was designed to investigate the effect of 6-BAP application on *R. collo-cygni* growth. 14 days old *R. collo-cygni* isolates of SC19 B5 were tested on PDA (Sigma, Dorset, UK) containing a 10-fold serial dilution of 0.1 mM, 0.01 mM and 0.001 mM 6-BAP, respectively. Stock solutions of 10 mM 6-BAP were prepared in a 1% 1M HCl solution (Sigma, Dorset, UK) in SDW and diluted down to 1 mM and 0.1 mM stock solution in SDW. The assay was carried out in 12-well plates (Thermo Fisher Scientific, Leicestershire, UK) containing 5 mL of PDA. Agar was allowed to cool down before 6-BAP stock solutions were added into the cooled down agar. Once the PDA solidified, a mycelial plug was added. Plates were closed, sealed with Parafilm (Amcor, Zurich, Switzerland) and incubated for 21 days at

18°C in the dark. Negative control consisted of SDW containing 1% of 1M HCl (Sigma, Dorset, UK). Fungal radial growth was monitored weekly over a course of three weeks and pictures were taken using Essential V6 imaging system (Uvitec, Cambridge, UK). Fungal radial growth was quantified using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). Data shows the average of three mycelium plugs ± standard error.

2.2.7 Evaluation of *Ramularia collo*-cygni symptom development and host physiology in barley seedlings

2.2.7.1 Visual assessment of Ramularia leaf spot

RLS symptom development was assessed to monitor the process of infection, from the asymptomatic growth phase to the necrotrophic phase of Ramularia. Visual assessments were conducted on the prophyll leaf of barley seedlings and disease severity was estimated as the percentage of leaf area covered by RLS. Data shows the mean ± standard error ribbon calculated in the linear mixed-effect model (LMEM) on three independent inoculation experiments comprising of 12 biological replications each.

2.2.7.2 Ramularia collo-cygni DNA quantification

In planta colonisation by *Rcc* during RLS development was assessed by taking via qPCR. Each biological replicate consisting of two prophyll leaves were snap-frozen in liquid nitrogen. Total *R. collo-cygni*-DNA was extracted based on the method described by Edwards et al., 1991. In detail, 7.5M ammonium acetate (Sigma Aldrich, Darmstadt, Germany) was used to precipitate proteins and ice-cold isopropanol (Fisher Scientific Ltd, Leicestershire, UK) was used to precipitate the DNA. Extracted DNA was washed with 70% ethanol and incubated in DNA/RNA free water over night.

DNA was then quantified, and quality evaluated using the Denovix Ds-11 Spectrophotometer (Cambridge Bioscience). DNA was diluted down to 20 ng/μL and *R.collo-cygni* DNA was quantified by qPCR as described by Taylor et al. 2010. Each biological replicate consisting of two leaves was quantified at least as a technical duplicate. Data in the delayed leaf senescence experiment shows the average ± standard error ribbon calculated in the LMEM on three independent inoculation experiments each consisting of six biological replicates, quantified in technical triplicates. Data in the N-experiment shows the mean ± standard error ribbon calculated in the LMEM on three independent inoculation experiments each consisting of two biological replicates of six leaves each (6 biological replicates in total).

2.2.7.3 Measurement of the relative chlorophyll content

In order to monitor the onset of senescence in the barley seedlings during RLS development, Soil Plant Analysis Development (SPAD) was taken as a measure of chlorophyll content using the portable SPAD reader FK-YL02 (Shandong Fangke Instrument Co., Ltd., China). Two SPAD measurements were taken on the adaxial side of the prophyll leaf of 21-day-old barley seedlings for each biological replicate. Data shown in this chapter shows the mean ± standard error calculated on 12 prophyll leaves from three independent experiments and are presented as the LMEM returned predictive values taking all responses and predictors into account.

2.2.7.4 Measurement of leaf nitrogen content

In addition to SPAD measurements, Carbon and Nitrogen (CN) analysis was performed to determine the leaf nitrogen content. Barley prophyll leaves were oven dried at 60°C for 48 hours and milled on a Qiagen TissueLyser LT® for 30min. Subsets of 15 mg ± 1μg for each sample were weighed using a Mettler Toledo microbalance prior to transferring into

8x5mm tin capsules (CE instruments). Two technical replicates per biological replicate were prepared and CN analysis was performed using an elemental microanalyzer (Thermo Fisher Scientific Flash SMART). Data in the delayed leaf senescence experiment represents the LMEM mean ± standard error ribbon calculated based on three independent experiments, each consisting of two biological replicates, each being run in technical duplicates. Data in the N- experiment shows the average ± standard error calculated from four technical replicates of two biological replicates from a single inoculation experiment.

2.2.8 Statistical Analysis and Visualisation

Data in this chapter were analysed in the open-source R software using version 4.2.3 (RStudio, 2020). Unless stated otherwise, all data were analysed using the LMEM) analysis via the restricted maximum likelihood algorithm (REML) in the "lme4" package (Bates et al., 2015). A LMEM regression was fitted for each measured response (Symptoms, *Rcc*-DNA, SPAD) using time and treatment as predictors and their interaction as fixed effects.

In the leaf nitrogen datasets of this study, the treatment predictors in the LMEM were separated into *R. collo-cygni*-inoculated and non-inoculated plants as well as separated by method to induce or delay senescence respectively. First, a two-way interaction LMEM was performed, however the interaction between time and treatments were not significant, while the main effects were highly significant. Therefore, the reason for the significance in the main effect was untangled to better understand the relationships and a three-way LMEM wa conducted on the dataset. The number of each independent experiment was the random effect in all LMEMs.

In the case of the Leaf N content assessment under nitrogen starvation, samples were only collected in the last independent inoculation experiment and therefore, for this dataset a linear regression model instead of the LMEM was used to calculate the predicators as no random effect had to be taken into account. Linear regression with response = Leaf N and predictors = time, inoculation (+*Rcc*/-*Rcc)* as well as nitrogen (-N/+N) was used to calculate the main effects to better understand the impact of the fungal inoculum on Leaf N.

The p-values in this chapter were calculated based on the models estimate by calculating the two tailed p-value (2*(1- pnorm(abs(estimated*t.value)) which gives the upper and lower tail of the normal distribution. The models' assumptions were checked each time by testing for homoscedasticity by comparing residuals to the fitted items and a QQ-plot was used to assess if the residuals were normally distributed. All graphs were performed using ggplot2 (Wickham, 2011).

2.3 Results

2.3.1 Investigating the effect of leaf senescence manipulation on Ramularia leaf spot development

2.3.1.1 Evolution of the relative chlorophyll content of *R. collo-cygni*-inoculated barley cv. Fairing post application with cytokinin

The onset of leaf senescence was monitored during RLS disease development using SPAD readings that represent the relative chlorophyll content in cv. Fairing, which were monitored for three weeks post application of 6-BAP (cytokinin). First SPAD readings were taken on the day of cytokinin treatment and time points are therefore indicated as days post treatment (dpt). A significant difference in SPAD readings was observed at 0 dpt between the non-inoculated (Mock) plants and *R. collo-cygni*-inoculated (I) plants (Figure 2.4). No significant difference was observed between the cytokinin treated (+CYT) and untreated (-CYT) plants non-inoculated (Mock) at 0 dpt. The non-inoculated plants exhibited SPAD values of 42.66 ± 3.97 (p-value: 1.07e^{-07}) and 43.77 ± 3.97 (p-value: 3.43e^{-11}) for cytokinin treated (Mock+CYT) and untreated plants (Mock-CYT), respectively (Figure 2.4). Over time the relative chlorophyll content decreased in both treatments (+CYT and -CYT) as time had a significant effect on the relative chlorophyll content (p-value: 0). Non-inoculated, CYT-treated plants (Mock+CYT) exhibited the slowest decrease in relative chlorophyll content over time compared to non-inoculated, untreated plants (Mock-CYT), indicating a delay in leaf senescence or a slowing down of the rate of senescence (Figure 2.4). Plants inoculated with *R. collo-cygni* exhibited SPAD values at 0 dpt of 38.60 ± 3.17 and 38.13 ± 3.12 for cytokinin treated (I-+CYT) and untreated plants (I-CYT), respectively (Figure 2.4). No significant differences were observed between cytokinin treated and untreated plants inoculated with *R. collo-cygni* at 0 dpt. Over time the relative chlorophyll content decreased in all treatments as time had a significant effect on the relative chlorophyll content (p-value: 0). Overall, the relationships between time and treatments on SPAD were significantly different from each other, with the I+CYT treated plants exhibiting the biggest effect with a decrease of 1.24 ± 0.12 per unit of time in SPAD (p-value: $6.93e^{-06}$). Moreover, both *R. collo-cygni*-inoculated plants (I+CYT; I-CYT) exhibited a faster rate of decline compared to the control plants (Mock+CYT; Mock-CYT), with the I+CYT showing the fastest decline overall (Figure 2.4).

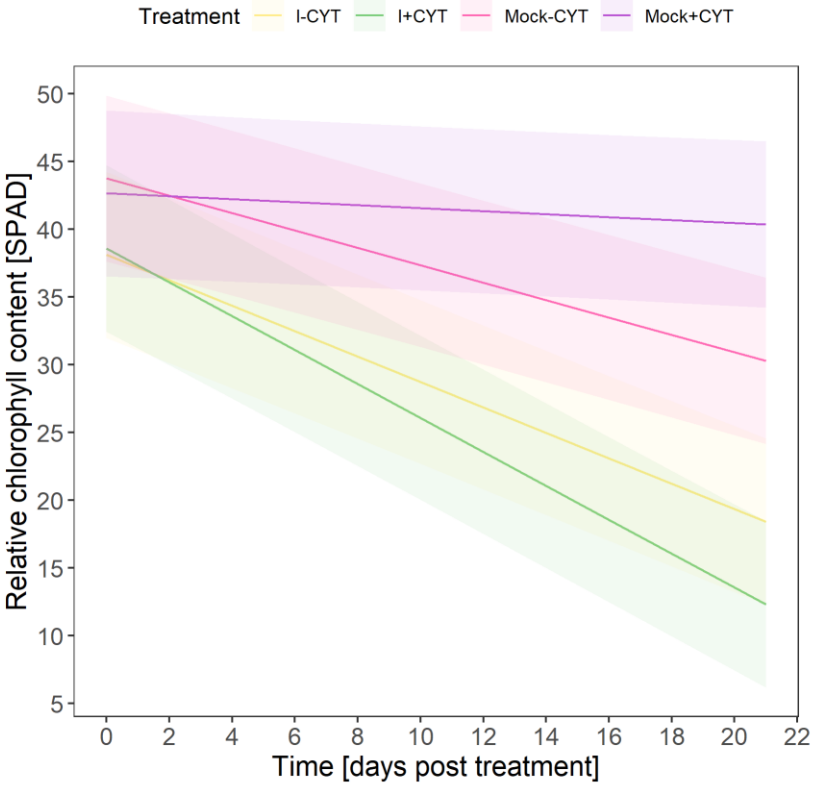

Figure 2.4: Relative chlorophyll content measured as SPAD units in *R. collo-cygni*-inoculated barley seedling cv. Fairing following foliar applications of cytokinin. LMEM of relative chlorophyll of *R. collo-cygni* inoculated plants (I+CYT: green; I-CYT: yellow) compared to non-inoculated plants (Mock+CYT: purple; Mock-CYT: pink) assessed via SPAD readings. Data represents mean ± standard error of three independent inoculation experiments, each representing 12 biological replicates.

2.3.1.2 Visual Ramularia leaf spot symptom development post cytokinin treatment

Assessments of RLS disease development were based on visual estimations of the percentage of leaf area covered by RLS symptoms. The first observation of "pepper spots" was made usually at 7 dpi when the 0.1 mM cytokinin treatment was applied (Figure 2.5 A). The typical reddish, brown lesions appeared after 10 dpi and increased continuously until 28 dpi regardless of treatment as time had a significant effect on symptom development (p-value: 0.0; Figure 2.5 B and 2.5 C). The interaction of time and cytokinin treatment was significant (p-value: $2.2e^{-16}$), with cytokinin treatment increasing the amount of symptoms seen over time. Symptoms developed faster in cytokinin treated plants compared to the non-treated plants (Figure 2.5.A). For every unit of time, the cytokinin treatment increases symptom development by 0.4 ± 0.03 % over the control treatment. The increase in RLS symptoms correlated positively in both treatments with a decrease in green leaf area (Figure 2.5 B and 2.5 C). This was accompanied by a faster decrease in green leaf area in cytokinin treated plants compared to non-treated plants. Moreover, it was noted that in the mock-inoculated plants treated with cytokinin did not undergo the same decrease in green leaf area compared to the mock-inoculated untreated controls, indicating that the cytokinin treatment delayed leaf senescence.

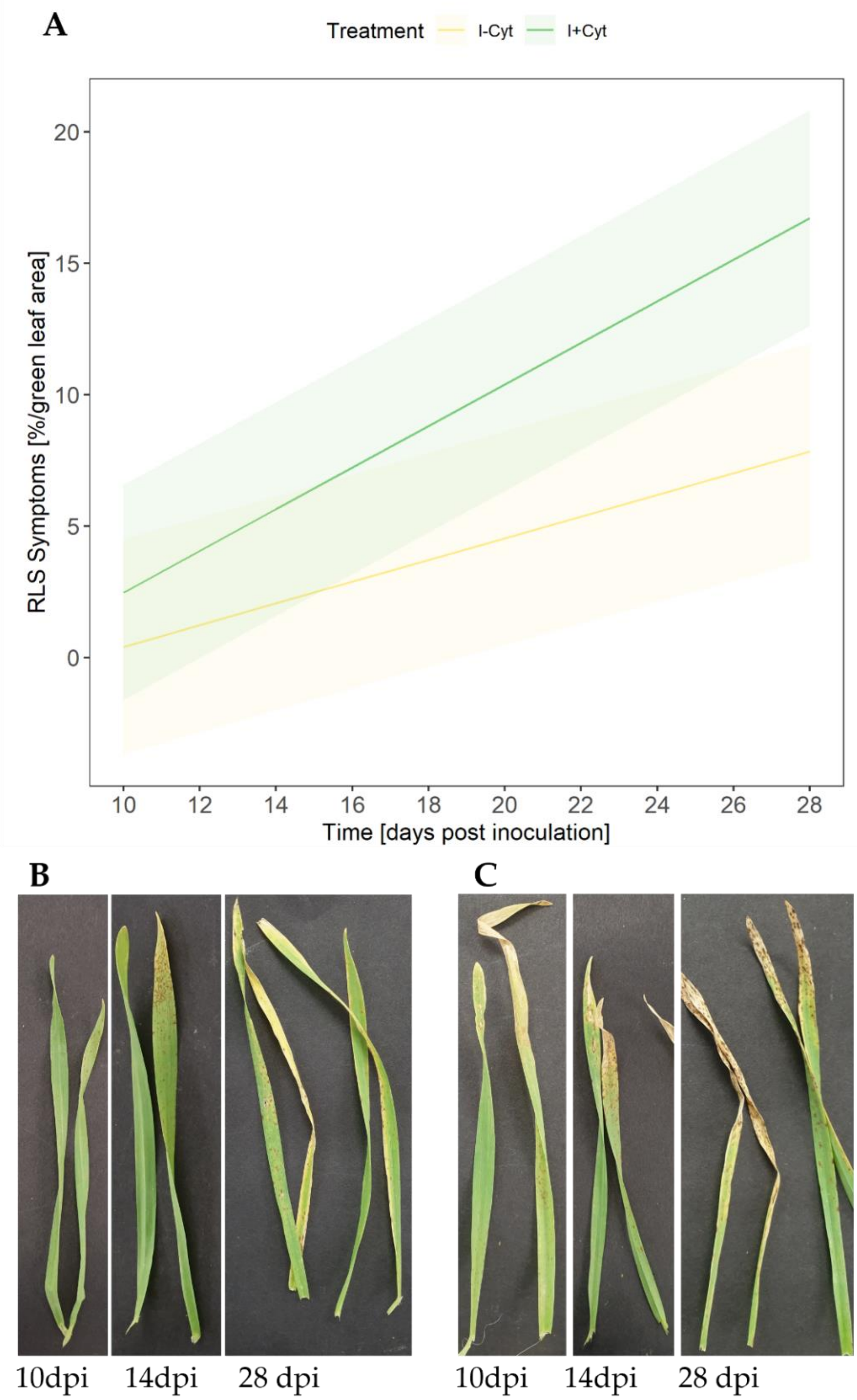

Figure 2.5: Effect of cytokinin treatment on Ramularia leaf spot disease development in barley seedlings cv. Fairing.

A: LMEM of symptom development (I+CYT: green; I-CYT: yellow). B: Plants were inoculated with *R. collo-cygni* (I) but did not receive cytokinin treatment (I-CYT, B). C: Plants were inoculated with *R. collo-cygni* (I) and treated with 0.1 mM cytokinin (I+CYT; C). Data represents three independent inoculation experiments, in which in total 36 prophyll barley leaves were assessed for RLS symptom development.

2.3.1.3 Fungal colonisation during disease development

To better understand the relationship between asymptomatic growth and necrotrophic disease development, *R. collo-cygni* DNA was quantified at six different time points during disease development (Figure 2.6). Although no symptoms were observed before 10 dpi, *R. collo-cygni* DNA was detected at 5 dpi amounting to 38.79 pg, suggesting a successful colonisation. After 10 dpi fungal DNA steadily increased until the end of the experiment at 28 dpi in cytokinin treated plants whereas it remained relatively constant in the untreated plants. The interaction was significant with cytokinin treatment significantly (p-value: $5.23e^{-09}$) increasing fungal DNA over time compared to the untreated plants. The amount of *R. collo-cygni* DNA increased by 6.87 ± 1.17 pg for every unit of time compared to the control treatment.

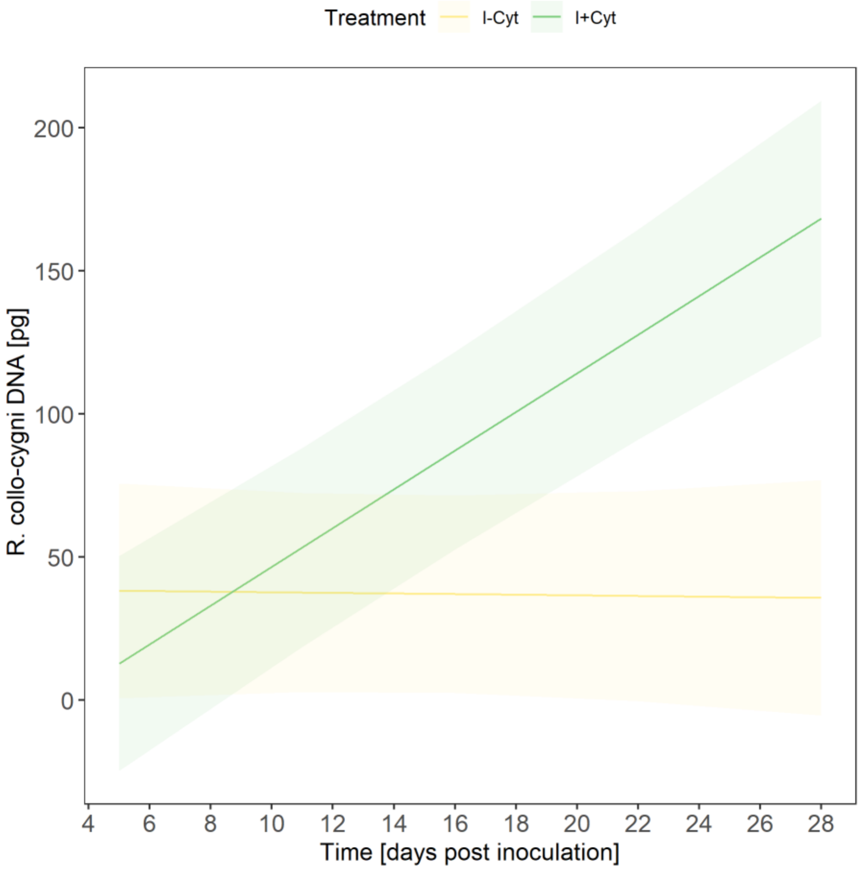

Figure 2.6: Quantification of *R. collo-cygni* DNA during RLS development in barley seedlings cv. Fairing.
Fungal DNA increased significantly over time in *R. collo-cygni*-inoculated (I) plants treated with 0.1 mM cytokinin (I+CYT: Green) compared to the untreated plants (I-CYT: Yellow). LMEM shows mean ± standard error of three independent inoculation experiments, consisting each of six biological replicates.

2.3.1.4 Leaf nitrogen content

The leaf N content was assessed in addition to the relative chlorophyll content at six different time points to better understand the relationship between RLS and age-related nitrogen mobilisation in barley plants. During the asymptomatic phase of RLS at 5 dpi the leaf N content was significantly higher (p-value: $2.99e^{-08}$) in non-inoculated plants (-Rcc) with a leaf N content of 4.99 ± 0.40 % compared to that of *R. collo-cygni*-inoculated plants (+Rcc) with a leaf N content of 4.46 ± 0.49 % (Figure 2.7 A). Over time, the leaf N content decreased under both treatments, with inoculated plants having the biggest effect on the decrease of leaf N. For every unit of time, *R. collo-cygni*-inoculated plants lost 0.53 ± 0.1 % leaf N. The cytokinin treatment did not influence the leaf N content as there were no significant differences (p-value: 0.5) in the relationship between treated (+CYT) and untreated (-CYT) plants ((Figure 2.7 B).

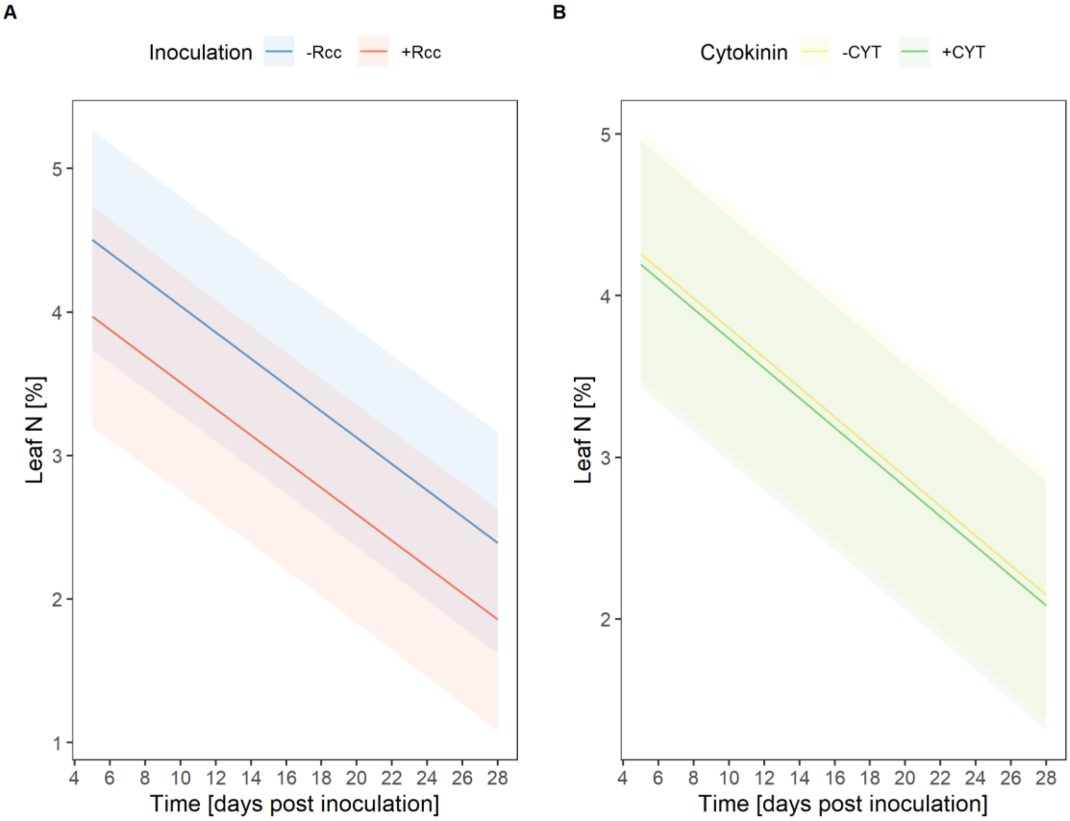

Figure 2.7: Evolution of leaf nitrogen content in barley seedlings cv. Fairing following *R. collo-cygni* inoculation (A) and cytokinin treatment (B).
A: LMEM separated into R. collo-cygni-inoculated (+Rcc; red) and mock-inoculated (-Rcc; blue). B: Pants either treated with 0.1 mM cytokinin (+CYT; green) or untreated (-CYT; yellow). Data shows mean ± standard error of three independent inoculation experiments, each representing two biological replicates.

2.3.1.5 The effect of cytokinin on the growth of *R. collo-cygni in vitro*

The effect of CYT was tested on *in vitro* grown cultures of *R. collo-cygni* to test whether the phytohormones treatments have a putative growth stimulating or inhibiting effect on the fungus. At 14 dpt, no significant (p-value: 0.65) differences on fungal were observed between CYT treated PDA compared to the untreated control (Figure 2.8). The growth of *R. collo-cygni* on 0.001 μM, 0.1 μM, and 10 μM CYT treated PDA amounted to 1.03 ± 0.068 cm^2, 1.05 ± 0.138 cm^2 , and 0.94 ± 0.082 cm^2 respectively compared to the control with 1.16 ± 0.149 cm^2 growth at 14 dpt. Altogether, it appears that CYT does not significantly impact the growth of *R. collo-cygni in vitro*.

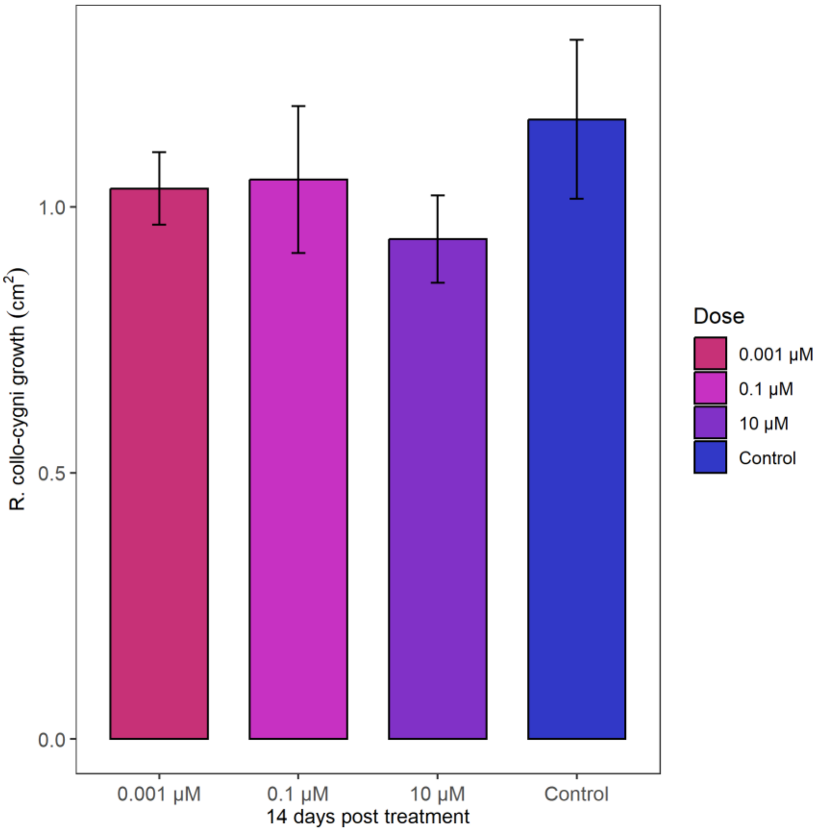

Figure 2.8: The effect of different cytokinin dosages on the growth of R. collo-cygni in vitro.
Bar plot represents the mean ± standard error of three measured *R. collo-cygni* mycelium in cm^2 via ImageJ after 14 days post treatment with 0.001 μM (magenta), 0.1 μM (pink), 10 μM (purple) and sterile distilled water (control: blue) respectively. No significant differences were observed between the treatments using the Kruskal-Wallis, non-parametric test.

2.3.2 Impact of nitrogen starvation on Ramularia leaf spot development

2.3.2.1 Evolution of relative chlorophyll content during Ramularia leaf spot disease development in barley grown under nitrogen starvation

The relative chlorophyll content was measured via SPAD in spring barley cv. Fairing during RLS disease development. Plants were monitored under nitrogen starvation (-N) and compared to control plants receiving a moderate nitrogen supply (+N). At 5 dpi the relative chlorophyll content was significantly affected by the nitrogen treatment as inoculated (I) and non-inoculated (Mock) plants grown under nitrogen starvation (I-N, Mock-N) exhibited lowest SPAD values compared to plants grown under normal nitrogen regime (I+N, Mock+N; Figure 2.9). Moreover, there was a significant difference observed between the non-inoculated plants grown under nitrogen starvation (Mock-N) compared to the plants grown under normal nitrogen regime (Mock+N) respectively, indicating that nitrogen starvation induced senescence in the control plants (Mock-N). Overall, the relative chlorophyll content decreased in all treatments as treatments had a significant effect on the relative chlorophyll content (p-value: 0). Plants inoculated with *R. collo-cygni* and grown with moderate nitrogen supply (I+N) influenced the relationship with SPAD and over time, exhibited significantly (p-value: $1.03e^{-06}$) lower relative chlorophyll content compared to non-inoculated plants (Mock+N). There was no significant difference observed between the nitrogen starved plants regardless of their inoculation status.

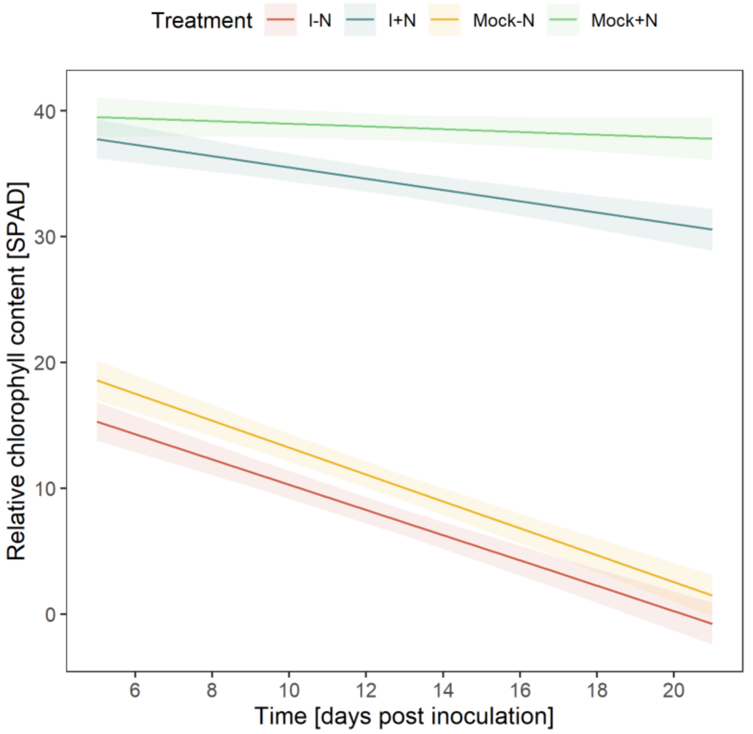

Figure 2.9: Evolution of the relative chlorophyll content in barley cv. Fairing grown under nitrogen starvation during Ramularia leaf spot development.
Data shown as Linear mixed regression of chlorophyll content that was assessed via SPAD measurements on *R.collo-cygni*-inoculated plants (I) as well as non-inoculated plants (Mock). Plants were either grown under nitrogen starvation (-N) or under normal nitrogen supply (+N) respectively. Data shows the mean ± standard error of three independent inoculation experiments, consisting each of 12 biological replicates.

2.3.2.2 Visual assessment of Ramularia leaf spot symptoms in barley plants grown under nitrogen starvation

RLS disease development was visually assessed as the percentage of leaf area covered by symptoms during the fungal infection process in spring barley cv. Fairing grown under different nitrogen conditions. Plants grown under moderate nitrogen supply were watered with a Hoagland solution containing 4 M of ammonium nitrate, whereas no nitrogen was supplied to plants grown under nitrogen starvation. The early signs of infection were observed at 7 dpi when small “pepper spots” became visible under both nitrogen regimes. Between 10 and 15 dpi the nitrogen starvation lead visually to a full loss of green leaf area compared to control plants (Figure 2.10 B-C). Typical symptom formation started at 15 dpi and increased over time in both treatments (Figure 2.10 A). The effect of nitrogen on symptom development was significantly higher under moderate nitrogen supply (I+N; blue) compared to nitrogen starvation (I-N; red). The interaction between time and nitrogen supply was highly significant, with I+N increasing symptoms by 0.54 ± 0.08 % (p-value: $4.59e^{-12}$) for every unit of time compared to I-N plants. Furthermore, visually observations revealed that plants under nitrogen starvation appeared very pale from the very beginning of the experiments (Figure 2.10 C), indicating that some form of nitrogen supply was perhaps required in this experiment for *R. collo-cygni* to transition into the necrotrophic growth phase. However, symptom development in control plants has been associated with a decrease of green leaf area (Figure 2.10 A-B). Together with the I-N observations it suggests that the fungus might induce the discolouration in barley plants.

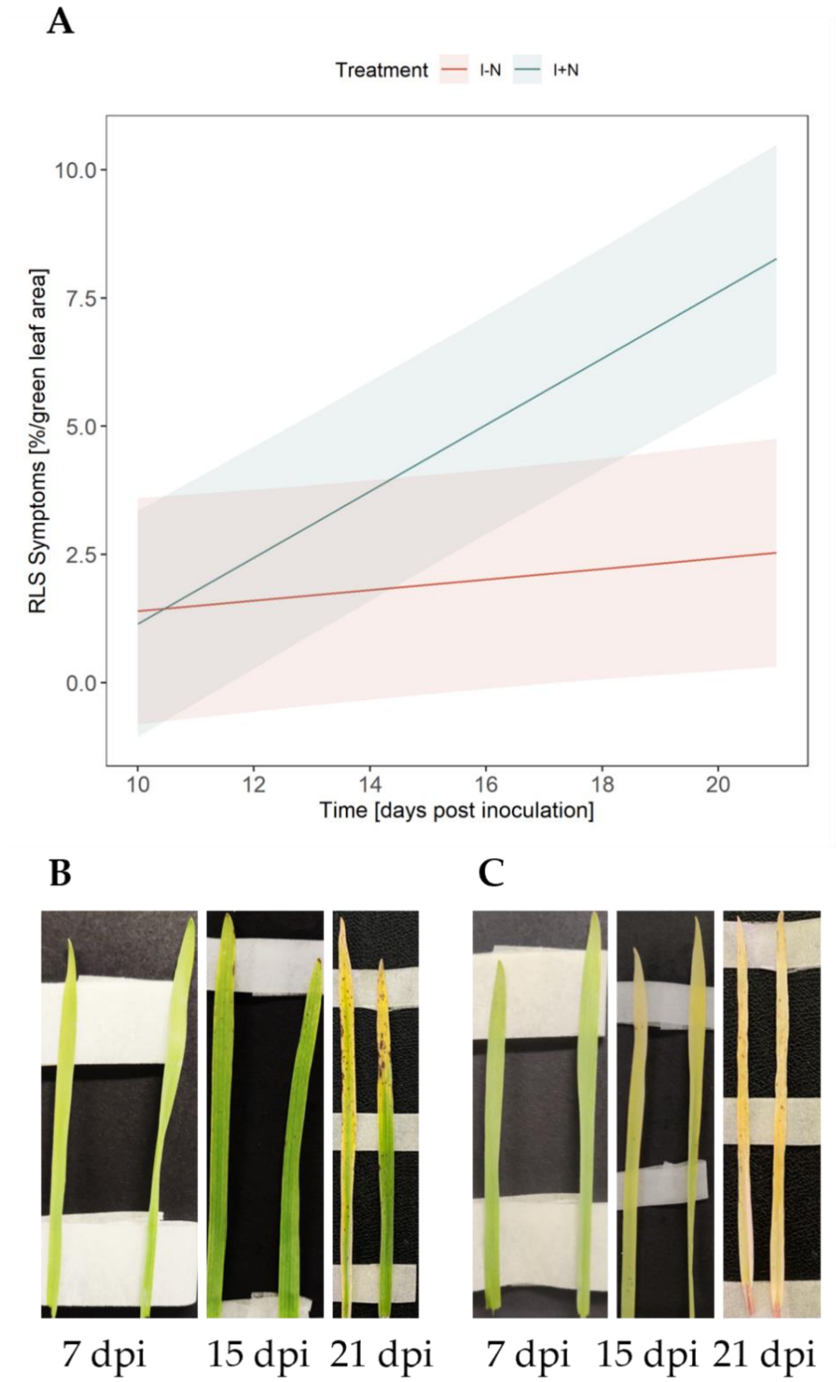

Figure 2.10: Visual assessment of Ramularia leaf spot symptoms development in barley seedlings cv. Fairing grown under different nitrogen regimes.

A: Disease symptoms development represented by the percentage of leaf area covered by RLS symptoms in barley seedlings grown under moderate nitrogen supply (I+N; blue) and nitrogen starvation (I-N; red). Data presented as LMEM of three independent inoculation experiments, each consisting of 12 biological replicates. B: Representative photographs of RLS symptoms observed on plants grown under moderate nitrogen supply. C: Representative photographs of RLS symptoms observed on plants grown under nitrogen starvation.

2.3.2.3 Fungal DNA quantification during Ramularia leaf spot development in plants grown under nitrogen starvation

R. collo-cygni DNA quantification was used to assess fungal growth during RLS infection and was monitored at six different time points post inoculation. Fungal DNA was present during the asymptomatic growth stage of *R. collo-cygni* and steadily increased over time regardless of the nitrogen supply. *R. collo-cygni* DNA in plants grown under nitrogen starvation increased significantly faster (p-value: $2.63e^{-03}$) than that observed in plants grown under moderate nitrogen levels (Figure 2.11).With every unit of time the inoculated plants grown under nitrogen starvation increased the fungal DNA by 27.26 ± 4.69 pg.

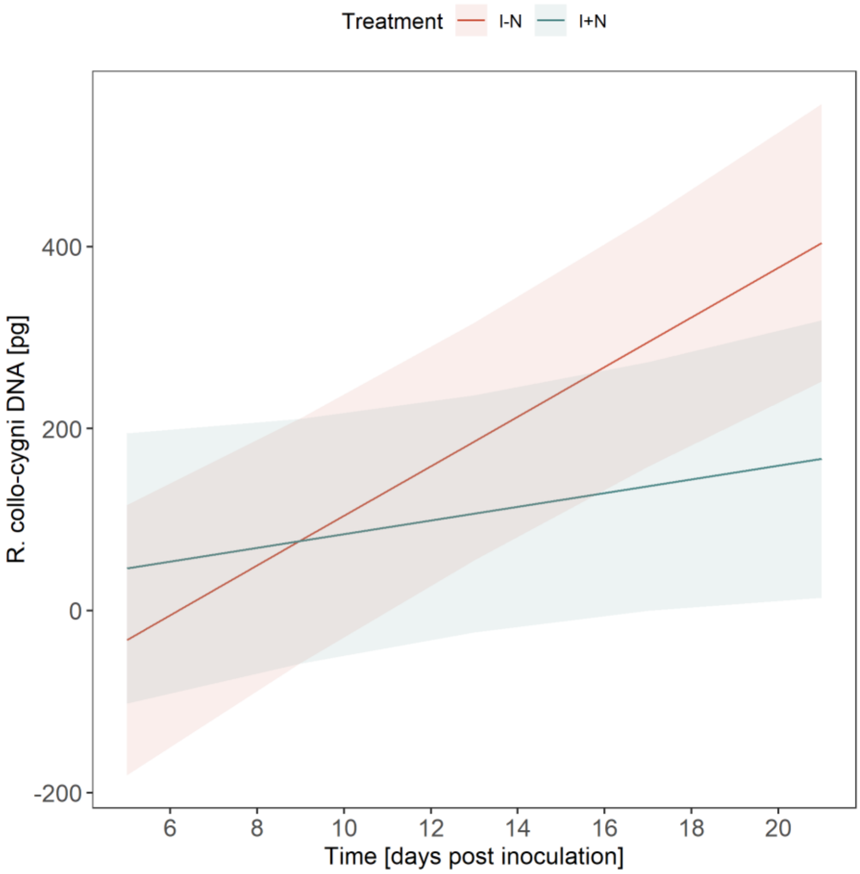

Figure 2.11: Quantification of *R. collo-cygni* DNA during Ramularia leaf spot development in barley seedlings cv. Fairing grown under nitrogen starvation.
Data represented as LMEM of *R. collo-cygni*-DNA levels over time in fungal inoculated (I) plants grown under plants grown nitrogen starvation (I-N: red) compared to control plants under normal nitrogen regime (I+N: blue). Data shown mean ± standard error of quantified fungal DNA of three inoculation experiments, consisting each of two biological replicates.

2.3.2.4 Evolution of leaf nitrogen content in plants grown under nitrogen starvation during Ramularia leaf spot disease development

The leaf N content was quantified via CN analysis at five different time points to better understand the relationship between RLS and age-related nitrogen mobilisation in barley plants. The amount of N started higher in plants that have been mock-inoculated with 2.28 ± 0.09 % leaf N compared to plants inoculated with *R. collo-cygni* with 1.91 ± 0.16 % leaf N. Overall, leaf N decreased steadily over time, however; *Rcc*-inoculated plants exhibited significantly lower leaf N (p-value: 5.40e^{-07}) throughout the experiment (Figure 2.12 A). This is supported by the observation that relative chlorophyll content was lower in inoculated plants compared to non-inoculated plants (Figure 2.4). Moreover, the effect of nitrogen starvation significantly decreased the leaf N content compared to control plants (Figure 2.12 B) suggesting a successful induced senescence by removing ammonium nitrate from the modified Hoagland solution.

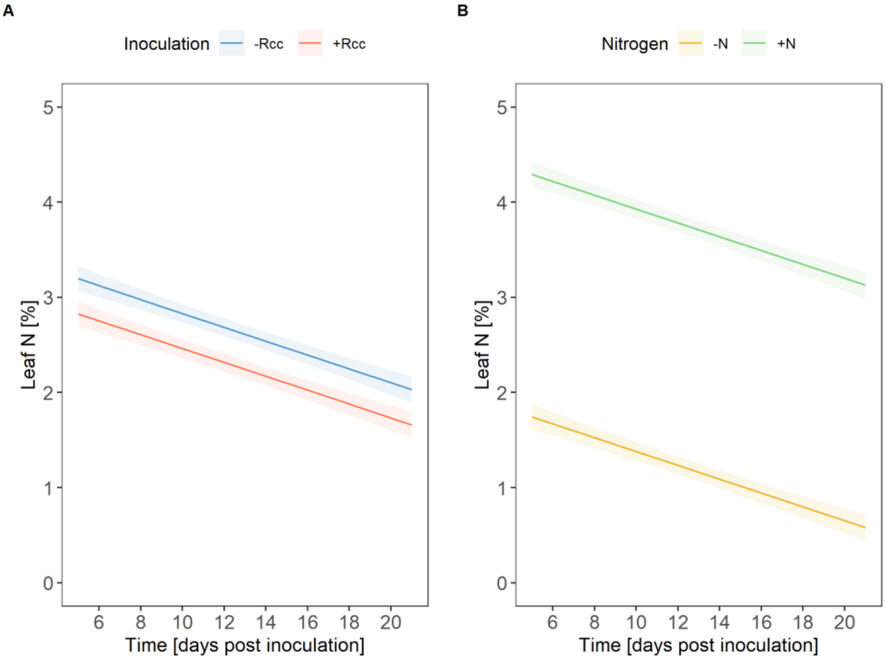

Figure 2.12: Evolution of leaf nitrogen content in barley seedlings cv. Fairing following R. collo-cygni inoculation (A) and nitrogen treatment (B).
Data shown as LMEM of leaf nitrogen content that was assessed via CN analysis. A: Plants following *R.collo-cygni*-inoculation(+Rcc) or non-inoculation (-Rcc). B: Plants grown nitrogen starvation (-N) or normal nitrogen regime (+N) respectively. Data represents the mean ± standard error of one independent inoculation experiment, consisting of 2 biological replicates.

2.4 Discussion

Key agronomic traits, including yield, nutrient-use efficiency and GPC are influenced by the timing and rate of senescence in the field (Distelfeld et al., 2014; Gregersen et al., 2013; Munier-Jolain and Salon, 2005). Monocarpic senescence in major crops such as wheat, rice, maize and barley is accompanied by nitrogen mobilisation, which forms the main components of nitrogen yield and grain nitrogen content (Distelfeld et al., 2014). Both abiotic stresses such as drought, high salinity, high temperature or nutrient deficiency and, biotic stresses can trigger monocarpic senescence (Bancal et al., 2008; Tan et al., 2023). Studies have often reported that foliar diseases accelerate or induce early senescence which is associated to yield losses due to the decrease in green leaf area (Bancal et al., 2016). However, not many studies distinguished between the development of local necrosis as a direct response to pathogen infection and the potential variability of leaf apical senescence as an integrated response of plants or response to the environment (Bancal et al., 2016).

The yield losses attributed to RLS infection due to reduction in green leaf area can range from an average of 20% to up to 70% (Walters et al., 2008). RLS symptoms generally appear late in the barley growing season, typically on older senescing leaves post-anthesis (McGrann and Havis, 2017). In some years, the fungus remains in its endophytic stage without causing disease epidemics (Havis et al., 2014). The unpredictable nature of RLS symptom development and subsequent disease epidemics has now been confounding barley growers and scientists for over 40 years (Dussart et al. 2020). The factors triggering *R. collo-cygni* to transition from its endophytic lifestyle to a necrotrophic one has been attributed to a combination of factors including abiotic stresses, the host genetics, as well as the processes involved in monocarpic senescence such as a loss of antioxidative capacity leading to the accumulation of ROS and senescence-induced nutrient mobilisation in the host (Huss et al., 2003; McGrann et al., 2014; McGrann and Brown, 2018; Schützendübel et al., 2008). In the field, it is known that a causal relationship exists between abiotic stresses, host physiological differences in elite spring barley cultivars and RLS symptom development as many abiotic and biotic factors influence crops during the growing season simultaneously. However; the relative importance and which roles each of these factors play independently, is not fully understood yet. The aims of this chapter were to investigate the impact of delaying leaf senescence on RLS symptom development and the impact of the delay in leaf senescence on host physiology and pathogen activity.

The aim to delay leaf senescence in seedlings of spring barley cv. Fairing was achieved by using exogenous application of 6-BAP (CYT; Figure 2.4). Early senescence was achieved by growing seedlings under N starvation and results on non-inoculated plants under N starvation demonstrate early senescence compared to plants grown under moderate nitrogen supply (Figure 2.9). The results in this study indicate that the delay in leaf senescence via exogenously applied CYT at 7 dpi leads to enhanced RLS disease symptom formation and increased *R. collo-cygni*-DNA levels compared to that observed in untreated plants (Figure 2.5 A and Figure 2.6). Congruently, induced early senescence resulted in reduced RLS symptom formation in *R. collo-cygni*-inoculated plants, however increased fungal DNA levels were observed (Figure 2.10 A and Figure 2.11). The present chapter gave contrasting results to the hypothesis that upon nitrogen starvation in spring barley plants are more susceptible to *R. collo-cygni* which could be promoted by the decline of antioxidant systems in the host (Schützendübel et al., 2008). The delayed senescence in barley was hypothesised to extend the endophytic stage of *R. collo-cygni* resulting in reduced symptom formation as observed in preliminary experiments performed by Clarinda Burrell, (2021). However, results in this study gave contrasting results to this hypothesis. RLS symptom development increased in response to delayed leaf senescence (Figure 2.5 A). The second stage of *R. collo-cygni* lifestyle is characterised as necrotrophic and, as seen in other necrotrophic pathogens, the fungus appears to induce premature senescence across both inoculation studies presented here (Figure 2.4 and Figure 2.9; Mengiste 2012). Furthermore, leaf N content decreased significantly upon *R. collo-cygni* infection, even in the plants grown under moderate nitrogen regime and those treated with CYT (Figure 2.7 and Figure 2.12). This suggests that premature leaf senescence was induced by *R. collo-cygni* as part of its infection strategy. This is supported by findings of Sjokvist et al. (2019) that observed an upregulation of TFs) associated with senescence during RLS development. The sister species, *Zymoseptoria tritici*, the causative agent for Septoria blotch of wheat, also triggers host senescence as part of its infection strategy (Bancal et al., 2016; Ma et al., 2018). Similar observation were made in the necrotrophic pathogen *Botrytis cinera* which induces host senescence as part of its infection strategy in tomato (*Solanum lycopersicum*) and *A. thaliana* (Swartzberg et al., 2008). Unlike the findings of the study presented here, the studies by Swartzberg et al. (2008) showed that *B. cinera*-induced disease severity was reduced in transgenic tomato plants that expressed delayed leaf senescence promoted by the expression of *IPT* under the control of the promotors *SAG12* and *SAG13* (Swartzberg et al., 2006). In winter wheat field trials, of genotypes exhibiting premature senescence of the culm, which is the joined stem of grass species, showed increased crown rot disease, caused by the necrotrophic pathogen *Fusarium pseudograminearum* compared to control genotypes (Knight et al., 2017). The findings on RLS symptom expression in this study (Figure 2.5 and Figure 2.10), however; appear to be more in line with findings on biotrophic pathogens, in which senescence is associated with increased disease resistance (Häffner et al., 2015). The wheat leaf rust 34 (*Lr34)* gene has been providing broad spectrum quantitative resistance to the biotrophic pathogens *Puccinia triticina* (causing leaf rust), *P. striiformis* (stem rust) and *Blumeria graminis* (powdery mildew) for over 50 years (Krattinger et al. 2009). *Lr34* encodes an ATP-binding cassette (ABC) transporter and is expressed in flag leaves during the grain filling stage (Krattinger et al., 2016, 2009). Based on results of Northern blot analysis of a barley gene *Hv40* that is upregulated during senescence, authors speculated whether *Lr34*-mediated resistance to rust might be the result of senescence-like processes (Krattinger et al., 2009). A second gene in wheat, *WHEAT KINASE START1* (*WKS1*), that confers partial resistance to *P. striiformis* is associated with accelerated senescence (Häffner et al., 2015). The *WKS1* resistance gene encodes a serine/threonine kinase and START lipid binding domains that is present in wild wheat cultivars, but not in hexaploid bread wheat (Gou et al., 2015). In the absence of *P. striiformis,* increased expression of *WKS1.1* in transgenic hexaploid wheat was associated with accelerated senescence of older wheat leaves and higher levels of H_2O_2 (Gou et al., 2015). McGrann et al. (2015) demonstrated that the overexpression of stress-responsive *SNAC1* TFs have led to reduced RLS symptom formation (Christiansen and Gregersen, 2014). *SNAC1* TFs are salt and drought responsive in barley and associated with the regulation of diverse developmental processes including senescence (Puranik et al., 2012). McGrann et al. (2015) hypothesised that resistance to RLS may be controlled by mechanisms controlled by *SNAC1* that are involved in tolerance to abiotic stress. In response to drought, plants accumulate ROS that lead to lipid peroxidation in the membrane of the chloroplast and protein degradation, which appears to play a role in premature leaf senescence in plants (Allu et al., 2014). Hoheneder et al. (2021) showed reduced RLS symptom levels in spring barley genotypes exposed to drought stress in field trials in Germany. However, McGrann and Brown (2018) found that abiotic factors leading to a slow breakdown of the chloroplast such as high light intensity and waterlogging led to enhanced disease severity in the susceptible cv. Braemar and Golden Promise, indicating that a gradual breakdown of the chloroplast and in turn, the antioxidant system promotes RLS symptom formation. McGrann and Brown (2018) also demonstrated that *R. collo-cygni* remained in the endophytic stage in experiments using *albostrians* and *bst1-7* mutants that completely lack chloroplast development and degrade chloroplasts, respectively. In the *bst1-7* mutant the level of fungal DNA was as high as in the wildtype cv. Bowman. These results are reminiscent of the findings in this study where *R. collo-cygni* DNA was steadily increasing in the plants grown under N starvation compared to plants grown under moderate N regime (Figure 2.11). Examining molecular processes of senescence that appear to determine either resistance or susceptibility to RLS disease development, will help to gain a better understanding of this relationship. Moreover, understanding how abiotic stresses affect the onset of senescence, could help understand the factors responsible for the increased suceptibility in delayed senescence plants and increased resistance under N starvation respectively.

In this study, no growth-promoting effect of cytokinin was observed on *R. collo-cygni* during *in vitro* growth on PDA (Figure 2.8). Cytokinins (CK) are not only known to be involved in delaying leaf senescence in plants, but are also associated with increased pathogen growth in plant/pathogen interactions, which is also referred to as cytokinin-induced suceptibility (Albrecht and Argueso, 2017). In *A. thaliana* plants, the exogenous application of 0.1 μM of benzyl adenine, an aromatic CK derivate, resulted in increased susceptibility to the oomycete *Hyaloperonospora arabidopsidis* (*Hpa*). However; increased resistance to *Hpa* was observed using a concentration of 10 μM of benzyl adenine (Argueso et al., 2012). Babosha (2009) also observed a multiphasic dose-response relationship between *Blumeria graminis* and wheat seedlings in response to exogenous application of zeatin, an isoprenoid cytokinin derivate, suggesting a dose dependent immune response.

Albrecht and Argueso (2017) attributed the phenomenon of CK-induced susceptibility to increased levels of biologically active CK in plants. Interestingly, plant pathogens have the ability to produce active CK and it has been demonstrated that fungal CK can act as virulence factors (Anand et al., 2022; Chanclud et al., 2016; Hinsch et al., 2015). For instance, the rice blast pathogen *M. oryzae* produces CKs *in vitro* and CKs act as defence signals in rice leaves to mobilise nutrients such as sugars, increase levels of photosynthesis, and activate SA-mediated immune responses (Jiang et al., 2013). Rice leaves inoculated with the *cks1* mutant isolate of *M. oryzae*, which is impaired in CK biosynthesis, exhibited fewer lesions compared to those observed on plants inoculated with the wildtype isolate (Chanclud et al., 2016). The *cks1* isolate was not affected in its development or asexual reproduction and *cks1* virulence was restored by exogenously applying a solution of cis zeatin, an isoprenoid CK derivate (Akhtar et al., 2020; Chanclud et al., 2016). These results suggest a role for fungal CKs acting as effectors to inhibit defence responses and to mobilise nutrients (Chanclud et al., 2016). The biosynthesis of CK via the tRNA pathway is well conserved in the fungal kingdom, including in *M. oryzae* (Chanclud et al., 2016), *Fusarium pseudograminearum* causing both Fusarium crown rot in wheat and Fusarium head blight in barley, as well as in *Claviceps purpurea* (Hinsch et al., 2015), the rye ergot fungus (Obanor et al., 2013). The biosynthesis via the tRNA pathway leads to the production of isopentenyladenine (iP) and tZ (Sørensen et al. 2018). In axenic cultures *F. pseudograminearum* produced three pyrrole-substituted purine derivates that are cytokinin like molecule and are called Fusarium cytokinins by the authors; fusatin, 8-oxo-fusatin and fusatinic acid, with fusatin and fusatinic acid detected in wheat 14 days after floret infection (Sørensen et al., 2018). A study carried out in the model monocot *Brachypodium distachyon* showed that methyl salicylate- (MeSA) and 1-Aminocyclopropane-1-carboxylate (ACC)-synthases, which are responsible for SA and ET biosynthesis, respectively, were upregulated following a treatments with fusatinic acid (Sørensen et al., 2018). In the ergot fungus, *C. purpurea* two parallel CK biosynthesis pathways were described by Hinsch et al. (2015), one comprising the tRNA-IPT pathway and a second *de novo* biosynthesis pathway consisting of an IPT-LOG fusion protein enzyme with a cytochrome P450 monooxygenase that catalyses the transformation of iP to tZ.

The biosynthesis of CKs is mostly associated with biotrophic pathogens as CKs seem to be involved in green island formation (Walters and McRoberts, 2006), and were identified to function as virulence factors in hemi-biotrophic pathogens (Chanclud et al., 2016; Hinsch et al., 2016). Apart from the necrotrophic pathogens *F. pseudograminearum* and *C. purpurea* which produces fungal CK, there was no indication for a crucial role of CK in the infection process (Sørensen et al., 2018). A fungal genome analysis by Hinsch et al., (2015) suggested that the bifunctional enzyme IPT-LOG required for the activation of CK nucleotides is unique to the species from the orders of *Hypocreales* and *Capnodiales* which include *F. pseudograminearum* and *R. collo-cygni*, respectively. The genome of *R. collo-cygni* contains a tRNA isopentenyl transferase and several cytochrome P450 (McGrann et al., 2016). The present study showed that delaying leaf senescence in barley leaves did not result in lower symptom expression by *R. collo-cygni*, but rather enhanced symptom formation (Figure 2.5), whereas early senescence led to the hypothesis to increased resistance to RLS symptom development (Figure 2.10). Examining whether *R. collo-cygni* has the ability to potentially synthesise CK, will help to understand if the fungus can manipulate the host response in barley as seen in *C. purpurea* and *M. oryzae* (Chanclud et al., 2016; Hinsch et al., 2015) and whether the fungus is favoured by the nutrient sink created by increased levels of CK (Walters and McRoberts, 2006).

The observation in the induced senescence study, with the lack of RLS symptom development, but increased *R. collo-cygni* biomass in plants under nitrogen starvation (Figure 2.10 and Figure 2.11), raises the question whether the fungus can sense changes in nitrogen availability in the host that might trigger the transition between asymptomatic colonisation and symptomatic disease development of RLS. As previously discussed, McGrann and Brown (2018) showed that a gradual breakdown of the chloroplast resulted in increased RLS disease development, while in barley mutants lacking chloroplast development, no disease symptoms were observed. The gradual breakdown of chloroplasts and N remobilisation is also seen during monocarpic senescence (Distelfeld et al., 2014) when RLS symptoms typically become apparent, indicating that these may be important factors in RLS symptom development. The nitrogen metabolism is a tightly regulated process in fungi that confers the ability to use diverse nitrogen sources when primary substrates are not available (Donofrio et al., 2006). A genome wide micro array experiment under nitrogen sufficient and nitrogen limited conditions in *Fusarium fujikuroi* showed that the majority of gene clusters involved in the biosynthesis of secondary metabolites (SM) are regulated by both nitrogen (Tudzynski, 2014). Nitrogen limiting conditions *in vitro* have been previously shown to induce the expression of effector genes during growth *in planta* (Coleman et al., 1997; Talbot et al., 1993). The *Mpg1* gene of *M. oryzae* was expressed under nitrogen and carbon starvation both *in planta* and *in vitro* (Talbot et al., 1993). Similarly, in the tomato pathogen *Cladosporium fulvum* the avirulence gene *Avr9* was strongly induced under nitrogen limited conditions, both *in planta* and in cultures (Coleman et al., 1997). Most nitrogen metabolism studies were performed on the model fungi *Apergillus nidulans* and *Neuospora crassa* and showed that ammonium, glutamine, and glutamate are the preferred assimilated forms of nitrogen, while nitrate, amino acids and proteins can function as secondary sources (Marzluf, 1997). Nitrogen regulators facilitate the utilisation of secondary nitrogen sources and the expression of many genes when primary sources are lacking (Bolton and Thomma, 2008). The GATA family of TFs, including *AreA* from *A. nidulans* (Kudla et al., 1990) and N*it2* from *N. crassa* (Stewart and Vollmer, 1986) are the most studied TFs. The activity of *AreA* is partially increased as a result of increased mRNA stability under nitrogen starvation (Tudzynski, 2014). In *F. graminearum, AreA* activity is mediated by nitrogen starvation-induced nuclear translocation of *AreA* and in turn, elevated *AreA*-dependent gene expression in *A. nidulans and F. fujikuroi* (Tudzynski, 2014). Interestingly, *R. collo-cygni* contains a putative homolog of *AreA,* which was identified in gene expression profiling during RLS development (Dussart et al., 2018a). The highest transcript level of *AreA* was observed during the asymptomatic growth stage at 5 and 7 dpi respectively, which is similar to secondary metabolite (SM) core genes (Dussart et al., 2018a). The biosynthesis of the backbone of the SM product usually involves core genes. Thus, a core gene is often responsible for the production of the first intermediate compound in the biosynthetic pathway of the SM. In this study, the effect of *R. collo-cygni* on the decreasing Leaf N status was greater than the applied treatments (Figure 2.7 and Figure 2.12), indicating that the observed Leaf N decline in *R. collo-cygni*-inoculated plants might be due to the catabolism of secondary nitrogen sources mediated by *AreA*. Future studies could focus on the impact on RLS development upon application of different nitrogen sources, such as individually supplying ammonium and nitrate and/or different concentrations. The expression of *AreA* could be monitored at different time points to investigate when nitrogen starvation is sensed and if this TF is differentially expressed under nitrogen-limiting conditions. The lack of any nitrogen supply in this study suggests that *R. collo-cygni* may have failed to use secondary sources of nitrogen to promote virulence (Figure 2.12). Two mutants of *M. oryzae* were identified that are non-allelic to *Nitrogen-uitlisation* 1 (*NUT1*), which is an ortholog of *AreA* and is required for full induction of MPG1 expression (Lau and Hamer, 1996; Soanes et al., 2002). The *nut* mutants failed to use a variation of nitrogen sources and were apathogenic on rice (Soanes et al., 2002). This result indicates a role for nitrogen regulation in plant infection and appears to be under the regulation of *AreA* (López-Berges et al., 2010). Future work could investigate putative *AreA*-binding sites in the previously identified SMs biosynthetic gene clusters by Dussart et al. (2018), whichcould help to understand the interaction between Leaf N content and *R. collo-cygni.*

Chapter 3:

Characterising genetic regions conferring resistance to Ramularia leaf spot in spring barley

3.1 Introduction

3.1.1 Quantitative trait loci

Most agricultural and economically important traits in crop plants including yield and quality attributes, such as malting quality and resistance to abiotic and biotic stresses are inherited quantitatively (Falconer and Mackay, 1996; St.Clair, 2010). Phenotypic variation are accomplished through underlying genetic complexity from multiple interacting loci and allelic effects influenced by external environmental conditions (Falconer and Mackay, 1996). The basis for determining the inheritance of those quantitative traits and using this information for developing plant breeding programmes and thereby, selection methods, goes back to the rediscovery of Mendel's laws of inheritance in 1900 (Hallauer, 2007). The dissection of quantitative traits into quantitative trait loci (QTL) was initiated in the 1980s with the development of DNA-based polymorphic markers (MacKay et al., 2009). A comprehensive review of the different genetic markers developed over the past decades to detect QTLs can be found in Collard et al. (2005). Generally, DNA or molecular markers arise from different classes of DNA mutation such as point mutation, insertions or deletions and errors in replication of tandemly repeated DNA (Paterson, 1996). Polymorphic markers represent differences between individuals of the same or different species and can be divided into dominant or codominant markers. This is based on their potential to discriminate between homozygotes and heterozygotes (Collard et al., 2005). The development of these polymorphic markers, along with advances of statistical methods for computing QTL mapping, enabled the discovery of associations between marker genotypes and quantitative variations to map QTLs (MacKay et al., 2009; St.Clair, 2010). Thus, a QTL can be described as a statistically significant association between allelic variation at a marker genotype locus and a phenotypic trait showing continuous variation in a genetically varying population (St.Clair, 2010).

3.1.1.1 Quantitative trait loci mapping

The principle of QTL mapping is based on the localization of QTLs by their genetic linkage to visible marker loci with genotypes that can be classified. Therefore, if a QTL is linked to a marker locus, the mapping population comprising different marker locus genotypes exhibits different mean values of the quantitative trait (MacKay et al., 2009; Sax, 1923). Currently the most commonly used markers are Single Nucleotide Polymorphism (SNP) markers, which arise through base pair mutations and by simple sequence repeats. QTL mapping can be roughly divided into 1) QTL mapping, also known as linkage mapping of families or segregating progeny of crosses and 2) association mapping in unrelated individuals of the same population (MacKay et al., 2009). There are two components in mapping QTLs, the detection, and the localization. The power to detect those QTLs is dependent on the effects and allele frequency. The effect can be described as the average difference between the phenotype of the trait, for example the disease level, and the marker allele genotype, respectively (MacKay et al., 2009).

In QTL mapping, usually bi-parental mapping population are used, in which parental genotypes exhibit contrasting phenotypes such as susceptible vs. resistant parent plants. This approach can be employed to different types of populations *e.g.* F_2, double-haploid (DH) populations, backcross (BC), or recombinant inbred lines (RILs) families, by using Amplified Fragment Length Polymorphism (AFLP), microsatellite, Simple Sequence Repeat (SSR) and SNP markers (Alqudah et al., 2020). The fundamental limitation in QTL mapping is that only alleles/QTLs for which the parental genotypes segregate are detected. Therefore, many important QTLs which may control the trait of interest might be missed (Comadran et al., 2011; Kulwal, 2018). The resolution of mapping population depends on the number of recombination events that occurred during the population development which further limits the approach (Mitchell-Olds, 2010). Moreover, the approach of developing homozygous lines for mapping populations is time-consuming (Alqudah et al., 2020). In conventional breeding, six to eight generation of introgression or selfing are required to form homozygous lines of RILs or near isogenic lines (NILs) populations, whereas two generations to form a DH with lower rates of recombination events compared to RILs (Yan et al., 2017). The resolution in the mapping population can also be improved by increasing the number of intercrosses using multiparent RIL. For example, it was used to map awn length using a barley cultivar combined with four wild accessions (Liller et al., 2017). Another approach to overcome these problems is based on the principle of linkage disequilibrium (LD) and is called association mapping or Genome-Wide Association Scan (GWAS; Kulwal 2018).

3.1.1.2 Principle of genome wide association studies

GWAS can provide not only insights into the genetic architecture of a trait, but also enables informed choice of parents for QTL analysis and suggest candidates for expression analysis, mutagenic or transgenic experiments (Korte and Ashley, 2013). The aim in performing a GWAS is often to determine causative/predictive allele(s)/loci for a given trait, which in turn can be used in breeding crops for adaptation and yield improvement (Alqudah et al., 2020). The strength of a GWAS is to detect true association between a SNP and a trait but its success depends on the phenotypic variance within the population explained by the SNP. The magnitude of the difference of the allelic variants as well as their frequency, determines the phenotypic variance (Korte and Ashley, 2013). Through the use of diverse germplasm collections instead of bi-parental populations, GWAS exploits ancestral recombination events occurring over the plants` evolutionary history (Zhu et al., 2008). The five most important factors affecting the outcomes of a GWAS were summarised by Alqudah et al. (2020) as follows: 1) phenotypic variation; being vigilant with outliers that might skew the natural diversity analysis, 2) number of individuals; increasing population size to improve power of associations, 3) population structure; essential to be accounted for as not all individuals are equally distantly related to each other and to avoid false positives, 4) allele frequency; rare alleles lead to a lack of resolution power but difficult to detect if allele frequency is low 5) LD; coefficient of LD is used to calculate how likely two loci are associated and share the history of mutation as well as recombination (Flint-Garcia et al., 2003). The main challenges in GWAS are represented by missing rare variants that are associated with economically important traits and small effect size (Crossa et al., 2017; Korte and Ashley, 2013).

3.1.1.3 Genome-wide association studies in barley

The diploid characteristic of barley makes this cereal crop in comparison to the more complex hexaploid wheat, a great genetic model for cereals. Barley is represented by over 400,000 accessions in gene banks and now has a new ordered high quality reference genome sequence assembly (Knüpffer, 2009; Mascher et al., 2017). The continuous advances in barley science, such as the launch of the barley 50k Illumina Infinium iSelect genotyping array representing 44,040 working assays (Bayer et al., 2017), the availability of map position over the barley genome (Beier et al., 2017), and the most recent published gene reference transcriptomic dataset (Milne et al.,

2021) as well as the pangenome assembly (Mascher et al., 2021), has provided researchers with a better toolkit for studying barley. Therefore, barley represents a suitable candidate for GWAS as it is highly autogamous, comprises a long history of recombination events and conserved LD (Comadran et al., 2011). *ANTHOCYANINLESS 2* (*ANT2*) was identified and subsequently cloned based on results from a GWAS (Cockram et al., 2010). Initially, a GWAS of 32 morphological traits including awn anthocyanin coloration was performed in over 500 cultivars of which 15 traits showed significant association. The fine mapping of *ANT2* was conducted in a biparental mapping population using contrasting phenotypes to develop DH mapping population. Resequencing of *ANT2* basic helix-loop-helix-protein1 (*HvbHLH1*) gene identified a deletion in a premature stop codon upstream of the basic helix-loop-helix domain that results in the lack of anthocyanin in the mapping population (Cockram et al., 2010).

More recently, GWAS studies were used to identify novel loci associated with natural variation of resistance to biotic stress responses such as *Pyrenophora teres f. teres*, the causative agent of net form net blotch (NFNB) of barley (Amezrou et al., 2018; Richards et al., 2017). A glasshouse study of 1050 barley lines comprising landraces, breeding lines and cultivars, investigated the resistance to three North American *Puccnia teres teres* isolates (Richards et al., 2017). Landraces represent locally adapted barley varieties that were intentionally selected based on desired traits (Bothmer et al., 2003). A total of 78 marker-trait-associations were found in the study of Richards et al. (2017), with 16 unique loci associated with resistance/susceptibility to the three studied isolates (Richards et al., 2017). Another GWAS study was conducted on over 336 genotypes selected by ICARDA's germplasm (panel AM-2014) that have been phenotyped in seven environments in Morocco during the adult stage as well as at the seedling stage in the USA to test NFNB resistance using two *P. teres teres* isolates (Amezrou et al., 2018). The study found 21 QTLs at the adult plant stage and 31 QTLs at the seedlings stage respectively, of which two conferred resistance to NFNB at both stages (Amezrou et al., 2018). Moreover, the authors observed a high variability among their studied accessions with a strong gene by environment (G x E) interaction effect (Amezrou et al., 2018). Both studies (Amezrou et al., 2018; Richards et al., 2017) confirmed the previously reported association of barley chromosome 6H with net blotch resistance that potentially harbours NFNB resistance genes (reviewed in Liu et al. 2011). These studies highlight the power of GWAS and its application in marker assisted selection (MAS) used in breeding programmes to enrich resistance alleles while eliminating alleles contributing to susceptibility.

Barley stripe rust, caused by *Puccinia striiformis f. sp. hordei* represents another major barley disease in South Asia and North Africa (Gyawali et al., 2021). The recent characterisation of a new *P. striiformis hordei* race 7S0 in India poses an emerging threat to barley production in the region as this new race represents the most virulent race (Gangwar et al., 2016). The ability of the fungus to spread via long distances and its ability to overcome resistance genes, highlights the importance to exploit new resistance sources of non-race specific resistance genes (Gyawali et al., 2021; Rimbaud et al., 2018). A GWAS performed on two- and six-row barley at the seedling and adult stage reported 17 and 25 resistance QTLs to six races of *P. striiformis hordei* at the seedling stage respectively (Gyawali et al., 2021). Moreover, Gyawali et al. (2021), reported several candidate genes involved in abiotic and biotic stress tolerance such as transcription factors, protein kinases, heat shock proteins, and β-glucanases. The putative β-glucanases were associated with QTL

QPsh-rQ-6R-1.1. Similarly, Visioni et al. (2018) reported several candidate genes involved in β-glucan biosynthesis in a GWAS study of 261 barley genotypes performed on resistance to stripe rust in India. Plant β-glucanases appear to be involved in defence responses by hydrolysing the cell walls of fungal pathogens usually in combination with chitinase isozymes (Balasubramanian et al., 2012) which supports the findings of Gyawali et al. (2021).

GWAS studies in barley remain a pivotal tool to develop resistant cultivars in order to identify novel alleles associated with resistance against barley diseases and will help to improve yield in future breeding programmes (Alqudah et al., 2020).

3.1.2 The potential for genetic control against Ramularia leaf spot in barley

At present, there are no known sources of plant genetic resistance to Ramularia leaf spot (RLS) in barley. Although shortly after being recognised as a serious threat to barley production in the late 1990's, a variation in susceptibility was observed in several countries including Denmark, Lithuania, Slovakia, the Czech Republic and the United Kingdom (Havis et al., 2015). A quantitative variation in resistance was observed in most cases, which led to the hypothesis that RLS resistance may be expressed as a quantitative trait (Havis et al., 2015). However, a link between the presence of the loss-of-function mutation in the barley *MILDEW LOCUS O (mlo)* gene and RLS susceptibility has been reported (Brown and Makepeace, 2009; Makepeace et al., 2007; McGrann et al., 2014). The wild-type *MLO* encodes a seven transmembrane-domain protein that interacts with calmodulin and negatively regulates plant defence responses including plant cell death, resulting in susceptibility to powdery mildew caused by *Blumeria graminis f. sp. hordei* (Büschges et al., 1997; Consonni et al., 2006; Kim et al., 2002).

Therefore, a loss-of-function mutation in *mlo* prevents fungal penetration and development inside the host (Piffanelli et al., 2002). This represented a major success in plant breeding and *mlo*-alleles have been providing resistance for over 40 years now (Jørgensen, 1992). A molecular analysis using AFLP markers of eleven mutagen induced *mlo* alleles confirmed that *mlo* resistance is a result of a loss of function of the wild type *MLO* gene (Büschges et al., 1997). Originally derived from an Ethiopian landrace, *mlo-11* is the most commonly used allele in European spring barley cultivars (Dreiseitl 2012). The frequency of *mlo-11* was 0.2-0.6% in total Ethiopian landrace material collected in 1937 and 1938 (Piffanelli et al., 2004). Piffanelli et al. 2004 performed a haplotype analysis of 91 barley accessions of modern, cultivated *H. vulgare,* undomesticated *H. spontaneum* ancestors as well as cultivation intermediate varieties represented by Ethiopian *H. vulgare* species. Three distinct haplotypes were observed in the cultivated barley accessions, whereas a greater number of haplotypes was found within *H. spontaneum.* The pattern of diversity can perhaps be explained by the small transfer of genetic diversity from *H. spontaneum* to cultivated barley (Matus and Hayes, 2002). PCR of *mlo11* genomic DNA uncovered tandem repeat units consisting of 1.1 kb *MLO* coding sequence flanked by 3.5 kb upstream sequence. A G-T dinucleotide separated those juxtaposed repeats, which are not present in wildtype *MLO* (Piffanelli et al., 2004). Several studies have shown that the introduction of *mlo* alleles was linked with enhanced susceptibility to necrotrophic pathogens, such as *Magnaporthe oryzae* (Jarosch et al., 1999), *Bipolaris sorokiniana* (Kumar et al., 2018) and *Fusarium graminearum* (Makepeace et al., 2007). Increased sensitivity to abiotic stresses were also reported, as cultivars carrying the *mlo* mutation were more susceptible to form physiological leaf spots (PLS; Wu and Von Tiedemann, 2004).

3.1.2.1 The trade-off between *mlo*-mediated resistance to *B. graminis f. sp. hordei* and susceptibility to *R. collo-cygni*

The widespread use of *mlo*-mediated resistance in spring barley cultivars and the emergence of RLS led to the hypothesis that the increased incidences of RLS in Europe may be associated with *mlo* deployment (Makepeace et al., 2007). However, there have been contradicting findings about the effect of *mlo*-alleles on the severity of RLS. Makepeace et al. (2007) showed that plants carrying the *mlo* mutation had lower RLS disease scores than the Near-isogenic lines with the wildtype *MLO* allele. A field study from 2010 to 2013 in Germany that assessed *R. collo-cygni* DNA levels in grains of barley cultivars Ingrid and Pallas did not observe a significant difference between wildtype *MLO* and near isogenic BC *mlo-5* plants (Hofer et al., 2015). In contrast, Brown and Makepeace, (2009) showed that plants carrying the *mlo-5* allele exhibited greater RLS severity compared to *MLO* NIL. This was further supported by the observation that spring barley cultivars carrying the *mlo* mutation developed on average more symptoms than plants carrying the wild type *MLO* (Pinnschmidt and Sindberg, 2009). Similarly, double haploid (DH) populations derived from crosses between susceptible and resistant lines showed that plants carrying the *mlo-11* allele had significantly higher RLS levels than plants carrying the wild type gene (McGrann et al., 2014).

Altogether, these results indicate that *mlo* contributes to the susceptibility of spring barley cultivars to RLS. However, the strength of this effect differs with the environment, location and the host genetic background (Havis et al., 2015). McGrann et al. (2014) observed a significant effect of trial site on RLS in DH populations derived from crosses between susceptible and resistant lines. In two different field sites in Germany, *mlo-11* lines had significantly more RLS than *MLO* alleles (McGrann et al., 2014). A QTL analysis has shown a single QTL for RLS severity in field trials in one DH population associated with the *MLO* locus at chromosome 4H and accounted for up to 37% of genetic variation at the site with the greatest difference (McGrann et al., 2014).

The *mlo*-mediated susceptibility to necrotrophic pathogens appears to be regulated by other genes as indicated by mutant analyses, and the host genetic background may strongly affect the efficiency of *mlo* resistance (Freialdenhoven et al., 1996; Lyngkjaer et al., 2000). Two genes were identified which appear to be required for *mlo*-mediated resistance against powdery mildew, *ROR1* and *ROR2* (Required for *mlo* Resistance; Freialdenhoven et al., 1996). In seedling assays with a barley cultivar Ingrid NIL population background, with *mlo5+ror1-2*, *mlo5+ror2* and *mlo-5* respectively, it was shown that *ror mutants* reduced the development of RLS. However, they did not reduce the amount of *R. collo-cygni* DNA in barley leaves (McGrann et al., 2014). Moreover, Hückelhoven et al., (2000) has indicated that mutation of either *ROR1* or *ROR2* moderates the hydrogen peroxide (H_2O_2) burst, but that both *mlo-5 ror* mutant genotypes accumulate more H_2O_2 in the mesophyll.

Investigations of the *mlo*-mediated susceptibility to *M. oryzae*, identified two loss of function mutations; *Enhanced Magnaporthe Resistance* (*emr1* and *emr2*) which resulted in partial resistance to *M. oryzae* without compromising on mildew resistance (Jansen et al., 2007). Seedling assays using double mutant *emr1 mlo-5* lines showed reduced RLS development similar to that of *mlo-5 ror* double mutant genotypes (McGrann et al., 2014). Similarly, the reduction in RLS symptoms did not correlate with a decreased fungal biomass in this mutant. Although no significant differences were observed in disease levels in field trials using the same genotypes, a trend for lower RLS levels was seen in the *mlo-5* genotype (McGrann et al., 2020). In contrast, *mlo-5* contributed to increased symptom development and fungal DNA in lesion mimic mutants under controlled inoculation experiments (McGrann et al., 2015). Lesion mimic mutants (LMM) can result in spontaneous necrotic lesions in absence of disease. The LMM phenotype develops as a result of mis-regulations in cell death processes such as the hypersensitive response (HR), which is part of the effector triggered immunity (ETI) defence response (Dangl et al., 1996; Jones and Dangl, 2006). LMM often show accelerated leaf senescence and altered reactive oxygen species (ROS) homeostasis (Lorrain et al., 2003). The study by McGrann et al. (2015), used necrotic (*nec)* mutants varying in the degree of leaf spotting, chlorosis and increased HR-induced gene expression (Rostoks et al., 2006) to study the susceptibility of these mutants to *R. collo-cygni*. Barley necrotic *nec1* plants containing a MITE insertion in the *Cyclic Nucleotide Gated Ion Channel 4(CNGC4)* (Rostoks et al., 2006) exhibit reduced basal resistance against powdery mildew and increased non-host resistance against *Pseudominas syringae* pv. *Tomato* (Keisa et al., 2011). The *nec1* mutants in the study by McGrann et al. (2015) exhibited reduced RLS symptom development, however, there was no effect on fungal DNA levels in leaves. Moreover, the study showed that the genes *NEC1* and *MLO* have a complex interaction. The impact of *nec1* on RLS appeared to depend on the allele at the *MLO* locus. Reduced RLS symptoms were observed in *nec1 MLO* plants but increased in *mlo-5* plants. Moreover, plants with *mlo-5* had increased fungal growth and RLS symptom development compared to *NEC1 MLO* and *NEC1 mlo-5* plants (McGrann et al., 2015b). Similar observations were made in *nec1 MLO* plants showing reduced susceptibility to powdery mildew (Keisa et al., 2011). Altogether the data suggests that there are genetic loci resulting in contrasting effects on pathways regulated by *mlo* as shown in the studies described above (McGrann et al., 2015b, 2020).

3.1.3 Aims and Objectives

The aims of this chapter were to investigate the potential for genetic control of RLS disease in spring barley and to identify genetic loci associated with increased resistance to RLS by:

- Performing a novel GWAS to investigate the association between spring barley genotypes and resistance/susceptibility to RLS and identifying putative candidate genes underlying identified quantitative trait loci.
- Identifying the ten most resistant and susceptible barley genotypes in the studied panel and testing adult plant resistance under field trials.
- Testing seedling resistance in controlled inoculation studies and investigating the link between senescence and RLS development.

3.1.4 Statement of collaboration

The GWAS study was performed in collaboration with the James Hutton Institute (JHI) in Dundee with Dr Mark Looseley, Dr Kelly Houston and Dr Joanne Russell who provided raw data from the CORACLE project which finished in 2013. Additionally, pedigree data of spring barley genotypes used this study as well as geographic data of barley landraces and wild barley accessions were provided by Dr Paul Shaw from JHI.

The field trials in Germany were conducted by KWS and led by Dr Klaus Oldach. Symptoms in Germany were scored in conjunction with Professor Neil Havis. DNA of field trials in Germany was extracted in the laboratory of Professor Ralph Hückelhoven at the Technical University of Munich and DNA buffers were provided by Felix Hoheneder.

3.2 Material and Methods

3.2.1 Genome-wide Association Study

3.2.1.1 Phenotypic data – disease scores for Ramularia leaf spot

The present study utilised data gathered in the CORACLE LINK project, which was a collaborative effort in 2013 between Scotland, Germany and Norway (Brown et al., 2015). A collection of 263 spring barley varieties were investigated at six trial sites. Disease symptom data was collected from four sites in Germany, one in Scotland and one in Norway. To avoid potential false positive associations due to inconsistencies across sites and lack of genotypic data, the dataset was reduced to 239 varieties before the GWAS was performed. Disease severity was visually assessed, and data were analysed as a percentage of the maximum possible Area Under the Disease Progress Curve (AUDPC; Jeger and Viljanen-Rollinson 2001). To normalise the variance of errors and make the errors independent of the expected values, data were logit transformed. Moreover, to avoid an undefined transformed value when AUDPC=0, an empirical logit transformation was used as follows:

$$\log_e[(\text{AUDPC}+0.25)/(\text{maxAUDPC}-\text{AUDPC}+0.25)]$$

3.2.1.2 Genotypic data

The genotypic data set used for the GWAS analysis is derived from the 50k Illumina Infinium iSelect genotyping array for barley (Bayer et al., 2017). It features 49,267 SNP marker that was reduced to 44,040 SNP working assays, of which 43,461 SNPs could be assessed in Genome Studio (Bayer et al., 2017). A total of 29, 151 SNPs of the 43,461 SNPs have physical positions on the barley genome (Mascher et al., 2017) and therefore was the number of genetic markers included in the GWAS.

The GWAS analysis was conducted in R-Studio 3.6.1 (RStudio, 2020) using the downloaded package GAPIT (Lipka et al., 2012). The efficient mixed model association (EMMA; Kang et al., 2008) was used in this GWAS as it was shown by a previous study to have a high statistical power compared to other mixed model methods (Xiao et al., 2017). Furthermore, population structure was accounted for through a principal component analysis (PCA=3) and for relationships among individuals using a kinship matrix (K; EMMA). Through a further model selection using the Bayesian Information Criterion (BIC), the optimal number of PC/covariates for each trait was tested. Heritability of the model, and the quality of the fit known as Quantile-Quantile-(QQ) plot of the predicted versus the observed p values were also calculated. For each phenotypic trait, the critical P values for assessing the significance in the naïve model were corrected for multiple comparison using a false discovery rate (FDR) of 0.1 (Benjamini and Hochberg, 1995). To account between common and rare variants and make the analysis more stringent, a minor allele frequency of 0.05 was applied.

3.2.1.3 Marker-trait association

Data were analysed in R-Studio 3.6.1 (RStudio, 2020). Analysis of variance (ANOVA) were performed using the R packages multcomp and tidyverse (Hothorn et al., 2008; Wickham et al., 2019). A Fisher-test was carried out to assess the homogeneity of variances and a Welch-t-test was subsequently performed to calculate the significance between two marker alleles associated with disease level of RLS for each site of the phenotypic dataset. Both tests were performed using the group by function from the package dplyr which is implemented in the tidyverse package of R (Wickham et al., 2019). All diagrams were created in R-studio using ggplot2 and ggpubr packages (Wickham et al., 2019).

As part of the further discussion of the marker-trait association, this study investigated the presence of the marker alleles in barley landrace and wild barley accessions based on SNP data published by Chen et al. (2022) and used map data derived from the James Hutton Institute (personal communication Kelly Houston).

3.2.1.4 Locus refinement by linkage disequilibrium analysis

A LD analysis was performed to examine the association between SNPs surrounding the identified QTL and the QTL. Analysis was performed on 700 SNPs on chromosome 4H, 500 SNPs up and 200 SNPs down the QTL of interest using the software TASSEL (Bradbury et al., 2007). LD of SNP markers was calculated as the correlation between marker pairs calculated as Pearson correlation coefficient (r). The degree to which LD patterns occurred were identified by selecting SNP blocks where $r^2 \leq 0.2$ was met at each of the side of the QTL of interest, with $r^2 \leq 0.2$ SNPs describing the LD decay (Zhu et al., 2008). Subsequently, genes underlying the QTL were extracted from the Barlex genome explorer (Home (ipk-gatersleben.de). Physical positions of the start and end of the identified SNPs marking the QTL loci on chromosome 4H were used in the Basic Alignment Search Tool (BLAST) against the Morex v3 genome dataset (Colmsee et al., 2015).

3.2.2 Investigating Ramularia leaf spot resistance in field trials

3.2.2.1 Field trial location and layout of plots in Scotland

Genetic resistance was tested among 28 spring barley genotypes in field trials in Scotland. Field trials were conducted at Boghall Farm, Midlothian over two consecutive seasons (2021 and 2022). Each trial consisted of fully randomised plot designs (3 m x1 m) with two plots per spring barley genotype in 2021. In 2022, the Scottish trial design was intended to be performed as in 2021 using two randomised plots per genotype. However, due to circumstances outside my control, the second plot per genotype was not sown. At the beginning of stem extension *i.e.* growth stage (GS) 30 the fungicides prothioconazole (Proline, Bayer Crop Science @ 0.4 l ha^{-1}) plus pyraclostrobin (Comet, BASF @ 0.63 l ha^{-1}) were applied to each plot to control against other fungal pathogens such as *R. commune* (Bingham et al., 2021; Zadoks et al., 1974).

3.2.2.2 Field trial location and layout of plots in Germany

To test the impact of the environment on genetic resistance, field trials were conducted in Germany. Field trials were carried out by the breeding company KWS Saat (Einbeck, Germany) and were performed in Bergen in 2021. Due to the lack of disease pressure in the first season, the trial was conducted in Seligenstadt by Würzburg in 2022. The field trial was designed as fully randomised plots (3 m x 1 m) with one plot per genotype, and cv. Barke was used as a randomised repeated control genotype. To avoid other fungal pathogens, a cover spray using 0.61l/ha azoxystrobin and tebuconazole were applied at GS 30 (Zadoks et al., 1974).

3.2.2.3 Plant material used in field trial studies

The ten most resistant and susceptible spring barley varieties were selected based on the phenotypic dataset of the CORACLE project in 2013. A list of all spring barley varieties that have been consistently scored over two consecutive seasons and countries is given Table 3.1. Variety identification number (ID No) 1 to 20 highlights the ten most resistant and susceptible varieties based on the scoring that was performed as part of the CORACLE project in 2013. Eight additional spring barley varieties were added to the list as currently marketed spring barley varieties both in Germany and Scotland. The selection was made regardless of the QTL allele. RGT Planet, Power, Quench, and Scarlett are varieties with moderate resistance rating. While Braemar, Decanter and Fairing are spring barley varieties often cited in literature as susceptible controls for RLS.

Table 3.1: Spring barley varieties used in field trials in Scotland and Germany
The table shows the presence of the QTL allele JHI-Hv50k-2016-266331: arley varieties carrying the A allele are associated with increased resistance, whereas the ones carrying the G allele are linked to increased susceptibility to RLS.

Variety ID No	**Spring Barley Variety**	**QTL allele**
1	Derkado	G
2	Brahms	A
4	Trebon	G
5	Chariot	G
6	Tankard	G
7	Hydra	G
9	Publican	G
10	Chieftain	G
11	Novello	A
12	Sabel	G
13	Aramir	A
15	Cocktail	A
16	Tocada	A
18	Spike	A
19	Franklin	A
20	Heather	A
21	Power	A
22	Braemar	G
23	Decanter	G
24	Quench	G
25	Barke	A
26	RGT Planet	G
27	Scarlett	A
28	Fairing	G

3.2.2.4 Assessment of disease levels in field trials

After GS 30 (stem extension) visual assessments were carried out regularly every four weeks (Zadoks et al., 1974) until GS 58 (ear completely emerged above flag leaf ligule) and thereafter, every two weeks. Plots were evaluated for RLS development on the leaf preceding the flag leaf (F-1) on a minimum of ten leaves. In both seasons, disease assessment was taken on the F-1 leaf as a percentage leaf area covered by RLS lesions. In Scotland, ten leaves per plot were scored at GS 80 (late milk to early dough; 2021) and GS 89 (fully ripe; 2022) and the same leaves were sampled to quantify *R.collo-cygni*-DNA levels. A second disease assessment was attempted in both years to calculate the area under the AUDPC, however, due to the difference in maturity in the spring barley genotypes and the dry season in 2021 which resulted in fast senescence in over half of the plots, this disease assessment did not take place. Whereas in 2022, the overall low disease pressure limited the number of disease assessments. Senesced leaves can contribute to a biased, incorrect disease assessment as a difference between other necrotrophic pathogens and physiological leaf spots becomes difficult. Even though, the AUDPC is the preferred way to show field trial data, the percentage of leaf area covered by RLS lesions is a valid substitute. For the field trial in Germany, disease symptoms were absent in 2021. In 2022, ten leaves per plot were assessed at GS 79 (late milk) and the same leaves were taken to quantify *R.collo-cygni* -DNA levels. Data shown for 202, represents the mean of two plots consisting of five biological replicates per plot ± standard error. Data shown for 2022 represents the mean of one plot per genotype consisting of five biological replicates ± standard error.

3.2.2.5 Monitoring *in planta* colonisation by *R. collo-cygni* in field trials

The colonisation of barley leaves by *R. collo-cygni* during the spring barley season was monitored to better understand the relationship between the asymptomatic fungal growth and symptomatic disease development. Five barley leaves per plot were harvested in Scotland at GS 30, GS 58, and GS 80 in 2021 and were stored at -20°C until processed. At GS 30 the F-3 leaf was harvested, while after full ear emergence, the F-1 leaf layer was harvested, to represent the most recent colonisation by the fungus as *R. collo-cygni* colonised vertically through the developing crop canopy (Havis et al., 2014). Leaves harvested at GS 80 were first visually assessed before they were pooled together, ground up under liquid nitrogen and DNA was extracted as previously described in Chapter 2, Section 2.2.7.2. Due to the inconsistent presence of *R. collo-cygni* in the first season and to reduce the number of samples, fungal colonisation was only observed at GS 65 and GS 89 in the F-1 leaf layer in 2022. In both seasons, the seeds from one plot per genotype were harvested and the presence of *R. collo-cygni* was quantified by milling 10 g per plot and extracting *R. collo-cygni*-DNA as previously described in Chapter, 2, Section 2.2.8.2. with the only difference using 2 g of milled seed powder. In Germany, the quantification of *R. collo-cygni* was limited to one time point at GS 72 in 2022 when crops were visually assessed due to the logistics of having to travel to Germany. DNA extractions were performed at the Technical University of Munich using the same method previously described in Chapter 2, Section 2.2.7.2 and DNA was quantified by qPCR using the method of (Taylor et al., 2010). Data shown from 2021 represents the mean of two plots per genotype consisting of three technical replicates per plot which contained five pooled leaves per plot ± standard error. Data shown from 2022 in Scotland and Germany represents the mean of one plot per genotype consisting of five technical replicates of five pooled leaves per plot ± standard error.

3.2.2.6 Relative chlorophyll content and leaf nitrogen quantification in field trials in 2022

Based on visual observations in the field in 2021 the plant age-dependent senescence in relation to RLS disease development was studied in eight different spring barley genotypes. Cultivars Fairing, Hydra, Chieftain and Quench were selected as susceptible spring barley varieties, whereas cv. Heather, Tocada, Barke and RGT Planet were selected as partially resistant varieties. Soil Plant Analysis Development (SPAD) measurements were taken from GS 72 onwards for four consecutive weeks with three measurements until visual assessment of RLS was performed in the field. Two SPAD measurements were taken on the F-1 adaxial leaf surface for each biological replicates. Data represents the mean of nine measurements ± standard error.

Due to the potential inaccuracy of SPAD readings in the field because of high barley brown rust pressure in that season that could absorb the wavelength used to measure the relative chlorophyll content, additional F-1 leaf samples were taken for subsequent leaf N analysis at the same time points. Leaf N was quantified as previously described in Chapter 2, Section 2.2.7.4. Data shown represents the mean ± standard error of three biological replicates each consisting of one F-1 leaf per genotype.

3.2.3 Evaluation of Ramularia leaf spot resistance under controlled inoculation studies

3.2.3.1 Plant material used in controlled inoculation studies

To assess disease resistance under controlled conditions compared to resistance to RLS observed in the field, a subset of spring barley genotypes differing in their resistance to RLS were selected. Spring barley cv. Fairing, Hydra and Chieftain with low resistance and Heather, Tocada and RGT Planet with higher resistance to RLS respectively were investigated. Seeds were pre-germinated, and grown in vermiculite soaked in Hoagland solution (Hoagland and Arnon, 1950) as described in Chapter 2, Section 2.2.1 and 2.2.2.

3.2.3.2 Barley seedling inoculation under controlled conditions

The six selected spring barley genotypes were inoculated using *R. collo-cygni* isolate SC19-B5 (+Rcc) and non-inoculated (+Mock) using PDB respectively, to test the resistance to RLS under controlled conditions. Seedling inoculation was performed on 14-day old seedlings as described in Chapter 2, Section 2.2.5. Inoculated seedlings were grown for 21 days in MC1000 high specification climate chambers (Snijders, Tilbourg, Netherlands) with a photoperiod of 16 h light (220 µmol m^{-2} s^{-1}) / 8 h dark and a temperature of 18°C / 12°C day / night respectively. Visual assessments were carried out between 10 and 21 dpi and scored as the percentage of green leaf area. Relative chlorophyll and leaf N contents were analysed to test the association between RLS disease development and host physiological traits in the selected genotypes. Colonisation by *R. collo-cygni* was assessed via qPCR during disease development to gain a better understanding how host physiological traits in different genotypes are linked to RLS symptoms expression and fungal growth. The presence of *R. collo-cygni* in +Mock plants was assessed at 21 dpi to show that *R. collo-cygni* presence in the seed did not contribute to the symptom expression under controlled conditions. All assessments were carried out at 5, 7, 10, 14, 18 and 21 dpi. SPAD readings and symptoms data represent the mean ± standard error of ten prophyll leaves from two independent inoculation experiments. Leaf N and *R. collo-cygni*-DNA data is shown as the mean ± standard error of two independent inoculation experiments which consisted each of two biological replicates consisting of three and two prophyll leaves respectively and were analysed in technical duplicates.

3.2.3 Statistical analysis

Data in this chapter were analysed using R version 4.2.3 (RStudio, 2020) using for all analyses the "tidyverse" packages (Wickham et al., 2019). A two-way ANOVA was performed to examine the effects of variety and QTL on symptom expression and fungal DNA respectively. Residual analysis was conducted to check the assumptions of the two-way ANOVA. Shapiro-Wilk's normality test was used to assess normality, and homogeneity of variances was assessed by Levene's test. Consequently, an analysis of simple main effects was performed for variety and QTL receiving a Bonferroni adjustment. If not stated otherwise, all statistical differences in the main effects groups were followed by pairwise comparisons by using emmeans which are estimated marginal means using the fitted model, within the "multcomp" package (Hothorn et al., 2016) with a confidence level of 0.95 and p-values were calculated using the Tukey method ($\alpha = 0.05$).

Linear mixed-effect model (LMEM) analysis was conducted via the restricted maximum likelihood algorithm (REML) in the "lme4" package (Bates et al., 2015). A LMEM regression was fitted for each measured response (Symptoms, *Rcc*-DNA, SPAD, Leaf N) using time and treatment as predictors and their interaction as fixed effects. The number of each independent experiment was the random effect. The models' assumptions were checked each time by testing the homoscedasticity by comparing residuals to the fitted items and a Shapiro-Wilk test was used to assess if the residuals were normally distributed. All graphs were produced using ggplot2 (Wickham, 2011).

3.3 Results

3.3.1 Genome-wide-association study

3.3.1.1 Phenotypic data generated in 2013

A wide range of the RLS levels was observed across the different sites (Figure 3.1), ranging from 1% at KWS in Germany to 60% disease on flag leaves at Secobra in Germany. A significant variation in RLS prevalence was observed between all sites except between Saaten Union (Germany) and SW (Germany), SRUC (Scotland) and SW (Germany) and Sejet (Norway) and Secobra (Germany). Considering that disease levels were assessed consistently across all sites, the high variation of RLS prevalence highlights the G x E effect. the barley variety Fontana was among the ten consistently high scoring RLS barley varieties across all sites. The cultivar Chariot showed high RLS scores across Scotland, and three sites of Germany (KWS, Saaten Union and SW). In Norway (Sejet) and two sites of Germany (Secobra and SW) cultivar Century was amongst the highest RLS disease levels. In contrast, the cultivar Proctor exhibited the lowest levels of disease across all sites except Norway. Barley variety Ricardo displayed consistently low RLS levels across Germany, whereas cultivar Franklin exhibited low RLS scores across the two sites, Saaten Union and Secobra in Germany and the site in Scotland. The identification of varieties which exhibit consistent RLS disease levels among sites is important to select for barley varieties in future studies and comparing the genetic background of those cultivars.

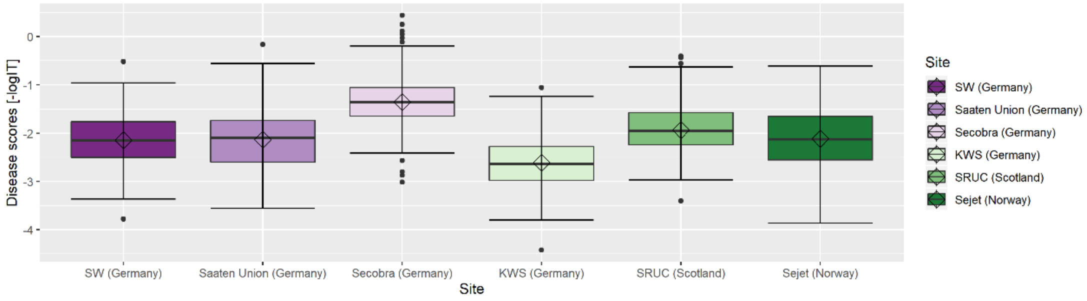

Figure 3.1: Ramularia leaf spot disease severity compared across different sites. Boxplots represent the range of disease severity observed in four sites in Germany, one in Scotland and one in Norway (x-axis). Data represented here were analysed as area under the disease progress curve (AUDPC) as a percentage of the maximum possible AUDPC (max AUDPC) in 238 spring barley genotypes. To normalise the variance of errors and make the errors independent of the expected values, data were logit transformed (y-axis).

3.3.1.2 Genome-wide association study to identify QTL associated with resistance to RLS

The results of the BIC based model selection indicated that no principal components were required to control for population structure. This can be explained as only a spring barley population was used in this study. Furthermore, the Q-Q-plots (Figure 3.2) supported the suitability of the GWAS model as the observed and expected p-values differed only for a few SNPs. In the presented GWAS, one main effect QTL for RLS sensitivity was detected at the site of SRUC in Scotland. The detected QTL is located at the end of Chromosome 4H with a -log(p) value of 6.05 (Figure 3.2). The significant marker identified in this study was JHI-Hv50k-2016-266331 and this SNP represents an A/G polymorphism. The observed QTL was not detected at any other site. The calculated pseudo-heritability is 17.6% with -2*log likelihood function of 339.07 as estimated by the kinship matrix from SNP data and represent the fraction of phenotypic variance that can be explained by the genetic relatedness between individuals (Korte and Ashley, 2013). The mapping of several known loci over the Manhattan plot reveals that the QTL detected in this study appears to be associated with the MLO locus which is also located on chromosome 4H.

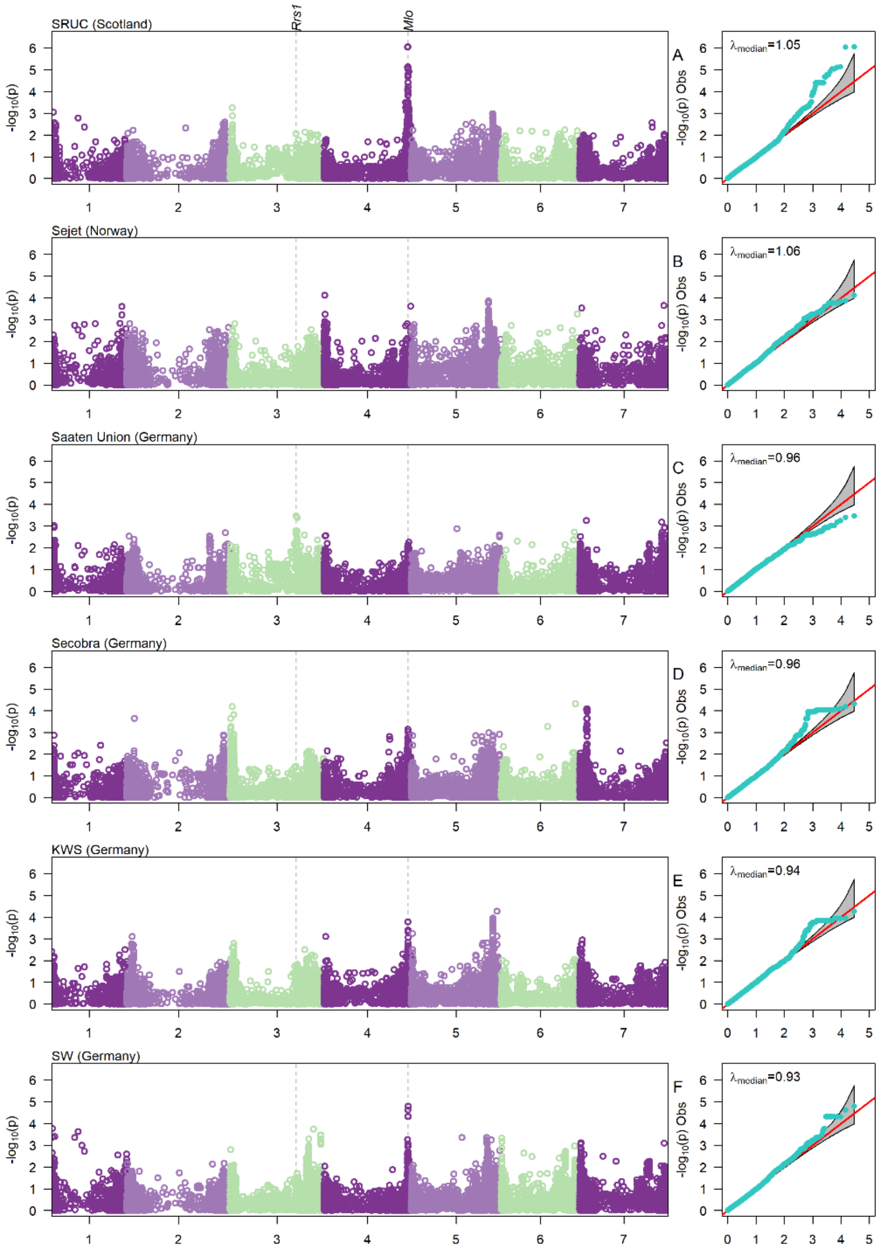

Figure 3.2: Genome wide association study of 238 spring barley varieties scored for Ramularia leaf spot symptoms across three countries.
Manhattan plots on the left divided by sites from top to bottom: SRUC (Scotland), Sejet (Norway), Saaten Union (Germany), Secobra (Germany), KWS (Germany) and SW (Germany) and their respective Q-Q-Plot on the right. One main effect QTL detected at the site of SRUC in Scotland with a p-value of 6.05 (SNP JHI-Hv50k-2016-266331). Phenotypic data derived from the CORACLE project completed in 2013 contained 238 spring barley cultivars. Genotypic data used in the GWAS derived from the 50k SNP dataset and included 29,151 SNPs.

3.3.1.3 Marker-trait association analysis of JHI-Hv50k-2016-266331 of the CORACLE Dataset

To determine the significance of the association between allelic variation at the genotypic marker JHI-Hv50k-2016-266331 (A/G) and RLS severity, the mapping population was segregated into their respective marker genotypes at each site (Figure 3.3). Although the effect of the QTL cannot be obtained from the Manhattan plots across all sites, the presented boxplots show a trend for the polymorphism with the G allele being associated with increased RLS disease levels. To ascertain the importance between A and G alleles, a Welch-t-test was conducted and estimated significances across all sites between both haplotypes and the disease levels. For all sites F-values <0.001 were obtained.

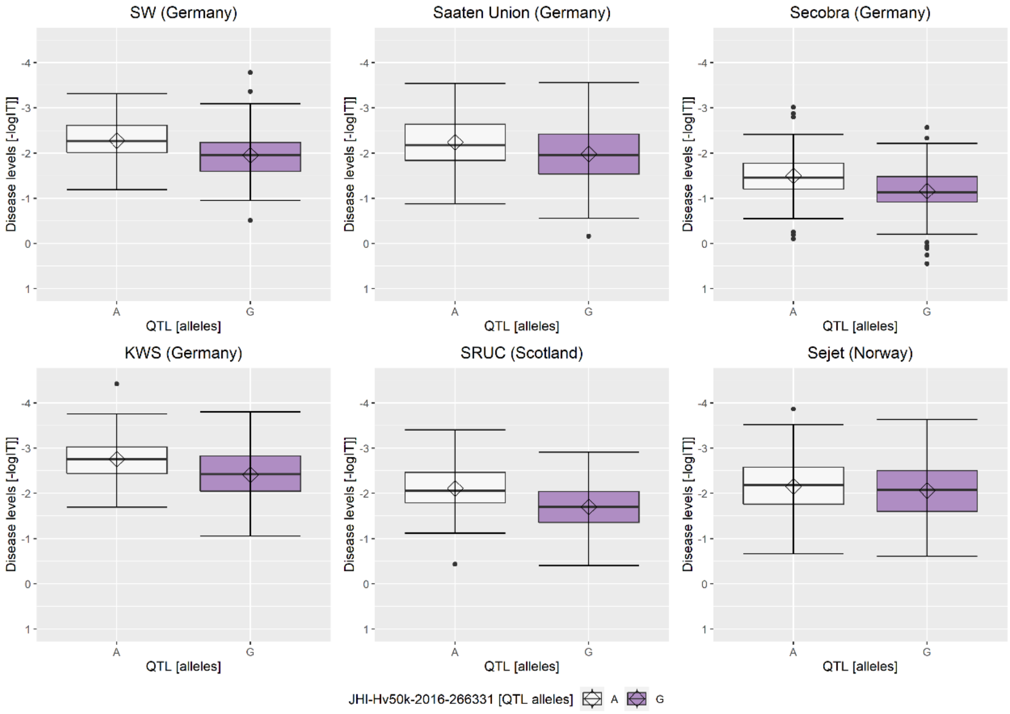

Figure 3.3: Effect of QTL on Ramularia leaf spot disease levels.
Data represent mean logit transformed disease levels of 238 spring barley varieties scored across six different sites segregated by the identified QTL alleles. QTL A allele: associated with increased resistance (white); QTL G allele: associated with increased susceptibility (purple).

3.3.1.4 Identification of candidate genes underlying JHI-Hv50k-2016-266331 in spring barley cultivars

The association between SNPs surrounding the identified QTL and the QTL of interest was examined via LD analysis. The SNPs JHI-Hv50k-2016-265563 and JHI-Hv50k-2016-268973 were identified as the start (AGP Start: 587,251,236) and end (595,541,550) of the QTL locus which covers around 8,290,314 base pairs. Putative candidate genes were extracted from the Barlex genome explorer, and 308 gene models were present within this interval on 4H, and a selected subset of 27 candidate genes associated with major plant abiotic or biotic stress responses are given in the Appendix A.1 and the remaining can be found in Table 3.3 in the Appendix. This includes six transcription factors, of which three are associated with jasmonic/ethylene responsive genes. Additionally, this subset of 27 genes includes those that encode enzymes related to the regulation of ROS status such as catalase, glutathione-S-transferase, superoxide dismutase, and peroxidase. Moreover, genes associated with the plant immune responses such as Leucine-rich repeat (LRR) family protein, Hypersensitive-induced response protein 1 and other kinases were found among the 27 genes of interest.

Table 3.2: List of putative candidate gene models underlying quantitative trait loci region JHI-Hv50k-2016-266331.

Results shown 27 of 308 Basic Alignment Search Tool (BLAST) results extracted from the Barlex Genome explorer. Horvu Morex version 3 using the physical positions of the single nucleotide polymorphism markers JHI-Hv50k-2016-265563 (587,251,236) and JHI-Hv50k-2016-268973 (595,541,550) were used as flanking markers. Genes are highlighted in each category separating the genes by their putative role in oxidative stress, as transcription factors, as part of the pattern- and effector triggered immunity and non-categorised genes.

Category	AGP Start	AGP End	ID	Description
Oxidative Stress-related	587479048	587482147	HORVU.MOREX.r3.4HG0409940.1	Catalase
	587479048	587482147	HORVU.MOREX.r3.4HG0409940.2	Catalase
	591669251	591671554	HORVU.MOREX.r3.4HG0411440.1	Superoxide dismutase [Cu-Zn]
	595214137	595215389	HORVU.MOREX.r3.4HG0412630.1	Peroxidase
	595218235	595222497	HORVU.MOREX.r3.4HG0412640.1	Peroxidase
	594615293	594615652	HORVU.MOREX.r3.4HG0412480.1	Glutathione S-transferase T3
	594617172	594621229	HORVU.MOREX.r3.4HG0412490.1	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
Transcription factors	587510757	587511530	HORVU.MOREX.r3.4HG0409950.1	AP2/B3 transcription factor family protein
	587746642	587747749	HORVU.MOREX.r3.4HG0410010.1	AP2/B3 transcription factor family protein
	588889254	588893529	HORVU.MOREX.r3.4HG0410400.1	MYB transcription factor-like
	588896608	588897882	HORVU.MOREX.r3.4HG0410410.1	WRKY transcription factor
	589053514	589054618	HORVU.MOREX.r3.4HG0410500.1	Myb transcription factor
	590704298	590704927	HORVU.MOREX.r3.4HG0411150.1	Ethylene-responsive transcription factor
	593375790	593376728	HORVU.MOREX.r3.4HG0412080.1	Myb transcription factor
	594964865	594967720	HORVU.MOREX.r3.4HG0412560.1	Basic helix-loop-helix transcription factor
	595540806	595546418	HORVU.MOREX.r3.4HG0412750.1	Basic-leucine zipper (bZIP) transcription factor family protein
PTI and ETI-related genes	588126591	588131540	HORVU.MOREX.r3.4HG0410090.1	Hypersensitive-induced response protein 1
	588596497	588597314	HORVU.MOREX.r3.4HG0410300.1	Defensin
	589397759	589402492	HORVU.MOREX.r3.4HG0410700.1	Calcium dependent protein kinase
	587406525	587409379	HORVU.MOREX.r3.4HG0409890.1	Calcium-dependent protein kinase
	588396936	588404092	HORVU.MOREX.r3.4HG0410190.1	Calcium-binding EF hand protein
	592690853	592691517	HORVU.MOREX.r3.4HG0411830.1	Leucine-rich repeat (LRR) family protein
	594094042	594095785	HORVU.MOREX.r3.4HG0412270.1	Receptor-like kinase
	594133390	594136381	HORVU.MOREX.r3.4HG0412300.1	Receptor-like protein kinase, putative
Others	592846881	592849408	HORVU.MOREX.r3.4HG0411870.1	Cysteine-rich repeat secretory protein
	589770425	589773203	HORVU.MOREX.r3.4HG0410880.1	NAC domain-containing protein
	595134588	595135935	HORVU.MOREX.r3.4HG0412610.1	Nascent polypeptide-associated complex alpha subunit, putative, expressed

3.3.2 Assessment of Ramularia disease levels in field trials

Disease levels of RLS were monitored in field trials between 2021 to 2022 in 28 different spring barley genotypes at one site in Scotland and one in Germany. The severity of RLS was based on visual disease assessments rated as the percentage of green leaf area. In addition to symptom assessment, the level of *R. collo-cygni* DNA was quantified in both seasons and environments of the scored F-1 barley leaves to examine the relationship between RLS and fungal colonisation. Moreover, in field trials in Scotland, the colonisation by *R. collo-cygni* was also monitored during the asymptomatic growth stage during both seasons. Spring barley cultivars in this study were selected based selected on three criteria: 1) the presented GWAS analysis, 2) currently used cultivars for malting in Europe and 3) on spring barley genotypes previously studied.

3.3.2.1 Ramularia symptom development in field trials from 2021 to 2022

Quantitative differences in RLS disease levels were observed among genotypes in Scotland 2021. Disease levels ranged from 11% to 1.5% (Figure 3.4 A). The germination rate of cv. Trebon and Sabel was poor in 2021 therefore, these cultivars were excluded from further disease interpretation. Furthermore, cv. Trebon was excluded from the 2022 season in Scotland due to the lack of seed stock.

The three most resistant varieties in 2021 were cv. RGT Planet with 3.9% RLS disease levels, cv. Heather with 4.4% RLS levels and cv. Scarlett with 4.5% RLS levels. The three most susceptible varieties were Barke, Hydra, and Chieftain which exhibited 8.7%, 9.3% and 11% RLS levels, respectively. In the 2022 season (Figure 3.4 B), RLS disease pressure appeared lower in Scotland and spring barley genotypes were mainly infected by brown rust and smut. RLS disease levels ranged from 0.6% to 12.8% with 88% of the varieties exhibiting less than 5% RLS symptoms compared to 30% in 2021. The three lowest RLS disease levels were observed in cv. Cocktail at 0.6%), Publican at 0.6% and Novello at 0.8%. The three most susceptible varieties in 2022 demonstrated disease levels above 5% RLS with Tankard at 5.6%, Derkado at 6.8%, and Fairing at 12.8% respectively. Similar to 2021, Chieftain with 4% and Barke with 3% were among the most susceptible varieties. The field season in Germany 2022 (Figure 3.4 C) showed similar trends to 2021 in Scotland. Quantitative differences in RLS disease resistance were observed which ranged from 0.2% to 9.4% with 33% of the varieties exhibiting RLS symptoms above 5%. The three most resistant varieties in

Germany were Aramir (0.2%), Trebon (0.3%) and Franklin (1%). Similar to 2021, Hydra (7%), Chieftain (8.4%) and Quench (9.4%) exhibited the highest disease scores in 2022 in Germany. Unlike what was observed during both seasons in Scotland, RGT Planet was in Germany among the most susceptible varieties with 6.8% RLS disease symptoms compared to 3.9% in Scotland in 2021 and to 1.4% in Scotland season 2022. Likewise, Quench showed greater resistance to RLS in Scotland 2021 with 1.8% RLS disease symptoms.

Overall, the observations made in these field trials indicate that genotypes used in this study containing the resistant, A allele were associated with increased resistance to RLS (Figure 3.4). However, consistent performance across trials was indicated to be more accurate for genotypes being associated with increased susceptibility. Moreover, it appears that the marker-trait association depends on the environment. The observations over two field seasons indicated that with medium disease pressure the quantitative difference among spring barley varieties appear to be stronger compared to low disease pressure seasons.

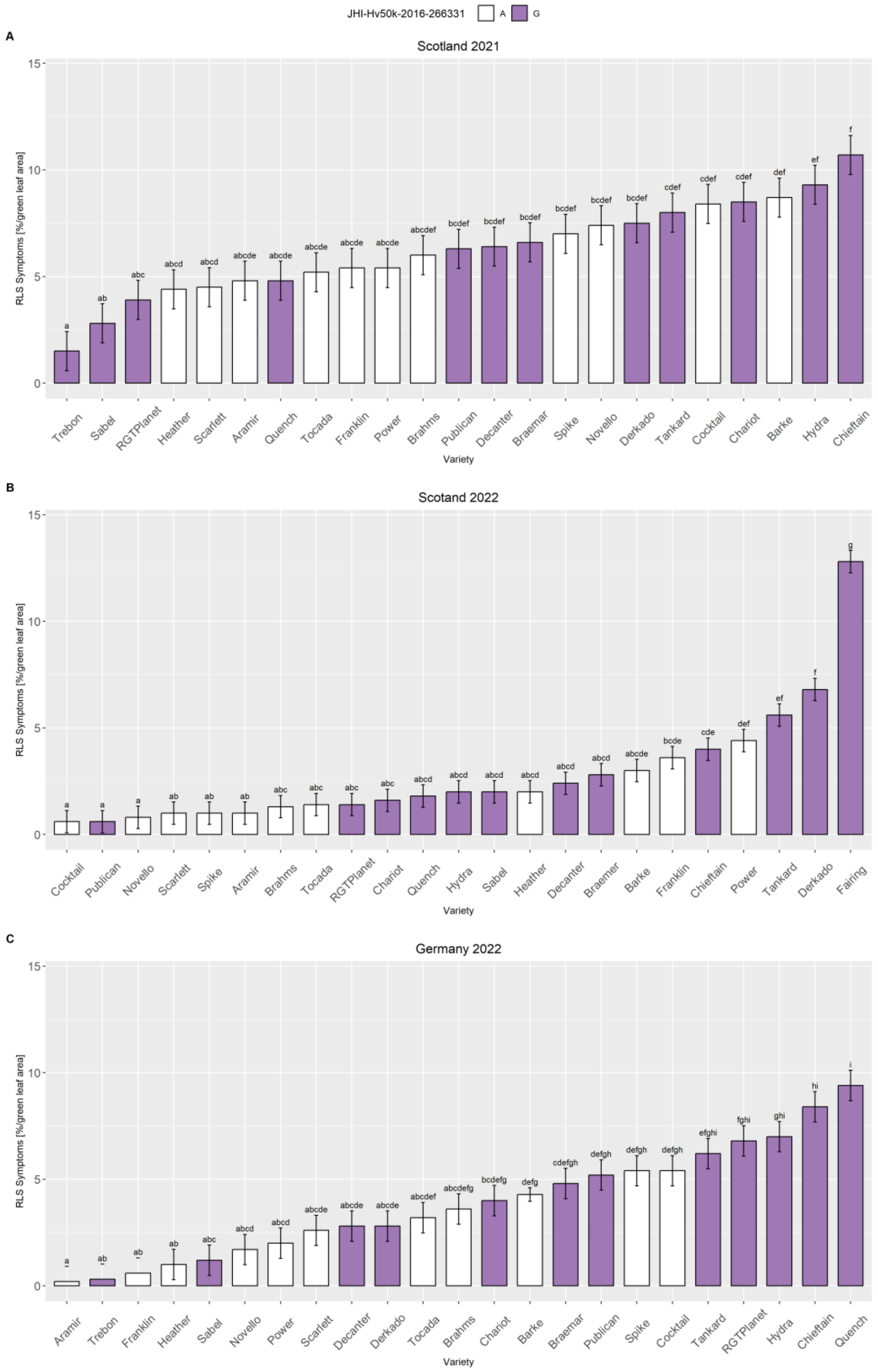

Figure 3.4: Ramularia leaf spot disease levels observed in different barley varieties during field trials in Scotland and Germany from 2021 to 2022.

Bar plots represent calculated emmeans disease levels ± standard error. A two-way ANOVA with interaction effect of variety*QTL was performed with symptoms as the response variable. Consequently, a pairwise comparison analysis using emmeans was performed between different varieties and significance levels were estimated using the Tukey method (alpha = 0.05). Compact letter design represents the significant differences among varieties, with varieties not sharing the same letter being different from each other. Varieties in purple contain the susceptible G allele and in white varieties with the resistant A allele of the identified QTL respectively. Disease levels are shown across two consecutive years: A: Scotland 2021; B: Scotland 2022; C: Germany 2022.

3.3.2.2 Varietal comparison between environments and years of Ramularia leaf spot field trials

The resistance to RLS within a variety was compared between the years 2021 and 2022 and between Scotland and Germany to test whether the performance of the respective variety behaved consistently (Figure 3.5). Cultivars Trebon, Tankard and Sabel exhibited consistently high resistance to RLS across environments and over two consecutive seasons. Cultivar Tocada, Scarlett, Heather and Braemar showed significant differences over the two consecutive seasons in Scotland, but not in comparison to the 2022 season in Germany. Cultivar RGT Planet differed significantly between Germany and Scotland in 2022, but not in comparison to 2021. Cultivars Chieftain and Hydra exhibited high susceptibility across environments over two consecutive years but were consistent between Germany 2022 and Scotland 2021 when disease pressure was medium/high. Overall, the interaction between variety and environment was significant with $p=5.65e^{-12}$, highlighting the importance to repeat field trials in different environments over several years.

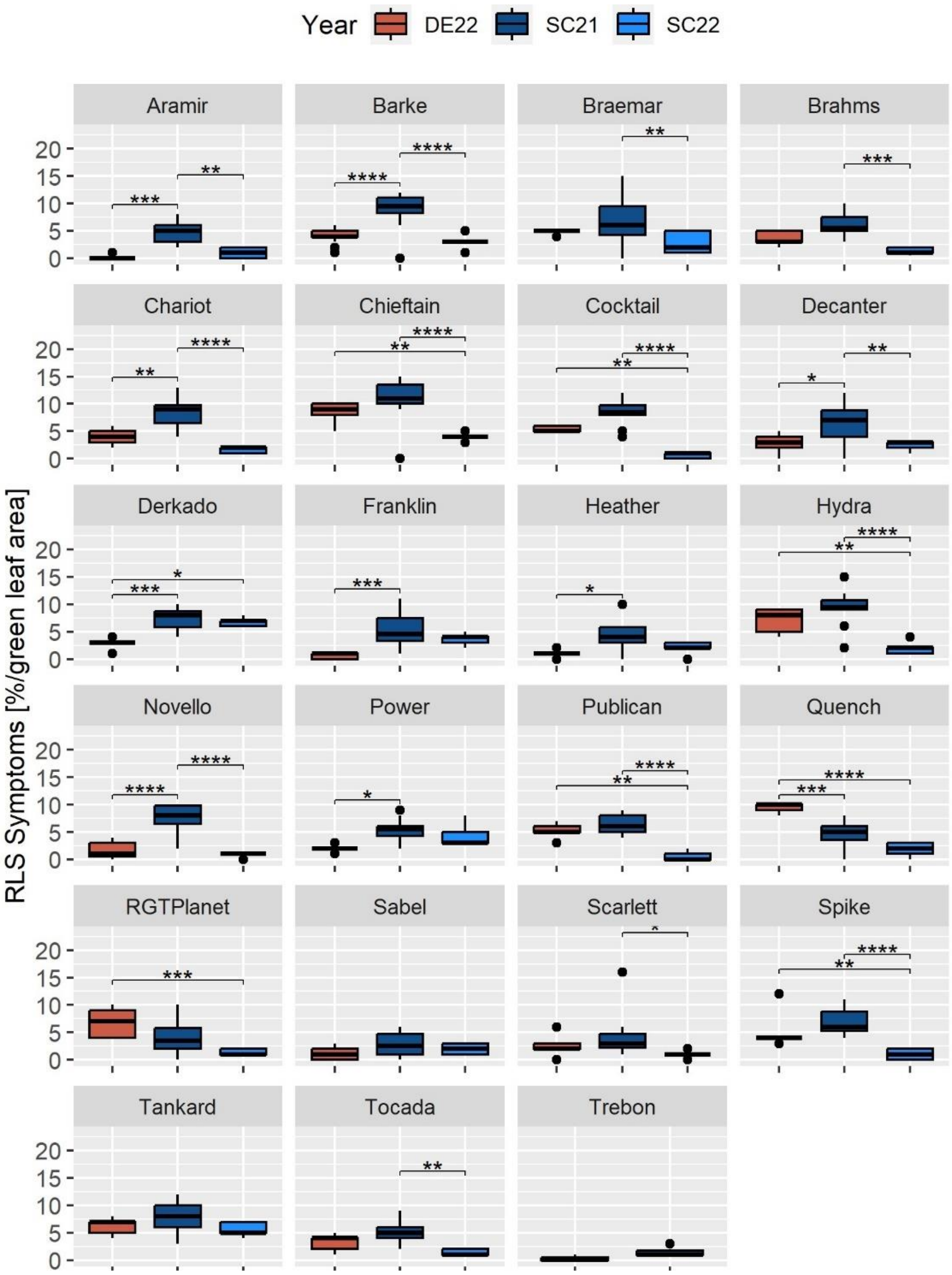

Figure 3.5: Ramularia leaf spot disease levels compared across environments from 2021 to 2022.

Box plots represent disease level based on five to 10 visually assessed leaves per plot in field trials assessed in Scotland in 2021 (dark blue), 2022 (light blue) as well as in Germany 2022 (red). A two-way ANOVA with interaction effect of variety*environment was performed with symptoms as the response variable. A pairwise comparison (pwc) analysis using emmeans was performed between environments within each variety. The shown significance levels were estimated using the Tukey method and adjusted p-values were calculated using the Bonferroni correction (alpha = 0.05).

3.3.2.3 Marker-trait association analysis of JHI-Hv50k-2016-266331 in 2021 and 2022

The association between allelic variation at the observed marker genotype JHI-Hv50k-2016-266331 and RLS severity in each field trial was determined by separating the scored spring barley genotypes into their respective alleles (Figure 3.7). The GWAS analysis (Figure 3.2) associated the A allele with increased tolerance to RLS and the G allele with increased susceptibility. To assess the significance between A and G alleles on disease severity in this study, a student t-test was conducted to estimate significances between both haplotypes and disease levels obtained in each field trial (Figure 3.6). In Scotland the association between genotypes with the A allele providing better resistance was not confirmed in field trials conducted in 2021 (Figure 3.6 A). Genotypes containing the A allele exhibited on average 6.1% RLS compared to the genotypes with the G allele with 6.3% RLS. In 2022, average RLS disease symptoms were 1.8% and 3.7% for varieties with the A and G allele respectively (Figure 3.6 B). The association of genotypes with the A allele providing lower disease symptoms was significant (p-value: 0.0004). The marker-trait association was also significant (p-value: $2.98e^{-4}$) in Germany 2022 (Figure 3.6 C), where average RLS disease levels were 3% in the tolerant genotypes compared to 5% in the susceptible genotypes.

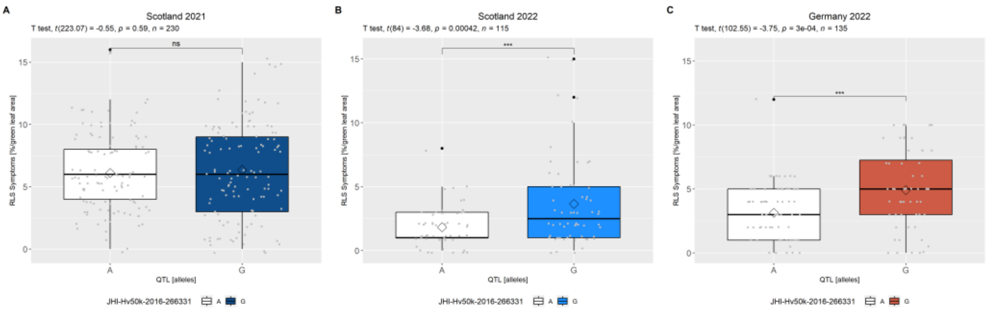

Figure 3.6: Marker-trait association of JHI-Hv50k-2016-266331 over two consecutive seasons in Scotland and one season in Germany.
Boxplots represent the range of Ramularia leaf spot (RLS) disease levels segregated by QTL marker JHI-Hv50k-2016-266331. Allele A represents genotypes with increased tolerance and allele G with increased susceptibility respectively. The light grey dots represent raw data points recorded in each respective genotype. Boxplots are separated by field trials conducted in A: Scotland in 2021; B: Scotland in 2022; C: Germany in 2022. A student t-test was conducted to estimate significances with adjusted p-values (p) using a Bonferroni correction of alpha= 0.05 between allelic variation and RLS disease levels and total number of scored leaves (n) per field trial.

3.3.2.4 Colonisation of *R. collo-cygni* in field trials between 2021-2022

The development of *R. collo-cygni* was quantified via qPCR in F-1 spring barley leaves that have been visually assessed for RLS symptoms (Figure 3.7). In field trials in Scotland, the amount of fungal DNA was also quantified during the asymptomatic development of RLS in 2021 and 2022, but the quantified DNA amounts obtained from these fell under the lowest concentration ($2e^{-6}$ ng*μL^{-1}) of the standard curve and hence, are not presented in this study.

The presence of *R. Collo-cygni* DNA was highest in the Scottish field trial in 2021. Fungal DNA levels varied among the genotypes with values ranging from 451.8 pg in cv. Braemar to 12.3 pg in cv. Trebon. There was no significant (p-value: 0.517) marker (JHI-Hv50k-2016-266331)-trait association observed in 2021 and neither was a correlation between RLS symptoms and *R. collo-cygni* DNA observed (R^2: 0.11). The highest fungal DNA levels were found in cv. Scarlett (368.3 pg) and cv. Tocada (268.2 pg), which exhibited lowest RLS disease symptoms in the same year (Figure 3.4 A). One of the lowest fungal DNA levels was found in cv. Hydra (71.1 pg) which exhibited one of the highest RLS disease scores in that year (Figure 3.4 A).

The presence of *R. Collo-cygni* DNA was lowest in the field trial in Scotland during the 2022 season (Figure 3.7 B), with values ranging from 0.59 pg in cv. Aramir to 15.17 pg in cv. Hydra. There was a significant marker-trait association (p-value: 0.04) observed in Scotland in 2022 with genotypes associated with the resistant, A allele containing on average 1.7 pg *R. collo-*cygni DNA compared to 2.8 pg in susceptible G allele genotypes. There was no correlation between fungal DNA and RLS symptom levels (R^2: 0.03) observed in Scotland during the 2022 season. Similar to 2021, cv. Braemar (4 pg) was in 2022 among the genotypes with the highest fungal DNA levels. In contrast, cv. Fairing (0.23 pg) was among the varieties exhibiting the lowest fungal DNA levels, however exhibited the greatest RLS disease scores (12.8%) in 2022 in Scotland (Figure 3.4 B).

In the 2022 season in Germany, fungal DNA levels varied among the different spring barley genotypes (Figure 3.7 C). *R. collo-cygni* DNA ranged from 24.4 pg in cv. Quench to 0.05 pg in cv. Aramir. There was no significant (p-value: 0.6) marker-trait association observed, and no correlation between DNA and symptom expression (R^2: 0.38). The highest amount of fungal DNA was found in cv. Quench (24.4 pg) and Chieftain (22.3 pg) which also exhibited the highest RLS disease scores that year (Figure 3.4 C). In contrast to 2021, cv. Scarlett (0.57 pg) and Tocada (2.11 pg) were found with the lowest levels of *R. collo-cygni* DNA and exhibited greater resistance to RLS (Figure 3.7 C).

Altogether, it was observed that there is no correlation between RLS symptom development and colonisation by *R. collo-cygni* within one season and that varieties perform differently across seasons. Moreover, it was shown that the marker-trait association depends highly on the environment.

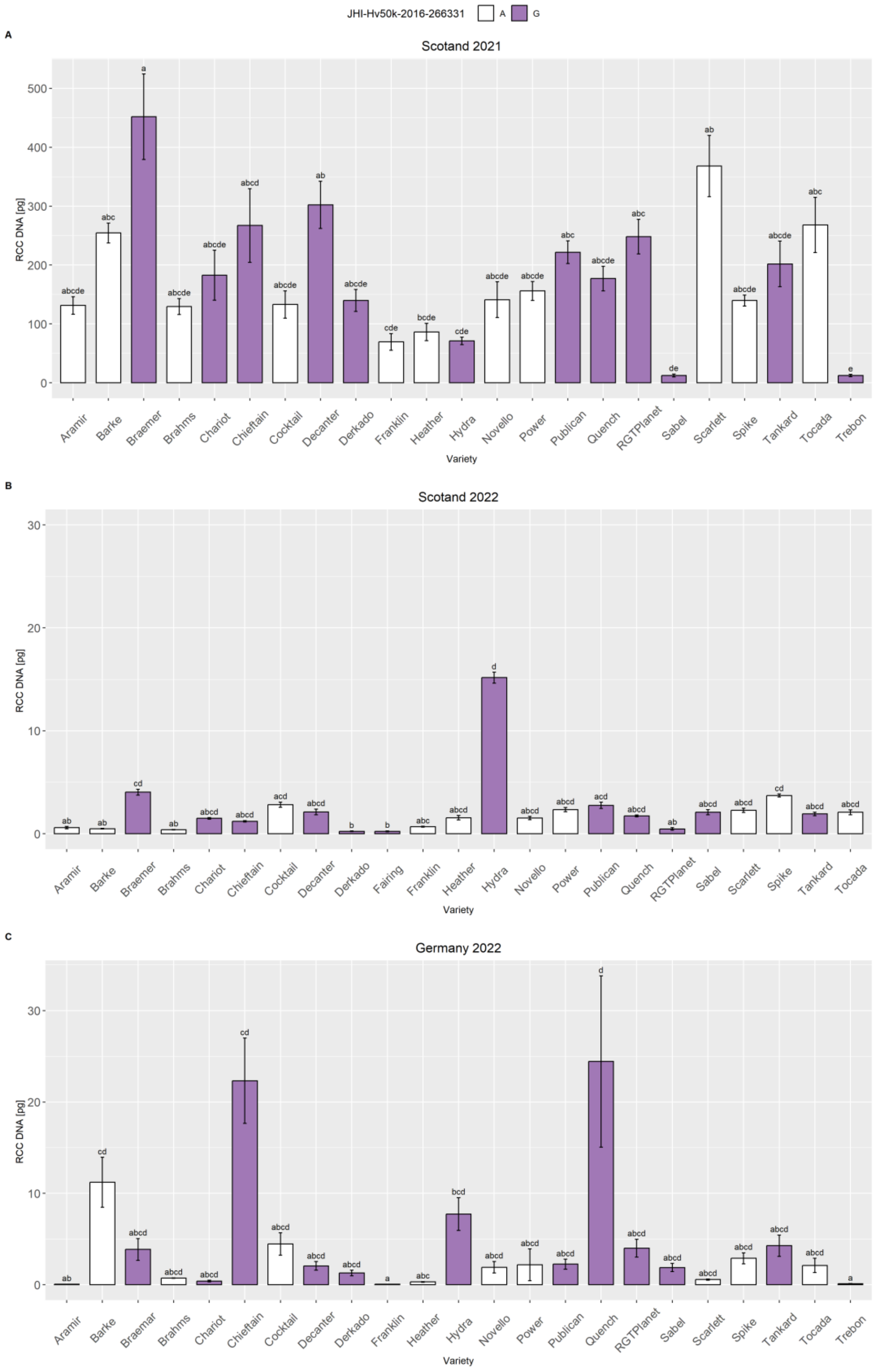

Figure 3.7: *R. collo*-cygni DNA levels in scored spring barley genotypes in field trials from 2021 to 2022.

The histograms represent the mean ± standard error of five technical replicates containing five leaves per plot. *R. collo-cygni* DNA quantified via qPCR. A non-parametric Kruskal-Wallis test was performed to determine whether there is a statistical difference between the medians of fungal DNA of the different genotypes followed by a Dunn's Test to determine the significances between the genotypes. Compact letter design was used to show the significant differences between the genotypes.

3.3.2.5 Leaf nitrogen content in a subset of spring barley cultivars in Scotland 2022

To test whether there is a relationship between senescence and RLS disease prevalence, the leaf N content was determined via C&N analysis in a subset of spring barley cultivars during the 2022 field season in Scotland. The age-related decrease in leaf N content was assessed over three consecutive weeks after GS 72 (early milk) until visual disease assessment at GS 89 (fully ripe). Overall, the leaf N content decreased over time in all eight varieties apart from cv. Barke and Hydra (Figure 3.8). The varieties Heather and Tocada were found to have the most significant impact on the leaf N content among the eight varieties as well the relationship between time and variety was significant (Figure 3.8). These two varieties exhibited the highest leaf N contents at the start of the assessments with intercepts of 3.02 ± 0.75 % and 2.7 ± 0.75 % leaf N for Heather and Tocada respectively. The steepest decline of leaf N content over the three weeks was observed in Heather (Figure 3.8). Quench exhibited the lowest leaf N content at the start of the assessment with 1.46 ± 0.75 % but was not significantly lower than the other six varieties. Altogether, the relationship between time and variety was not significant among cv. Barke, Chieftain, Fairing, Hydra, Quench and RGT Planet (Figure 3.8). The leaf N contents in these six varieties were observed to be lower from the start of the assessment but did not decrease significantly over the following three weeks. Apart from cv. Chieftain (4%) and Fairing (12.8%), all other six varieties exhibited lower than 3% RLS symptom severity (Figure 3.4 B).

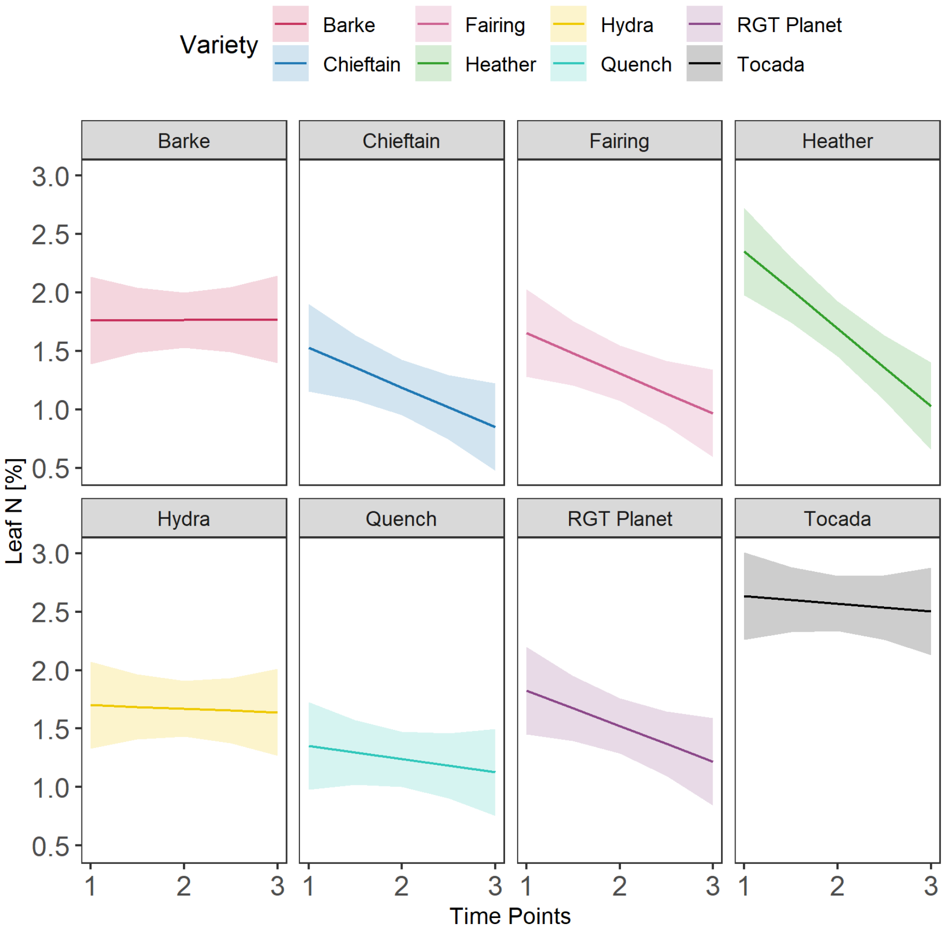

Figure 3.8: Leaf nitrogen content in spring barley genotypes post flowering.
Assessments of leaf nitrogen content were taken over three consecutive time points 1:GS 72; 2: GS 80, 3: GS 89 post flowering. Leaf N content was analysed via carbon and nitrogen analysis and is expressed as a percentage of weighted mass. Linear mixed effect models with response = Leaf N and predictors = time, variety was used to calculate the main and interaction effects to better understand the relationship between time and variety on leaf N. LEM shown represents the mean ± standard error of three biological replicates each consisting of one F-1 leave per genotype.

3.3.3 Susceptibility to Ramularia leaf spot under controlled inoculation experiments in a subset of spring barley varieties

In this study, a subset of spring barley varieties differing in RLS disease tolerance were assessed to examine whether adult susceptibility observed in the field trials of this study can be replicated in controlled inoculation studies using barley seedlings. Disease susceptibility was visually assessed over a period of 21 dpi and *R. collo-cygni* colonisation was quantified via qPCR over the same period. Additionally, the decline in leaf chlorophyll content was monitored via SPAD readings and leaf N contents were quantified at three time points: 7, 14 and 21 dpi.

3.3.3.1 Ramularia leaf spot symptoms development in different spring barley cultivars

Disease development was visually assessed as the percentage of RLS symptoms covering the green leaf area (Figure 3.9). Symptoms first appeared at 10 dpi as pepper spots and steadily increased during the course of the infection. The smallest, significant increase in RLS symptom development over time was observed in cv. Heather (p-value: $2.42e^{-03}$) and Tocada (p-value: $1.94e^{-03}$; Figure 3.9A). The average disease level was 5% and 4% respectively, at 21dpi (Figure 3.9 B). The variety RGT Planet exhibited the greatest symptom development with 21% RLS at 21 dpi (Figure 3.9 B). However, the relationship between time and varieties on symptom response was not significantly different for cv. Chieftain, Fairing, and Hydra (Figure 3.9 A).

Overall, the study indicated that cv. Tocada and Heather exhibited high resistance to RLS as seen in field trials (Figure 3.4). Similarly, cv. Chieftain, Fairing and Hydra appeared to have low resistance to RLS under controlled conditions and exhibited high disease levels under field experiments (Figure 3.4 and Figure 3.9) which indicates a host genetic susceptibility to RLS. While RGT Planet had high tolerance to RLS in in both seasons in Scotland, it showed low disease tolerance during the 2022 field trials in Germany and during artificial inoculation under controlled conditions (Figure 3.4 and Figure 3.9).

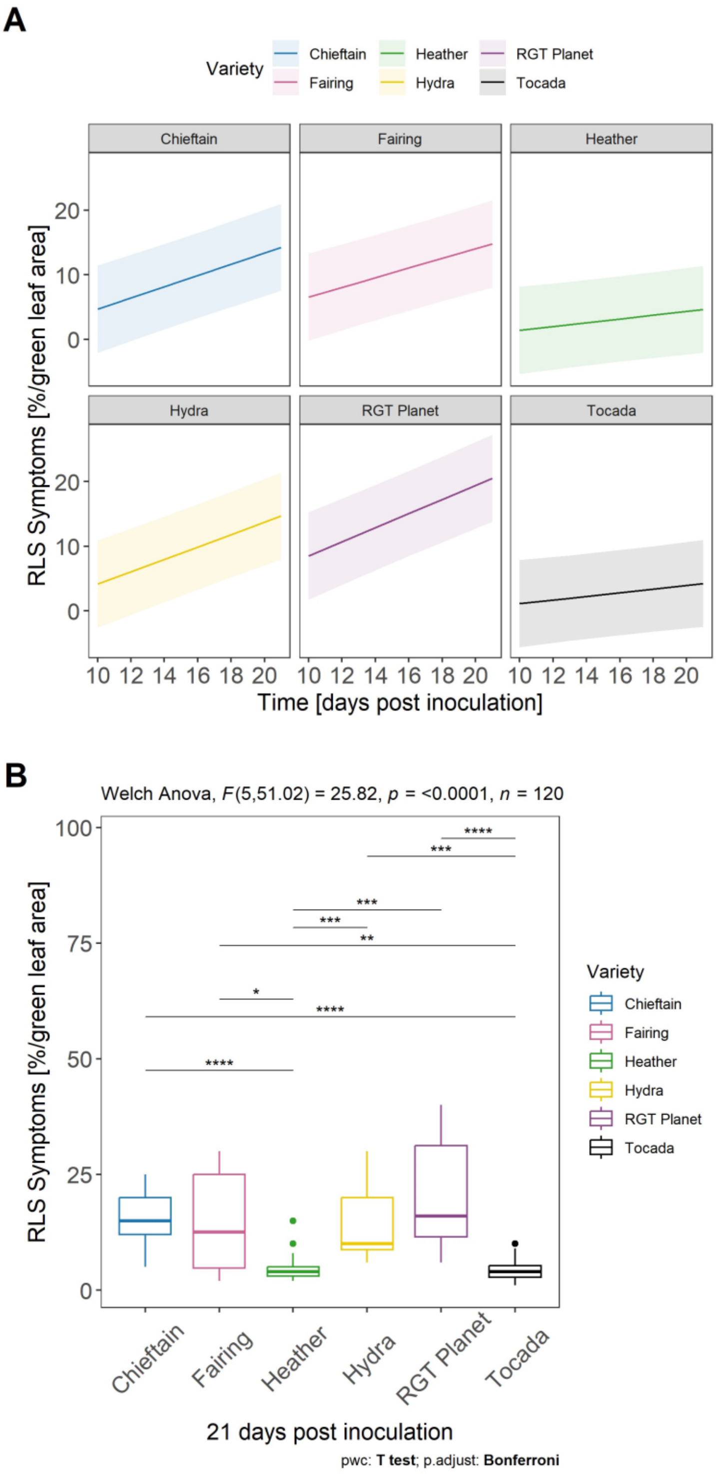

Figure 3.9: Disease severity of Ramularia leaf spot in controlled inoculation studies.

A: RLS disease symptoms development increase in all 6 varieties over the course of the bioassay. Disease levels are expressed as the percentage of green leaf area covered by RLS symptoms. Data in this study contains the results of two independent inoculation experiments where 10 prophyll leaves were scored in each independent experiment. LMEM was used to determine the relationship between time and variety as predictors on RLS symptom development in percent per total leaf area as the response variable. The repetition of the experiment was accounted for by using the experimental number as a random effect. B: Boxplots of the range of disease severity at the end of the experiment at 21-days inoculation are given to highlight the difference among the spring barley varieties. Data show the average of 20 leaves ± standard error. A two-way ANOVA followed by a Tukey post *ad hoc* test was used to determine the significant effects between the mean of groups. The p-value was adjusted using the Bonferroni correction with alpha=0.5.

3.3.3.2 *R. collo-cygni* DNA levels in a subset of spring barley cultivars during artificial inoculation experiments

In addition to visual disease assessments, the amount of *R. collo-cygni* DNA was quantified via qPCR during disease development (Figure 3.10). The fastest increase of fungal DNA was observed in cv. Chieftain (Figure 3.10 A) which contained 562 ± 104 pg of *R. collo-cygni* DNA at 21 dpi (Figure 3.10 B). RGT Planet, which showed a slightly slower but steady increase, contained 392 ± 125 pg of *R. collo-cygni* DNA after 21 dpi. The lowest increase in fungal DNA was observed in cv. Tocada with 123 ± 42.1 pg, Hydra with 146 ± 49 pg and Heather with 150 ± 29.2 pg at 21 dpi (Figure 3.10 B).) Comparing the *R. collo-cygni* DNA levels in cv. Hydra and Fairing (160 ± 53.9 pg) to the RLS symptom development results, in which the varieties exhibited 15% and 14% RLS respectively at 21 dpi, the fungal DNA levels are similarly low compared to the partially resistant cv. Tocada (4% RLS) and Heather (5% RLS) at 21 dpi (Figure 3.9 B and Figure 3.10 B). Overall, this study indicates that the increase of fungal DNA does not appear to follow the trends for varieties observed in symptom development (Figure 3.9). This supports the results observed in the field experiments where no correlation was found between fungal DNA and disease severity (Figure 3.4 and Figure 3.7). Taken together, the results in this study indicate that both field experiments and controlled inoculation studies give similar results for RLS symptom expression and fungal DNA and by this, providing potential candidate varieties for controlled molecular response studies to *R. collo-cygni*.

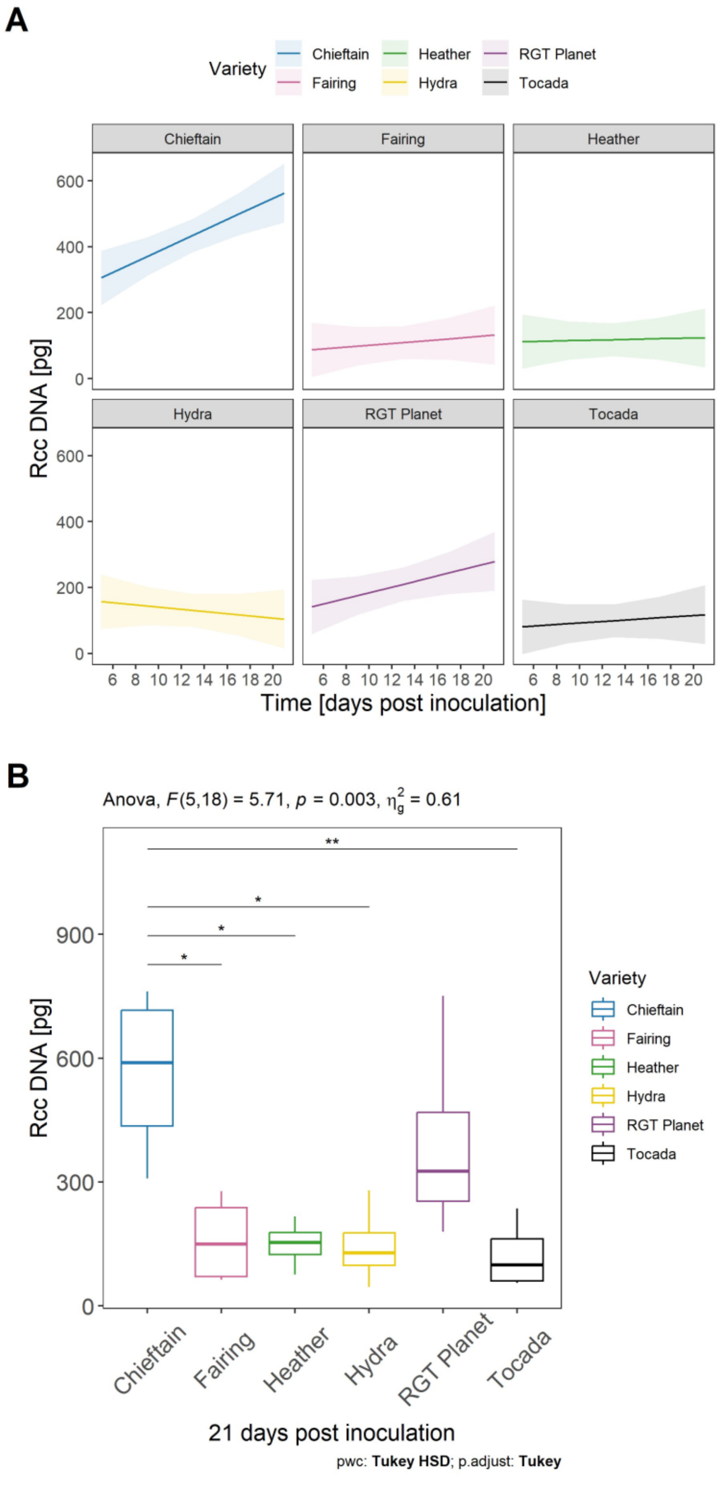

Figure 3.10: *R. collo-cygni* DNA levels during disease development during artificial inoculation of barley seedlings under controlled conditions.

DNA was quantified via qPCR and data show the average of two independent inoculation experiments. A: *R. collo-cygni* DNA levels under controlled inoculation studies increase over time in the performed bioassay. LMEM with time and variety as predicators and *R. collo-cygni*-DNA as the response variable. The experimental number was the random effect to account for the variability of the independent experiments. B: Boxplots of the range of fungal

DNA at the end of the experiment at 21-dpi are given to highlight the difference among the spring barley varieties. Data show the average of fungal DNA in 4 biological replicates ± standard error. A two-way ANOVA followed by a Tukey post *ad hoc* test was used to determine the significant effects between the mean of groups.

3.3.3.3 Relative chlorophyll content in a subset of spring barley seedlings during artificial inoculation studies

The relative chlorophyll content was monitored via SPAD reading during RLS disease development to test whether *R. collo-cygni* induces senescence under controlled inoculation conditions. Overall, the study showed a decline in relative chlorophyll content in all varieties over time regardless of treatments (Figure 3.11). However, the relative chlorophyll content has been observed to be lower in all inoculated varieties from the start of the measurements during the asymptomatic growth stage of *R. collo-cygni* (Figure 3.11). The greatest difference in relative chlorophyll content was observed in RGT Planet, in which the difference amounted to 16.15 SPAD units at the intercept. The variety Chieftain exhibited the lowest relative chlorophyll content in the control (24.6 SPAD units) and in *R. collo-cygni*-inoculated plants (15.3 SPAD units). In comparison, the varieties Hydra, Heather, Fairing and Tocada started with higher relative chlorophyll contents in both treatment groups and the decrease in relative chlorophyll content appeared to have not undergone the same rate of decline as Chieftain and RGT Planet (Figure 3.11 B). Comparing these findings to the results obtained from the fungal colonisation via DNA quantification (Figure 3.10), in which cv. Hydra, Heather, Fairing and Tocada exhibited a steady, low amount of fungal DNA, it appears that *R. collo-cygni* might not have colonised those varieties to the same extend as cv. Chieftain and RGT Planet.

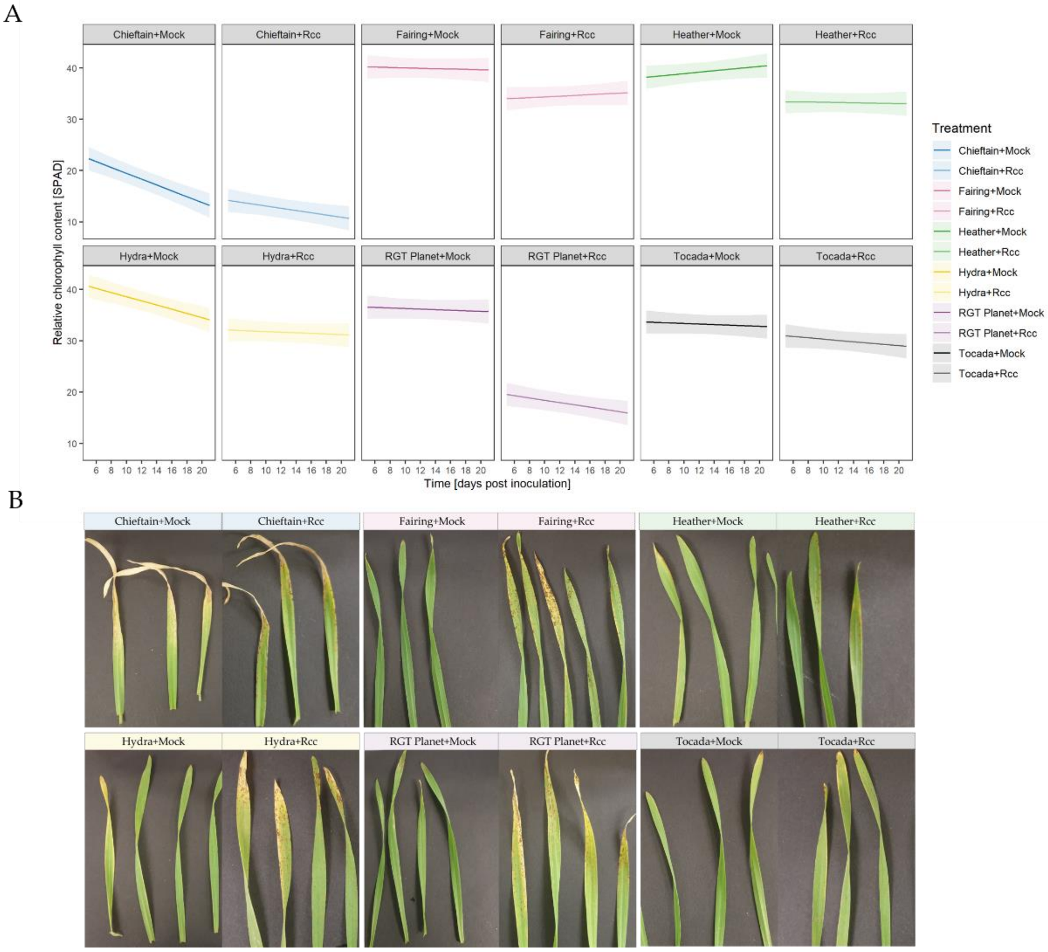

Figure 3.11: Relative chlorophyll content in spring barley seedlings under controlled inoculation studies.

Legend terms: +Mock: inoculated control with potato dextrose broth; +Rcc: inoculated seedlings with ten days old mycelium suspension of *R. collo-cygni*. A: Data show the mean of two independent inoculation experiments. The mean represents 20 disease assessed prophyll leaves ± standard error. A linear mixed effect model was used to determine the relationship between time and variety as predicators and relative chlorophyll content in SPAD units as the response variable. Number of experiments was used as a random effect to account for the variability among the two experiments. B: Representative pictures of the prophyll leaves of spring barley seedlings at the end of the experiment at 21 dpi highlighting RLS disease severity and decline of green leaf area between varieties.

Altogether, the fungal inoculation had a significant effect (p-value: 0) on the response of the relative chlorophyll content in this study (Figure 3.12). Similarly, time was found as a significant component (p-value: 0.004) in the response of SPAD reading. This indicates that *R. collo-cygni* appears to have the ability to decrease the relative chlorophyll content in the studied spring barley seedlings (Figure 3.12). Moreover, it appears that a decline in relative chlorophyll content occurs already at the asymptomatic growth stage (Figure 3.11 A) and the rate of decline appears to reflect the trend in fungal DNA levels (Figure 3.10 A).

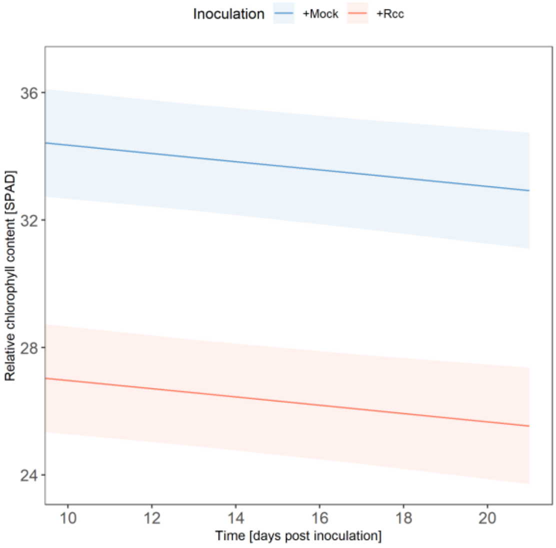

Figure 3.12: The effect of *R. collo*-cygni infection on the relative chlorophyll contents in spring barley varieties.
Figure shows the effect of *R. collo-cygni* inoculation on relative chlorophyll content in the investigated spring barley varieties. Dataset was segregated into the treatment groups with blue showing control plants (+Mock) and red showing the fungal inoculated plants (+Rcc). A linear regression model was performed on the dataset with treatments and time as the main effect and relative chlorophyll content as the response variable. The independent experiments were used the random effect to account for any variation between the two independent inoculation experiments.

3.3.3.4 Leaf nitrogen content in spring barley seedlings during controlled *R. collo-cygni*-inoculation studies

In this study the leaf N content was quantified via C&N analysis at three different timepoints during RLS disease development. The results show that in both *R. collo-cygni* inoculated- and non-inoculated plants, the leaf N content mass in percent decreases over time with time having a significant effect on leaf N content (Figure 3.13). Overall, similarly to the decline observed in the relative chlorophyll content, the leaf N content appeared to be lower in *R. collo-cygni*-inoculated varieties during the asymptomatic phase of RLS development. However, after 21 dpi, the leaf N content appeared to be higher in the inoculated varieties. The steepest decline in leaf N content was observed in the non-inoculated cv. Fairing with an initial leaf N content of 6.3% and decreasing per unit of time by 0.2%. The lowest leaf N content was observed in *R. collo-cygni*-inoculated cv. Chieftain and RGT Planet at 7 dpi with 3.9% and 3.6% respectively. However, it appears that the rate of decline is faster in Heather and Tocada, which also exhibited lower RLS symptom levels compared to the other four varieties (Figure 3.9).

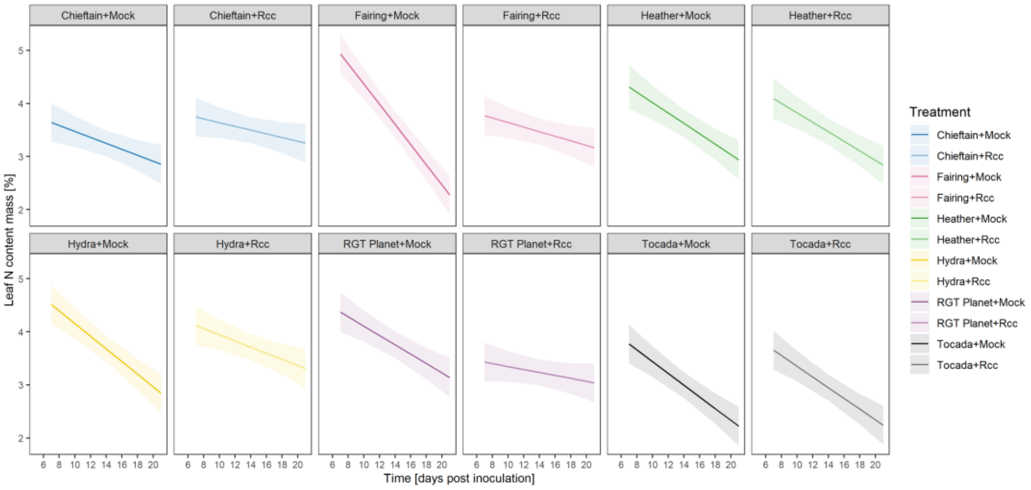

Figure 3.13: Leaf nitrogen content in artificially inoculated spring barley seedlings during Ramularia leaf spot development.
Figure shows the evolution of the leaf N content in *R. collo-cygni*-inoculated (*+Rcc)* and non-inoculated (+Mock) spring barley genotypes. Data show the mean of four biological replicates, consisting of two leaves each quantified via C&N analysis of two independent inoculation experiments. LMEM with time and treatments as predictor variables and leaf N content [%] as response variable was analysed to test the main and interaction effects. The experimental number was the random effect to account for any biological variability in the independent experiments.

Overall, the average effect of *R. collo-cygni* inoculation in the studied spring barley varieties has indicated an initial lower leaf N content, however, leaf N content in inoculated plants did not decrease as quickly over the course of this study as that observed in the non-inoculated plants (Figure 3.14). Over time, the non-inoculated varieties decreased in leaf N content by

0.9% compared to 0.06% in *R. collo-cygni*-inoculated plants. It appears that between the asymptomatic and symptomatic growth stage of *R. collo-cygni*, leaf N levels do not reflect the same decline as observed in the relative chlorophyll content (Figure 3.11). The progressing colonisation by the fungus in spring barley varieties and increasing RLS symptom development (Figure 3.9 and Figure 3.10), does not appear to be reflected in the decrease of photosynthetic available nitrogen.

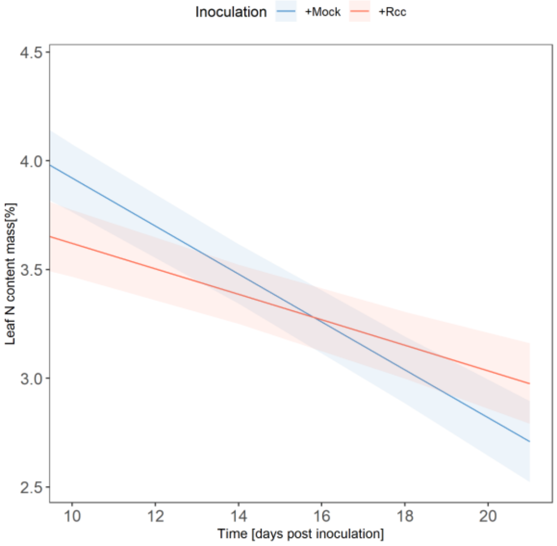

Figure 3.14: The effect of fungal inoculum on the leaf nitrogen content in spring barley cultivars during Ramularia leaf spot development.
Figure shows the effect of *R. collo-cygni*-inoculation on the leaf N content mass in percent in the investigated spring barley varieties. Dataset was segregated into treatment groups with blue showing control plants (+Mock) and red showing the fungal inoculated plants (+Rcc). A linear regression model was performed on the dataset with treatments and time to test main and interaction effects on the leaf N content mass [%] as the response variable. The experimental number was used the random effect to account for any variation between the two independent inoculation experiments.

3.4 Discussion

Improving our understanding of complex genetic traits that shape the phenotypic variation is an ongoing objective of small grain cereal yield and adaptation improvement programmes. GWAS studies have become a powerful approach to unravel the molecular basis underlying these natural occurring phenotypic variations (Alqudah et al., 2020). Understanding the relationship between the genotypic variation and variation in phenotypes will help us better predict disease risks (MacKay et al., 2009). Therefore, a GWAS approach was used in this study to better understand the genetic basis of resistance to RLS disease in barley.

The GWAS identified one major QTL (JHI-Hv50k-2016-266331) at the end of chromosome 4H at the site in Scotland (Figure 3.2) and significant marker-trait associations across all sites for this allele were found (Figure 3.1). The marker-trait association of the studied spring barley panel showed the G allele to be associated with increased susceptibility and the A allele with increased resistance to RLS. The field trials performed between 2021 and 2022 in Scotland and Germany confirmed the hypothesis of RLS being partially controlled by a quantitative trait (Brown et al., 2015; McGrann et al., 2014). Differences in RLS disease levels were observed both at the adult plant stage (Figure 3.4) and at the seedling stage (Figure 3.9). The marker-trait association of spring barley genotypes with the A allele being associated with increased resistance both at the adult and seedling stage was confirmed. While on average the QTL allele had a significant effect on RLS symptom expression in 2022, overall, it was found that each individual genotype appeared to be inconsistent in the resistance to RLS between the two sites and years at the adult plant stage (Figure 3.5 and Figure 3.6). At the seedling stage under controlled inoculation, the performance of the individual genotypes was consistent. Altogether, this indicates that the effect of the QTL on RLS symptom development depends on environmental conditions, which is consistent with previous studies (Brown et al., 2015; Hoheneder et al., 2021; McGrann et al., 2015a; McGrann and Brown, 2018). Environmental conditions were long hypothesised to play a role in the transition from endophytic to necrotrophic growth of *R. collo-cygni* (McGrann and Havis, 2017). This was supported by the observations that in some years, the fungus remains in the endophytic stage without developing symptoms (Havis et al., 2014). The findings of this study showed that there is no correlation between RLS symptom expression and *R. collo-cygni* colonisation at the adult or seedling stage ((Figure 3.7 and Figure 3.10). These observations support the hypothesis that the colonisation by *R. collo-cygni* and a susceptible genotype are not the only factors leading to RLS disease development. In addition, disease development appears to be influenced by environmental factors.

Abiotic stresses such as high light intensity and waterlogging are known to increase RLS symptom formation under controlled conditions (Makepeace et al. 2008; Peraldi et al. 2014; McGrann and Brown 2018). Recently, Hoheneder et al. (2021), studied the tolerance to RLS under controlled drought, irrigation and field conditions between 2016 and 2019. The study revealed strong variations in RLS severity between the rainfall shelter and drought conditions as well as genotypic variations. Plots grown under long-lasting drought periods exhibited lower fungal DNA levels compared to that observed in irrigated plots and exhibited increased resistance to RLS (Hoheneder et al., 2021). Under irrigation, cv. Quench, Barke and RGT Planet exhibited on average above 10% disease levels, which supports the association of susceptibility in varieties carrying the G allele (Hoheneder et al., 2021). Likewise, cv. Quench and RGT Planet exhibited high disease levels in this study during the 2022 field season in Germany (Figure 3.4). In contrast, Scarlett which carries the resistant A allele, exhibited high disease levels under irrigation (Hoheneder et al. 2021) but was a consistently resistant variety in this study. The prolonged period of drought was accompanied by observations of premature senescence before the appearance of necrotic spots (Hoheneder et al., 2021) and led to the hypothesis that drought-induced senescence could inhibit the formation of RLS symptoms in the field. McGrann et al. (2015) showed that overexpression of the barley *stress-responsive NAC* (*HvSNAC1*) transcription factor increased resistance to RLS under controlled conditions in the absence of stress and reduced fungal DNA in the plant. *SNAC1* is involved in the regulation of salt and drought stress in rice and barley (Hu et al., 2006). Understanding how abiotic stresses affect the host response to plant pathogens is important considering that all climate change models forecast an increase in abiotic stress to crop plants (Röhrig and Dussart, 2022).

The development of elite crop varieties with stable yields across different environments is an important goal of breeding programmes. Current elite cultivars derive from relative narrow germplasm pools that are adapted to high input agriculture but less to unpredictable climatic events (Newton et al., 2009). Cereal landraces represent a valuable resource of genetically diverse, locally adapted crops and are associated with traditional farming systems (Newton et al., 2009). One of the major successes in plant breeding derived from an Ethiopian landrace is the loss of function mutation *mlo* which confers broad spectrum resistance to powdery mildew (Piffanelli et al., 2004) and is now present in around 70% of elite material (Dreiseitl 2012). However, the introgression of *mlo* into elite cultivars appeared to have come with trade-offs as increased susceptibility to necrotrophic pathogens has been observed (Jarosch et al., 1999; Makepeace et al., 2007; McGrann et al., 2014). Assessing if the newly identified QTL associated with resistance to RLS already occurred in barley landraces and wild barley may help better understand the natural variation to RLS. Therefore, the spatial distribution of the identified QTL allele associated with increased resistance/susceptibility was investigated in barley landraces and wild barley accessions derived from the James Hutton Institute (Chen et al., 2022). Although not found in wild barley (Figure 3.15 B), the resistant allele (A) is present in 29% of landraces (Figure 3.15 A).

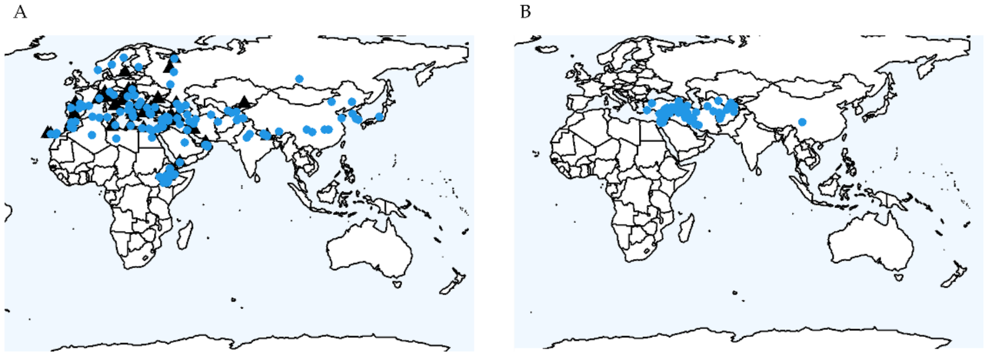

Figure 3.15: Spatial distribution of the identified QTL (JHI-Hv50k-2016-266331), associated with resistance/susceptibility to RLS, in barley landraces and wild barley accessions.
Data points show the spatial distribution of 241 landraces (A) and 206 wild barley (B) accessions by longitude and latitude segregated by the identified QTL marker allele present in the accessions; in blue: accessions with G allele and black: accessions with A allele.

Geographical clustering suggests that resistance arose in the primary (Fertile Crescent) and secondary (Ethiopia) centres of barley diversity until wider deployment in Europe and West Asia. A vast majority (70%) of resistant landraces were found in modern day Turkey, Iran, Iraq, Syria, Israel, Jordan, Ethiopia, north-east of Egypt and Morocco. This indicates that the resistance allele arose with the beginnings of domestication (Nevo, 1992) and might suggest that *R. collo-cygni* has been present before the first reports in Italy in 1898 (Cavara, 1893). Hence, global barley landrace accessions represent a valuable source of resistance to RLS yet to be exploited by breeders and barley geneticists. Recently, Looseley et al. 2020 exemplified the value landraces from Syria and Jordan provide, as the study identified the first diagnostic marker *Rrs1*$_{Rh4}$ on chromosome 3H based on SNPs against *Rynchosporium commune*, the causative agent of leaf scald. Later, Thauvin et al. 2022 narrowed down the physical intervals using GWAS for the previously published resistance QTLs and the study identified kinases and receptor-like kinases (RLKs) associated with 3 QTLs. Both studies highlighted that the absence of *Rrs1*$_{Rh4}$ in the reference Morex genome, makes it difficult to identify true resistance genes and requires resequencing of varieties carrying markers associated with rynchosporium resistance (Looseley et al., 2020; Thauvin et al., 2022). Furthermore, Thauvin et al. 2022 found that in Ethiopian landraces the highest number of resistance QTL to rynchosporium were present, which highlights the valuable resource these landraces bear to be included in pre-breeding programmes.

In this study, 308 genes were identified to underlie QTL- JHI-Hv50k-2016-266331 at the end of chromosome 4H. Based on the literature, the genes more likely to contribute to the phenotype can be broadly grouped into three categories 1) oxidative stress related 2) transcription factors and 3) pattern- and effector triggered immunity related (PTI; ETI; Table 3.2) all of which play a role in plant-pathogen interactions. The QTL interval contained a leucine-rich-repeat (LRR) family protein, a RLK and a receptor-like protein (RLP)-kinase which all belong to a family of pattern-recognition receptors (PRRs) that recognise conserved pathogen--associated molecular patterns (PAMPS) and form the first tier of the plant immune defence (Couto and Zipfel 2016; Jones and Dangl 2006). A transcriptome analysis in barley during RLS infection showed that the expression of RLKs was induced, indicating a recognition of *R. collo-cygni* PAMPS (Sjokvist et al., 2019). In wheat, RLKs and wall associated kinases (WAKs) confer resistance to Septoria tritici blotch (STB) caused by *Z. tritici* (Fones and Gurr, 2015). The first gene to be cloned conferring STB resistance was *Stb6* (Arraiano and Brown, 2006) which was found to consist of a WAK receptor protein conferring qualitative resistance in a gene-by-gene manner though recognition of an avirulent (Avr*)* effector (Saintenac et al. 2018). The second gene, *Stb6q* conferring broad-spectrum STB resistance was the recently cloned from a DH biparental mapping population and encodes a cysteine-rich receptor like kinase RLK sub-family (Saintenac et al., 2021). Whether RLK-mediated gene-by-gene resistance is conserved among the cereal crops recognising PAMPS of *Mycosphaerellaceae*, will need further fine mapping of the QTL locus in barley and cloning of those genes, but those might be promising genes considering *Z. tritici* and *R. collo-cygni* are closely related species (McGrann et al., 2016).

ROS and reactive nitrogen species accumulate upon abiotic and biotic stress responses (Suzuki et al., 2014). ROS interact with apoplastic signalling, cytosolic kinases, phosphatases, redox-sensitive proteins, and transcription factors (Foyer and Noctor, 2013; Mata-Pérez and Spoel, 2019; Mignolet-Spruyt et al., 2016). To protect their cells from oxidative damage, plants have evolved ROS-scavenging enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) as well as antioxidants including ascorbic acid and glutathione (GSH; Mittler et al. 2004; Miller et al. 2010). The QTL on 4H in the current study contained all three ROS-scavenging enzymes: two PODs, two CATs and two Cu/Zn SODs. Two markers were identified for one of the Cu/Zn SOD, JHI-Hv50k-2016-267907 ($-\log_{10}p$: 3.2) and JHI-Hv50k-2016-267912 ($-\log_{10}p$: =2.65) at the Scottish site, indicating a potential haplotypic variation associated with RLS disease resistance.

McGrann and Brown (2018) studied the role of ROS during RLS disease development under controlled inoculation experiments and found that in the partially resistant variety Power no change in the gene expression of ROS-scavengers was observed. Whereas, in the two susceptible varieties Braemar and Golden Promise the superoxide scavenger *CAT1* and *Glutathione Peroxidase 1* were upregulated. Furthermore, *Cu/ZnSOD1 (CSD1)* was upregulated in Braemar at 21 dpi. However, no effects were observed on RLS development in a catalase deficient RNAi Golden Promise or *HvCSD1* lines. Unlike Braemar, Golden Promise carries the resistant QTL allele, potentially explaining why there was no effect of *HvCSD1* demonstrated. Lightfoot et al. (2017) showed that *HvCSD1* expression increased in both susceptible and resistant responses to *P. teres teres* causing NFNB in barley but was higher in the resistance response. Moreover, they observed reduced symptom formation of in RNAi knockdown lines. However, symptoms appeared to be greater than in the resistance response and *HvCSD1* expression was still induced (Lightfoot et al., 2017). This suggests that the observed correlation between *HvCSD1* expression and disease severity to *P. teres teres* could be involved in the observed lack of cell death (Able, 2003; Lightfoot et al., 2017). Infiltrations of barley leaves with ROS scavengers did not have a negative impact on *P. teres teres* growth *in planta,* indicating that fungal growth is not impaired with increased levels of ROS. In McGrann and Brown (2018), infiltrations of catalase or hydrogen peroxide (H_2O_2) in *R. collo-cygni*-infected barley leaves led to increased symptom formation in susceptible varieties, however, the trend was not as consistent as the effect of abiotic stress treatments such as high light and waterlogging on increasing symptom formation. *In vitro* studies further demonstrated that *R. collo-cygni* growth is not impaired by high H_2O_2 levels, whereas *M. oryzae* and *Botrytis* *cinera* growth was inhibited (McGrann and Brown, 2018). Similarly, the infiltration of wheat leaves with catalase resulted in increased susceptibility to *Z. tritici*, whereas H_20_2 infiltration led to increased resistance (Shetty et al., 2007). However, the resistance could have been a result of the observed lower tolerance of *Z, tritici* to H_20_2 (McGrann and Brown, 2018; Shetty et al., 2007). Taken together these results suggest that manipulating the host redox status appears to change the host-pathogen interaction.

The ROS status can also be affected by the host ontogenetic status such as the antioxidative breakdown occurring during monocarpic senescence (Foyer and Noctor, 2015; Heiser et al., 2003; Schützendübel et al., 2008). In the field, symptoms of RLS typically become apparent post flowering when barley crops start to senesce. Previous studies linked leaf senescence, a decline of the associated leaf antioxidant status and a subsequent accumulation of ROS to symptom expression in barley seedlings and adult plants (McGrann et al., 2015b; Schützendübel et al., 2008). Consistent with previous findings, a decline in the leaf nitrogen status that is associated with the nitrogen remobilisation occurring post flowering, has been observed in the Scottish 2022 field trial in 2022 between GS 72 to GS 89 in the most susceptible varieties (Figure 3.9). Likewise, leaf N content decreased over time in controlled inoculation studies both in non- and *R. collo-cygni*-inoculated plants (Figure 3.14). During the asymptomatic growth phase of RLS, between 5 to 7 dpi, the relative chlorophyll content was lower in *R. collo-cygni*-inoculated varieties (Figure 3.12). The greatest decline was observed in the two most susceptible varieties Chieftain and RGT Planet under controlled conditions (Figure 3.9; 3.12). These results support the hypothesis that the fungus induces premature leaf senescence (Sjokvist et al., 2019). The higher leaf N content at 21 dpi may be explained by the colonisation of fungus contributing to higher nitrogen levels as total N content was measured. Pathogen infection often leads to the induction of *Senescence-Associated Genes (SAGs)* (Espinoza et al., 2007) suggesting an overlap of molecular responses to processes regulating senescence and plant defence (Mason et al., 2016). A proteomic comparison study between one early-senescencing barley variety 10_11, a NIL to the late senescencing cv. Karl, showed that PR1 proteins, intracellular and membrane receptors including nucleotide binding site (NBS)-LRRs, and LRR-RLK were upregulated in the early-senescencing phenotype (Mason et al., 2016). The phenotype is controlled by *HvNAM-1* which belongs to the *NAC* transcription factors and the phenotype is characterised by accelerated leaf chlorophyll degradation as well as flag leaf proteins including Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO; Jukanti et al. 2008; Jukanti and Fischer 2008). *NAC* and WRKY transcription factors are involved in senescence regulation as well as responses to abiotic and biotic stresses (Kumar et al., 2018; McGrann et al., 2015a; Puranik et al., 2012). Both NAC and WRKY were upregulated during *R. collo-cygni* colonisation of the apoplast at 7 and 12 dpi (Sjokvist et al., 2019) and also appear to be associated with the QTL- JHI-Hv50k-2016-266331 (Table 3.2). Overexpression of *HvSNAC1* in barley led to reduced RLS disease symptoms and fungal growth under controlled conditions, but it did not affect the regulation of ROS-scavengers (McGrann et al. 2015b). Likewise, in *B. sorokiniana tolerant* (*bst)* barley mutant lines that are resistant to spot blotch and in which chloroplast degradation occurred slowly, RLS symptoms were expressed similarly high to the wild-type Bowman (McGrann and Brown, 2018). This raised the hypothesis that a gradual breakdown of the chloroplast leading to increased levels of ROS perhaps plays key signalling to trigger the transition of *R. collo-cygni* to become necrotrophic (McGrann and Brown, 2018). In support of this hypothesis, it is well known that abiotic stresses such as salt and drought stress can lead to premature leaf senescence followed from an accumulation of ROS, subsequent lipid peroxidation in the membranes of the chloroplast and protein degradation (Alamri et al., 2020; Allu et al., 2014). Congruent with the hypothesis, abiotic stress treatments using high light intensity led to early leaf senescence and increased formation of RLS symptoms in susceptible barley varieties under controlled conditions (McGrann and Brown, 2018). Altogether, this study supports the hypothesis that ROS-mediated processes leading to senescence such as biotic stresses and abiotic stresses may trigger the necrotrophic transition of *R. collo-cygni* (McGrann and Brown, 2018). To summarise, this study has demonstrated that *R. collo-cygni* appears to induce premature leaf senescence under controlled conditions before symptoms become apparent, but it also appears that the extent of it depends highly on the genotype (Figure 3.11). While there appears to be a causal relationship between senescence (Figure 3.11), rate of symptom formation (Figure 3.9), and fungal colonisation (Figure 3.10) for the most susceptible genotype Chieftain at the seedling stage, it appears less apparent for other genotypes. Hydra and Fairing were both highly susceptible at the adult (Figure 3.4) and at the seedling stage (Figure 3.9), yet the rate of senescence (Figure 3.11) and the amount of *R. collo-cygni* DNA were comparable to the two most resistant varieties, Tocada and Heather. Further insights are needed to untangle the effects of ROS, senescence, and abiotic stresses on epidemiology of *R. collo-cygni,* and this study provides a good foundation for developing mutant barley lines to further test these hypotheses.

Two MLO-proteins were found within the QTL JHI-Hv50k-2016-266331. MLO proteins are seven transmembrane-domain proteins encoded by the wildtype *MLO* gene, which interacts with calmodulin and negatively regulates plant defence responses including plant cell death, resulting in susceptibility to powdery mildew caused by *Blumeria graminis f. sp. hordei* (Büschges et al., 1997; Consonni et al., 2006; Kim et al., 2002). Fungal penetration and development inside the host is prevented in loss-of-function mutation in *mlo* (Piffanelli et al., 2002). One of the MLO proteins is associated with two SNPs: JHI-Hv50k-2016-266643 (C/T) and JHI-Hv50k-2016-266644 (G/A) respectively. The other MLO protein is associated with SNP JHI-Hv50k-2016-266730 (C/T). MLO proteins and MLO-like proteins were upregulated at 3 dpi after infection with the Danish *R. collo-cygni* isolate DK05 in varieties containing the wildtype *Mlo* gene, suggesting an early response during the asymptomatic phase of RLS development (Sjokvist et al., 2019). Moreover, Lemcke et al. (2021) found variations in MLO transcripts between two barley varieties differing in their suceptibility to RLS in a transcriptomic analysis. The more susceptible cv. Tipple exhibited significantly increased MLO transcripts at 3 dpi, whereas increased MLO transcript levels in the partially resistant cv. Fairytale were observed at 7 dpi. Among the 28 investigated genotypes in this study, three haplotypes exist for the MLO marker combination; H1: C/A/C, H2: T/G/C and H3: T/G/T. H1 represents the most abundant haplotype with all of the susceptible associated varieties representing this haplotype and several partially resistant genotypes. The susceptible cv. Trebon represents H2 and exhibited consistently low disease levels in the field (Figure 3.4). H3 is present in cv. Aramir, Brewster, Spike, Tocada and Heather of which all carry the RLS resistant A allele. Apart from cv. Spike that exhibited only partial resistance in the field, and Brewster that did not germinate, Aramir, Tocada and Heather had consistently low disease levels (Figure 3.4). Cultivars Aramir, Spike, Trebon and Heather also exhibited consistent low *R. collo-cygni* DNA levels (Figure 3.7) suggesting that this haplotype might impair fungal colonisation. Piffanelli et al. (2004) identified three haplotypes of the MLO locus in cv. Ingrid which were characterised by four SSR markers (10267, 10517, 11679, 11240) that were later described as barley oligonucleotide pool assay (BOPA) markers 12_30987, 12_30988, 12_30990, 12_11139 respectively (Figure 3.16) and are all present in the locus of QTL JHI-Hv50k-2016-266331. Three of BOPA markers were used as diagnostic markers for *mlo-11*: 12_30987, 12_30988, 12_30990 respectively (Piffanelli et al., 2004). 12_30987 has not yet been genotyped as part of the 50k iSelect SNP dataset (personal communication Luke Ramsay). The genotyping of the third marker is currently underway at the James Hutton Institute as it is essential to identify which *Mlo* genes are present in the varieties used in this study. Due to the covid-19 panedmic this could have not been carried out previously. Based on Piffanelli et al. (2004), cv. Chariot, Derkado and Tankard all carry the *mlo-11* based and also contain the susceptible G allele. While cv. Tankard exhibits consistently high RLS disease levels but not fungal DNA levels, cv. Chariot and Derkado vary greatly by year and environment in symptoms and fungal colonisation (Figure 3.5; and Figure 3.7). Taken together these results suggest that *mlo-11* appears to contribute to susceptibility but is not the only driver.

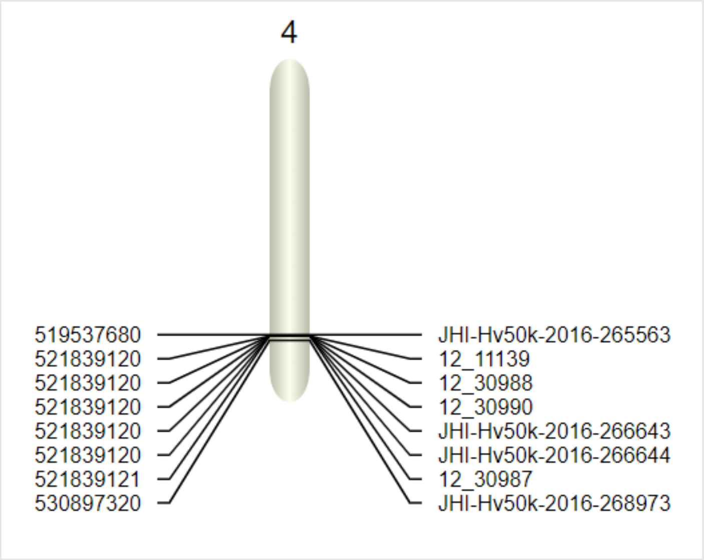

Figure 3.16: Position of previously characterised MLO markers on chromosome 4H relative to the flanking markers of the QTL.
Flanking markers for the start JHI-Hv50k-2016-265563 and JHI-Hv50k-2016-268973 end of the QTL. With following markers previously associated with MLO proteins in barley (Piffanelli et al., 2004): 12_30987, 12_30988, 12_30990, 12_11139 as well as JHI-Hv50k-2016-266644 and JHI-Hv50k-2016-266643 identified in this study.

Several studies have linked the presence of *mlo* to susceptibility to necrotrophic pathogens, such as *B. sorokiniana,* (Kumar et al., 2001) *F. graminearum* (Makepeace et al., 2007) and *M. oryzae* (Kumar et al. 2001; Makepeace et al. 2007; Jarosch et al. 1999). Although, the majority of resistance to powdery mildew arises from specific resistance genes associated with the *Mla* locus on chromosome 1H and to date more than 38 resistance genes have been described (Czembor et al., 2022; Dreiseitl, 2020). The link between *mlo* on chromosome 4H and RLS has been studied both in the field and under controlled conditions using *mlo-11* and *mlo-5* mutant lines as well as genes influencing the effect of *mlo* such as *ror1, ror2*, and *emr1* (Brown and Makepeace, 2009; Hofer et al., 2015; McGrann et al., 2014, 2020; McGrann and Brown, 2018; Pinnschmidt and Jørgensen, 2009). Overall, it appears that the effect of *mlo* on RLS severity differs greatly depending on the environment and with the plant genetic background (Havis et al., 2015).

This study supported the G x E effect on RLS development as observed over two seasons in Scotland and Germany (Figure 3.5). Moreover, the GWAS also identified an association with the *Mlo* locus (Figure 3.17). However, narrowing down of the identified QTL allele using a biparental population would be required to investigate how closely linked those two loci are. McGrann et al. (2014), showed in field trials using the DH Power (*Mlo*) and Braemar (*mlo-11*) that lines carrying the *mlo-11* allele exhibited significantly more RLS disease symptoms than *Mlo* lines. These results contrast with that of the present study, where Decanter (*mlo-11)* and Cocktail (*Mlo)* did not show an overall effect with *mlo-11* across all sites on RLS severity. However, while in both sites in Germany *mlo-11* exhibited more RLS than *Mlo,* in Scotland, in the Decanter X Cocktail (DECO) population, *mlo-11*-lines exhibited less symptoms compared to *Mlo* (McGrann et al., 2014). Similar results are observed in this study, while Power and Braemar were consistently resistant and susceptible respectively, Cocktail and Decanter differed greatly by year and site (Figure 3.4 and Figure 3.5). Interestingly, Power (*Mlo)* and Braemar (*mlo-11*) contain the resistant A and susceptible G allele respectively whereas Decanter (*mlo-11*) carries the resistant A allele and Cocktail (*Mlo)* carries the susceptible G allele. This supports the idea that the effect of *mlo* depends on other genes, and the susceptible effect of *mlo-11* on RLS might be partially compromised in plants carrying the A allele. In *mlo5+ror1-2*, *mlo5+ror2* NIL backgrounds, resistance to *R. collo-cygni* was increased compared to the Ingrid-*mlo5 ROR1 ROR2* (McGrann et al., 2014). The *mlo* locus was also effected in lesion mimic mutants in which *nec1* reduced RLS symptoms in wildtype *Mlo* barley plants, while *mlo-5* restored the susceptibility in *nec1* to *R. collo-cygni* (McGrann et al., 2015b).

Studies have also shown that abiotic stresses such as high light intensity led to increased RLS severity under controlled conditions (Makepeace et al., 2008b), and the response was indicated to be enhanced by the presence of *mlo* alleles (Brown and Makepeace, 2009). This could explain the difference observed between years and sites (Figure 3.5). The decline in relative chlorophyll content and accumulation of ROS production associated with the onset of senescence appears to be associated with RLS symptom development (McGrann and Brown, 2018; Schützendübel et al., 2008). Similarly, the loss of *Mlo* enhances the cell-wall-restricted H_2O_2 burst (Peterhänsel et al. 1997; Piffanelli et al. 2002). The enhanced mesophyll cell death in *mlo* plants has been suggested to contribute to the increased susceptibility to *M. oryzae* and *B. sorokiniana* (Jansen et al., 2007; Kumar et al., 2001). In contrast, in a dwarf barley mutant line that results in increased DELLA protein accumulation, which in turn delays ROS-induced cell death, increased susceptibility to both *R. collo-cygni* and *B. graminis* was observed (Saville et al., 2012). The widespread use of *mlo*-alleles in elite spring barley cultivars with its pleiotropic effect on other barley pathogens and the effect of abiotic stresses on RLS and *mlo*, highlight the importance of untangling this effect and trying to understand which genes interact with the *Mlo*-locus (Brown and Makepeace, 2009; Jansen et al., 2007; Kumar et al., 2001; Makepeace et al., 2007).

To summarise, this study has not only highlighted the complexity of the genetic basis of RLS disease resistance, but also identified a small effect QTL that appears to be influenced by environmental stimuli as shown in the field studies. The GWAS and the subsequent adult and seedling stage experiments have built a foundation for follow up studies to choose parents for population studies in different environments across Europe. The population study could not only narrow down the QTL region to identify candidate genes, but also be used to select genotypes in order to generate NILs to study the effect of different abiotic stresses under controlled *R. collo-cygni* inoculation studies.

Chapter 4:

Ethylene-mediated immune responses may suppress the development of Ramularia leaf spot in barley

4.1 Introduction

4.1.1 The basic principle of the plant immune response to pathogens

Although, plants are resistant to the majority of putative pathogens, plants pathogens have evolved diverse life strategies to overcome preformed defences to take advantage of the provided nutrient niche of plants (Dangl et al., 2013).This has required the development of a plethora of pathogenicity and virulence factors in pathogens. The co-evolution of host and its pathogen led to a high degree of adaption to a limited number of plants (Hückelhoven et al., 2013; Jones and Dangl, 2006). The response to an infection follows a two-tiered innate immune system comprised by 4 phases that represent the evolutionary timescale of plant pathogen interactions. The evolution of the plant immune system and pathogen effectors has been described as the so called *zigzag* model by Jones and Dangl, (2006). The first tier of the plant immune system is formed by extracellular pattern-recognition receptors (PRRs) that recognise evolutionary conserved pathogen (or microbial)-associated molecular patterns (PAMPs or MAMPs). Moreover, plants can detect the release of endogenous molecules from non-self-activities, such as cell-wall or cuticular fragments, known as damage-associated molecular patterns (DAMPs) (Dangl et al., 2013; Spoel and Dong, 2012). PAMPs typically represent components of pathogens, such as bacterial flagellin, fungal chitin or oomycete β-glucan (Dodds and Rathjen, 2010). PRRs that detect these ligands are typically leucine-rich repeat (LRR) kinases, lysine motif (LysM) kinases or transmembrane receptor-like proteins that lack a signalling domain (Dangl et al., 2013; Dodds and Rathjen, 2010). The stimulation of PRRs leads then to pattern-triggered immunity (PTI), which comprises diverse physiological responses including reactive oxygen species (ROS) accumulation, mitogen-activated protein kinase (MAPK) activation, calcium signalling, transcriptional reprogramming and the production of phytohormones (Yuan et al., 2021).

The second tier of perception is comprised by intracellular receptors that detect virulence molecules, commonly known as effectors, deployed by successful pathogens (Dodds and Rathjen, 2010). These effectors interfere with PTI, leading to effector-triggered susceptibility (ETS; Jones and and Dangl 2006). Through co-evolution between plants and pathogens, plants have evolved intracellular nucleotide-binding leucine-rich repeat receptors (NLRs), which are often encoded by *Resistance* (*R*) genes, and the detection of those pathogen 'avirulence' signals induces effector-triggered immunity (ETI; Spoel and Dong 2012; Ngou et al. 2022). Unlike PTI, which is widely conserved across families, ETI represents a high diversification of receptors both within and between species, and thus, is highly specific (Dodds and Rathjen, 2010). Characteristically, ETI leads to a hypersensitive response (HR) associated with programmed cell death of infected cells and production of antimicrobial molecules, such as phytoalexin, in the surrounding tissue (Spoel and Dong, 2012). This leads to a local resistance to the pathogen, mainly against biotrophic pathogens that feed on living host tissue (Glazebrook, 2005). While cell death is initiated for immunity against biotrophs, cell death can also be induced by toxins produced by necrotrophs that actively kill hosts to acquire nutrients (Bürger and Chory, 2019; Glazebrook, 2005). Following initial infection, different immune responses can be activated in plants which involve phytohormones that trigger extensive transcriptional reprogramming regulating defence responses (Berens et al., 2017). For example, systemic acquired resistance (SAR) is mediated by the hormone salicylic acid (SA), which triggers the transcriptional activation of *Pathogenesis-Related (PR)* genes (Berens et al., 2017). In *A. thaliana*, SA is a positive regulator of resistance against biotrophs and hemibiotrophs, while jasmonic acid (JA) positively regulates the immunity against necrotrophic pathogens (Glazebrook, 2005) and triggers induced systemic resistance (ISR; Ton et al. 2002). The JA pathway in *A. thaliana* can be divided into the MYC and Ethylene Response Factor (ERF) branch (Pieterse et al., 2012). While the MYC branch involves MYC-type transcription factors and the JA-responsive marker gene *Vegetative Storage Protein2* (*VSP2*). The ERF branch in *A. thaliana* is co-regulated by ethylene (ET) and involves members of the Apetal2/Ethylene Response Factor (AP2/ERF) family of transcription factors including ERF1 and Octadecanoid-Responsive Arabidopsis59 (ORA59; Pré et al. 2008; Pieterse et al. 2012) as well as the JA-responsive marker gene *Plant Defensin 1.2* (*PDF1.2;* Pieterse et al. 2012). The two branches of JA signalling are antagonistically regulated via MYC2 and ERF1 respectively, with Jasmonate Zim Domain (JAZ) proteins repressing the MYC2 and ERF1 independently (Bürger and Chory, 2019). Thus, disease resistance mediated by phytohormones comprises complex interactions between different phytohormones, including SA, JA, and ET, and enables plants to tailor their inducible defence responses against a wide diversity of pathogens (Aerts et al., 2021; Gupta et al., 2023).

4.1.2 Ethylene biosynthesis and signalling

ET is a gaseous hydrocarbon that is known to be involved in several aspects of plant developmental and physiological processes, including seedling emergence, senescence, fruit ripening and response to abiotic and biotic stresses (Abeles et al., 1992). Methionine constitutes the essential molecule for the ET biosynthesis (Figure 4.1; Adie et al. 2007). The first step comprises the conversion of methionine via the enzyme S-Adenosylmethionine (SAM) synthase into SAM (Bürger and Chory 2019).

Following, SAM is enzymatically converted into 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase which is considered to be the rate-limiting step in ET biosynthesis (Adie et al., 2007). The final step is the conversion of ACC into ET via ACC oxidase (ACO; Ju et al. 2015).

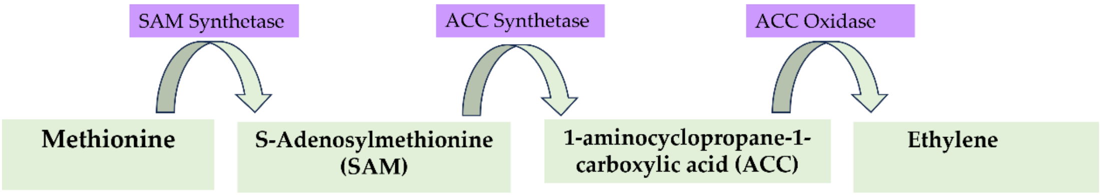

Figure 4.1: Ethylene biosynthesis pathway.
Adapted from (Bürger and Chory, 2019)

In *A. thaliana*, multiple receptors perceive ET, including the histidine kinase proteins Ethylene-Responsive 1 and 2 (ETR1; ETR2), Ethylene Response Sensor 1 and 2 (ERS1;ERS2) as well as the Ethylene Insensitive 4 (EIN4) (Bürger and Chory, 2019; Shigenaga and Argueso, 2016). The activation of these histidine kinases inhibits a downstream serine/threonine kinase Constitutive Triple Response 1 (CTR1). In the absence of ET, the receptors activate CTR1, which in turn, suppresses the ET response via phosphorylation of EIN2 (Merchante et al., 2013). In presence of ET, EIN2 is dephosphorylated, cleaved and translocated into the nucleus, where it contributes to stabilising EIN3 and EIN3-like (EIL) transcription factors that induce ET-controlled gene expression (Shigenaga and Argueso, 2016).

4.1.2.1 Ethylene responsive transcription factors

The Apetala 2 (AP2) family of transcription factors represents one of the largest groups that respond to abiotic and biotic stresses (Nakano et al., 2006; Riechmann et al., 2000). The AP2 family has been hypothesised to have originated from a horizontal transfer of bacterial or virus HNH-AP2 endonuclease (Magnani et al., 2004). The AP2 family is characterised by conserved protein domains that have been well studied in a variety of plants, including *A. thaliana*, pear, rice and barley (Guo et al., 2016; Li et al., 2018; Nakano et al., 2006). Depending on the protein domain, the AP2 family is classified into the AP2 subfamilies, Related to Abscisic Acid Insensitive 3/Viviparous 1 (RAV), Drought-Responsive Element Binding factor (DREB) and ERF (Sakuma et al., 2002). The AP2 subfamily is involved in developmental events and contains two AP2/ERF domains (Nakano et al., 2006). The RAV subfamily is characterised by one AP2/ERF domain and one B3 domain, while one single AP2/ERF domain is found in the DREB and ERF subfamilies (Liu et al., 1999). DREB transcription factors recognise a Dehydration-Responsive Element (DRE)/C-repeat(CRT) element and mainly found to be expressed during abiotic stress responses (Sakuma et al., 2002; Thirugnanasambantham et al., 2015). By contrast, ERF proteins are responsive to abiotic and biotic stresses, due to their ability to bind to the GCC box (AGCCGCC) present in promotors of ethylene-inducible genes (Ohme-Takagi and Shinshi, 1995) and JA/ET-responsive genes (Gutterson and Reuber, 2004), as well as to DRE/CRT elements (Eini et al., 2013). The activation of *ERF* genes follows from EIN3 binding to a response element present in the *ERF promotors* (Bürger and Chory, 2019).

4.1.3 The role of ethylene and ethylene response factors in plant-pathogen interactions

Depending on the pathogen, environmental conditions and plant species, ET can either contribute positively or negatively to host immunity (Berens et al., 2017). In *A. thaliana* it was shown that the transcription factor *AtERF1* was synergistically inducible by JA and ET and constitutive expression of *AtERF1* led to activation of *PDF1.2* (Lorenzo et al., 2003). Berrocal-Lobo et al. (2002) showed that *AtERF1* was induced upon *Botrytis cinera* infection and that the overexpression of *AtERF1* conferred resistance to the necrotrophic pathogens *B. Cinera* and *Plectosphaerella cucumerina*. Transcription factors can also negatively regulate host immunity depending on the pathosystem as demonstrated by Son et al. (2012) who demonstrated that *A. thaliana, AtERF5* appeared to negatively regulate resistance to the necrotrophic pathogen *Alternaria brassicola* causing black spot disease on a wide range of hosts, via the repression of chitin-mediated defense responses. The same study showed that *AtERF5* appeared to positively regulate resistance to the bacterial pathogen *P. syringae* via the SA signalling pathway (Son et al., 2012; Thirugnanasambantham et al., 2015).

ERFs were also shown to respond to both biotic and abiotic stressed due to their ability to bind to GCC-box of JA/ET inducible genes and DRE/CTR elements of abiotic stress responsive genes (Eini et al., 2013; Ohme-Takagi and Shinshi, 1995). Increased tolerance to high salt stress was seen in transgenic *A. thaliana* plants overexpressing the barley transcription factor *Hv root abundant factor* (*HvRAF*; Jung et al. 2007). The study also found increased resistance to the soilborne, root pathogen *Ralstonia solanacearum* responsible for bacterial diseases on a wide host range (Genin and Denny, 2012). Moreover, the study has indicated that both biotic and abiotic stress-responsive genes, including *PDF1.2, PR1, PR5, GSH1,* and the salt responsive *cold inducible 2* (*KIN2;* Kurkela and Borg-Franck 1992), were induced upon *HvRAF* overexpression in *A. thaliana*, explaining the increased resistance to both stressors (Jung et al., 2007). The transcription factor *HvRAF* was shown to be inducible by SA, ET, Methyl Jasmonate (MeJA), but not ABA, further indicating an ABA independent pathway (Jung et al., 2007).

The findings in *A. thaliana* led quickly to discoveries in the most important crops such as wheat and rice to study whether resistance to major pathogens can be conferred by modulating the host immune response. For example, in hexaploid bread wheat (*Triticum aestivum)* transcription factor, *pathogen-induced ERF1* (*TaPIE1*) was found to be involved in host responses to sharp eyespot caused by *Rhizoctonia cerealis* and freezing tolerance (Zhu et al., 2014). The overexpression of *TaPIE1* enhanced significantly resistance to the fungal pathogen and freezing stresses, while under-expression led to susceptibility to both stresses, indicating that *TaPIE1* acts as a positive regulator for the synergistic cross talk (Zhu et al., 2014). Moreover, the study by Zhu et al. (2014) found that stress-related genes, such as the ROS scavenging gene peroxidase (*POX2)* and ethylene biosynthesis genes like ACO2, as well as defense related genes *PR10* and *chitinase* (*CHI2*), were upregulated by *TaPIE1* overexpression and upon *R. cerealis* infection. Similarly, exogenous ACC application increased *TaPIE1* activity and in turn, increased transcript levels of these defense- and stress-responsive genes. However, ACC application was not able to recover the decreased tolerance of *TaPIE1*-underexpressing plants to freezing and the fungus (Zhu et al., 2014), indicating that ethylene is not the only factor contributing to increased resistance in the overexpression plants. Similarly, the rice ERF, *OsERF922* has been shown to be inducible by abiotic stress such as salt treatment, and by treatments with the phytohormone ABA as well as the rice blast fungus *Magnaporthe oryzae* (D. Liu et al., 2012). The study by Liu et al. (2012) has demonstrated that the overexpression of *OsERF922* led to increased susceptibility to rice blast fungus, while RNAi knockdown plants exhibited decreased *OsERF299* expression along with lower ABA levels, but increased resistance to *M. oryzae* (D. Liu et al., 2012). However, the ET-releasing compound ethephon (2- Chloroethyl phosphonic acid) appeared to induce resistance against *M. oryzae* and was reported to exhibit similar resistance compared to flooding-induced blast resistance (Singh et al., 2004).

Altogether, these examples highlight that ET-mediated susceptibility or resistance represents a dichotomy that can probably be explained by complex modulation of SA, JA and ET signalling (Berens et al., 2017).

4.1.3.1 Ethylene and Ramularia leaf spot in barley

In transcriptome analysis of the barley cv. Fairytale/Ramularia leaf spot (RLS) disease interaction under controlled conditions, it was recently found that upon stomatal penetration by *R. collo-cygni* at 3 dpi an early ET response was present as manifested by high induction of ERFs and ethylene biosynthesis genes, such as *ACS* (Sjokvist et al., 2019). The early response during the asymptomatic growth stage of the fungus included the upregulation of more than 99 genes involved in phytohormone metabolism and signalling, with ET, JA as well as auxin, and gibberellic acid (GA) representing the majority (Sjokvist et al., 2019). The activation of JA and ET mediated signalling and the upregulation of ERFs may indicate the switch from the biotrophic to the necrotrophic growth stage of *R. collo-cygni* as observed for other necrotrophic fungi (De Vleesschauwer et al., 2014). At later stages of *R. collo-cygni* infection, differentially regulated genes (DEGs) found in Sjokvist et al. (2019) appear to represent mainly *PR* genes, including *PR1,* chitinases and LRR-domain proteins that were expressed. Lemcke et al. (2021) reported that during the necrotrophic growth phase of *R. collo-cygni* at 12 dpi in two barley varieties differing in disease levels of RLS, the more susceptible variety Fairytale showed overall a stronger response to infection than the partially resistant variety Tipple. The study found that genes mainly involved in defense responses such as chitinases, dirigent-like proteins, kinases, as well as JA-, GA-, and ET biosynthesis genes were upregulated during late infection with the fungus (Lemcke et al., 2021). These two studies (Lemcke et al., 2021; Sjokvist et al., 2019) together with the finding of this study that one *HvERF* (HORVU.MOREX.r3.4HG0411150) was identified among the genes underlying the major QTL on chromosome 4H (Chapter 3), suggests a putative role for ethylene in the disease response to RLS and might be a potential candidate gene for genetic resistance to RLS in barley.

4.1.4 Aims and Objectives

The aims of this chapter were to investigate whether ethylene or ethylene responses may shape the relationship between barley and *R. collo-cygni* by:

- Testing the effects of the ethylene precursor ACC via exogenous, foliar application on RLS symptom expression and *R. collo-cygni* growth in controlled inoculation experiments.
- Monitoring physiological traits to assess the impact of ACC and *R. collo-cygni* on leaf senescence and leaf nitrogen content.
- Examining the ethylene responsiveness of a subset of spring barley varieties to test whether susceptible/resistant spring barley varieties exhibit differential responses.

4.2 Material and Methods

4.2.1 Plant Material

Spring barley cultivar (cv.) Fairing was used to investigate the effect of exogenous application of the ethylene precursor 1-Aminocyclopropane-1-carboxylic acid (ACC) on the development of RLS. Moreover, the hypothesis whether ethylene responsive genes are expressed differentially in barley cultivars differing in their susceptibility to RLS were tested in six spring barley cultivars. Fairing and RGT Planet are known susceptible and resistant varieties respectively and were therefore, chosen as control varieties. Based on the presented genome wide association scan (GWAS; Chapter 3), cv. Tocada and Heather carry the resistant A allele, whereas Hydra and Chieftain carry the susceptible G allele respectively.

4.2.1.1 Growing Conditions

Germinated seedlings were grown with their shoots pointing upwards in 20.5 cm x 15.4 cm x 5.2 cm half trays containing 1-3 ⌀ mm vermiculite Vermiculite was soaked with 500 ml nutrient solution and 250 ml distilled water to provide all seedlings in this experiment with homogenous nutrient and water supply. The nutrient solution used in these experiments was based on the recipe published by Hoagland and Arnon, 1950 (see appendix). Seedlings were grown in MC1000 high specification climate chambers (Snijders, Tilbourg, Netherlands) for the entire duration of the experiment. Plants were exposed to A photoperiod of 16h light (220 μmol m^{-2} s^{-1})/8h dark and a temperature of 18°C/12°C day/night respectively. Relative humidity was kept at 90% throughout the day/night cycle. Barley seedlings were watered with 10ml/plant nutrient solution every second day.

4.2.2 Fungal Material

Two-week-old plate cultures of *R. collo-cygni* SC19 B5 were used to prepare the mycelium inoculum used to infect barley seedlings in this experiment.

4.2.3 Seedling Inoculation with *Ramularia collo-cygni*

Fungal cultures in the experiment of this chapter were grown for two weeks in the dark at 18°C on potato dextrose agar (PDA; Sigma, Dorset, UK) containing 5 μg mL^{-1} streptomycin. Fungal inoculum was prepared as previously described in Chapter 2, Section 2.2.4. All experiments described in chapters contain mock-inoculated control plants (+Mock) by using potato dextrose broth (PDB; Sigma, Dorset, UK) instead of fungal inoculum(+*Rcc*). Two-week old barley seedlings were +Mock/+*Rcc* inoculated respectively using an artist's air brush (Clarke Wiz Air®, Clarke International, Essex, UK) at a rate of 0.5 mL per seedling. Barley seedlings were sprayed from every direction to obtain an equal inoculation. Inoculated seedlings were placed in 2 L nutrient solution filled plant propagators (50×40×5 cm) covered with a clear plastic lid. Lids were sealed to ensure maximum humidity and incubated in the dark with a temperature regime of 18°C for 16 hours and 12°C for 8 hours respectively. The light regime was restored after three days post- +Mock/+*Rcc* inoculation (dpi)and after five dpi clear plastic lids were removed and day/night regime was restored to 16 hours light and 8 hours dark respectively. Inoculation experiments were three times independently repeated.

4.2.4 Barley seedling treatments with Aminocyclopropane-1-carboxylic acid (ACC)

ACC is a precursor of the phytohormone ethylene and is since its discovery used to stimulate ethylene responses in plants (Fa Yang and Hoffman, 1984). Concentrations of ACC were based on preliminary experiments by Francesca Piatti's honours project (results not shown here). In the present study, ACC (Sigma, Dorset, UK) was dissolved in sterile distilled water (SDW) to obtain a solution of 10 mM. Solutions were always freshly prepared on the day of application and pH was kept at pH6.5. 21 days post sowing 10 mM ACC solution or SDW as a control was applied using an artist's air brush (Clarke Wiz Air®, Clarke International, Essex, UK) at a rate of 0.5 mL per seedling.

4.2.5 *In vitro assay with* Aminocyclopropane-1-carboxylic acid (ACC) on Ramularia collo-cygni growth

To investigate the putative growth inhibiting effect by application of ACC in a *Rcc in vitro* a plate assay was designed to ensure that effects on RLS symptom production are attributed to the host/pathogen interaction. 14 days old *Rcc* isolates of SC19 B5 were tested on PDA (Sigma, Dorset, UK) containing a serial dilution of ACC with final concentrations containing 10 mM, 0.1 mM, 0.01 mM respectively. Stock solution of 100mM ACC was prepared in SDW and diluted down to 10mM and 1mM stock solution in SDW respectively. Stock solutions were prepared in autoclaved Duran bottles the day before and kept in the fridge until usage. The assay was carried out in 12-well plates (Thermo Fisher Scientific, Leicestershire, UK) containing 5 ml of PDA. Agar was allowed to cool down before ACC stock solutions were added into the cooled down agar. Once the PDA solidified, a *Rcc* mycelium plug was added with the mycelium facing downward using the end of a sterile 200µl pipette tip. Plates were closed, sealed with Parafilm (Amcor, Zurich, Switzerland) and incubated for 21 days at 18°C in the dark. As negative control using SDW was used in triplicates. *Rcc*-radial growth was monitored every week over a course of three weeks and pictures were taken using the visualised on an Essential V6 imaging system (Uvitec, Cambridge, UK). Fungal radial growth was quantified via ImageJ. Data shows the average of three mycelium plugs ± standard error measured in technical triplicates.

4.2.6 Evaluation of *Ramularia collo*-cygni symptom development, growth, and physiological impacts in barley seedlings

All assessments in this chapter were performed on *R. collo-cygni* inoculated seedlings treated with ACC or SDW respectively to compare the effect on Ramularia development independent of ACC. However, the impact of ACC was also tested on +Mock inoculated seedlings to demonstrate that the effect of ACC on RLS does not reflect a toxic effect on barley seedlings itself. A summary of all assessment groups can be found in Table 4.1.

Table 4.1: Assessment overview

Abbreviation	**Treatment description**
+*Rcc* + ACC	+ *R. collo-cygni* inoculum treated 10 mM ACC
+*Rcc* – ACC	+ *R. collo-cygni* inoculum with SDW
+Mock + ACC	+ potato dextrose broth + 10 mM ACC
+Mock – ACC	+ potato dextrose broth + SDW

4.2.6.1 Visual assessment of Ramularia leaf spot

RLS symptom development was assessed between 5, 7, 10, 12, 15, 18 and 21 dpi to monitor the process of infection at the very start of the asymptomatic growth phase up to the necrotrophic development. Visual assessments were conducted on the barley prophyll leaf and disease severity was estimated as the percentage of green leaf area covered by RLS. Data show the mean ± standard error calculated on three independent inoculation experiments comprising of 12 biological replications each.

4.2.6.2 *Ramularia collo-cygni* DNA quantification

In planta colonisation by *Rcc* during RLS development was monitored at 5, 7, 10, 14, 21 and 28 dpi by destructively taking six biological reps consisting each of two prophyll leaves and snap-freezing them immediately in liquid nitrogen. *Rcc*-DNA was extracted as previously described in Chapter 2, Section 2.2.7.2. *Rcc* DNA quantification was performed using qPCR described by Taylor et al. 2010. Results shown represent the mean ± standard error calculated on three independent inoculation experiments, with each experiment consisting of six biological replications (18 in total). Each biological replicate consisting of two leaves.

4.2.6.3 Measurement of the relative chlorophyll content and leaf nitrogen content mass

The onset of senescence in the barley seedlings was monitored during RLS development, Soil Plant Analysis Development (SPAD) was taken as a measure of chlorophyll content using the portable SPAD reader (FK-YL02, Shandong Fangke Instrument Co., Ltd., China). Two SPAD measurements per leaf were taken at 7, 8, 10, 12, 14, 16, 18, 21, 22, 24, 26 and 28 dpi on the adaxial prophyll leaf surface. Data show the mean ± standard error calculated of 12 prophyll leaves per experiment. In total three independent inoculation experiments were performed, amounting it to 36 leaves in total. Additionally, the leaf nitrogen content (CN) in percent was quantified at 5, 7, 10, 14, 21, 28 dpi during RLS development. CN was analysed by using an elemental microanalyzer (Thermo Fisher Scientific Flash SMART) as previously described in Chapter 2, Section 2.2.8.4. Data represents the average ± standard error calculated of two biological replicates. Each biological replicate consisted of two prophyll leaves. CN analysis data represents the mean ± standard error of three independent inoculation experiments each consisting of two biological replicates (6 in total), measured in technical duplicates.

4.2.7 Gene expression analysis of putative ethylene responsive genes in a subset of barley seedlings

Selected barley *ethylene responsive factor* (*ERF*) genes characterised by Guo et al., (2016) were assessed based on the closest homology to the well-studied ethylene inducible *A. thaliana ERF1* gene (Nakano et al., 2006). Transcript levels of barley *ERF* genes were assessed post application with 10 mM ACC and SDW respectively in the absence of *R. collo-cygni*. At 6-, 12-, 24-, and 48 hours post treatments two biological replicates consisting of two prophyll leaves were harvested, pooled together and snap frozen in liquid nitrogen. Plant material was ground up in liquid nitrogen and subsequently, total RNA was extracted using the RNAeasy Mini Kit (Qjagen Ltd, Hilden, Germany) following the manufacturer's instructions. Total extracted RNA was followed by TURBO DNAse and 10x TURBO DNAse Buffer treatment (Invitrogen™ TURBO DNA-free™ Kit; Thermo Fisher Scientific Ltd., Leicestershire, UK) following manufacturer's instructions to ensure total removal of genomic DNA. Confirmation of the removal of genomic DNA was evaluated by qPCR using a 10-fold diluted RNA as template. DNA-free RNA was quantified, and its quality assessed using the Denovix Ds-11 Spectrophotometer (Cambridge Bioscience). Consequently, a total of 500 ng RNA was used for subsequent cDNA synthesis using the SuperScript III First

Strand Synthesis System (Invitrogen™, Thermo Fisher Scientific Ltd., Leicestershire, UK) following manufacturer's instructions. Transcript levels were quantified using 10-fold diluted cDNA in molecular grade water in a reverse transcription PCR (qRT-qPCR) system. The thermocycler AriaMx300P (Agilent Technologies, Santa Clara, CA, USA) was used for qRT-PCR amplification and melting curve analysis using the SybrGreen Jump Start ™ Taq system (Sigma, Dorset, UK) following manufacturer's instructions. Three barley reference genes (Cai et al., 2018) were tested for each treatment group. Elongation factor 1 α (EF-1α), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and α-tubulin (α-Tub) were assessed for transcript stability using Reffinder (Xie et al., 2012). The list of primer sequences is given in the Appendix. The most stable reference gene *EF-1*α was chosen to normalise the gene expression in each variety per each time point and treatment (Table 4.2).

Gene specific primers for barley genes of interest were designed using primer3 (Rozen and Skaletsky, 2000). The following criteria were based on the eleven golden rules of qPCR (Udvardi et al., 2008): primer length between 18- 22 bp, GC content 50±10%, Tm+60±1°C, product size between 80-160 bp. The list of primer sequences is listed in the Appendix. A pooled cDNA dilution series of 1/10, 1/100, 1/1000 and 1/10000 in SDW of the six differently treated barley varieties was used to calculate the efficiency for each primer pair using qRT-PCR. Primers with amplification efficiency ranging between 80 to 110% were used for qRT-PCR gene expression analysis. Based on the efficiency, three gene specific primers for the genes *HvERF3.8* on chromosome 2H, *HvERF2.16* on 4H and *HvERF5*.2 on 5H respectively were selected for quantifying the transcript level. Transcript levels were normalised against transcript levels of EF-1α in each treatment group as following:

$$Relative\ gene\ expression = 2^{\Delta Ct} = 2^{Mean\ Ct\ (gene\ of\ interest) - Mean\ Ct\ (EF-1\alpha)}$$

Data presented is given as abbreviations as stated in Table 4.2. Each Treatment group represents the mean normalised expression value ± standard error of two biological replicates which was quantified as technical duplicates. Each biological replicate consisted of two pooled prophyll leaves in a pool of 20 seedlings per treatment group.

Table 4.2: List of Treatments

Variety/Abbreviation	**QTL allele**	**Treatment**
Fairing + ACC	G	10 mM ACC
Fairing -ACC	G	SDW
RGT Planet + ACC	G	10 mM ACC
RGT Planet – ACC	G	SDW
Hydra + ACC	G	10 mM ACC
Hydra – ACC	G	SDW
Chieftain + ACC	G	10 mM ACC
Chieftain – ACC	G	SDW
Tocada + ACC	A	treated 10 mM ACC
Tocada – ACC	A	SDW
Heather + ACC	A	treated 10 mM ACC
Heather – ACC	A	SDW

4.2.9 Statistical analysis

Data presented in this chapter was analysed in the open-source R software using version 4.2.3 using for all analyses the “tidyverse” packages (Wickham et al., 2019). A Linear mixed-effect model (LMEM) regression was fitted for each measured response (Symptoms, *Rcc*-DNA, SPAD, Leaf N) using time and treatment as predictors and their interaction as fixed effects.

The number of each independent experiment was the random effect. Model analysis was conducted via the restricted maximum likelihood algorithm (REML) in the "lme4" package (Bates et al., 2015). The models' assumptions were checked by testing the homoscedasticity via comparing residuals to the fitted items and a Shapiro-Wilk's was used to test if the residuals are normally distributed. All graphs were performed using ggplot2 (Wickham, 2011). Additionally, *R. collo-cygni*-DNA level, visual RLS disease symptoms and the ACC *in vitro assay* were tested for significant differences between treatment groups at the end of each experiment by conducting a two-way-anova. . Assumptions of the two-way anova were tested by residual analysis. Shapiro-Wilk's normality test was used to assess normality and homogeneity of variances was assessed by Levene's test. Consequently, pairwise-comparison was performed using the Tukey post hoc test.

Gene expression results were not tested for statistical differences as the number of replicates ≤ 3 did not justify an appropriate method, and thus, results provided are to be taken as preliminary results.

4.3 Results

4.3.1 Exogenous application of 1-Aminocyclopropane-1-carboxylic acid reduced visual Ramularia leaf spot symptoms

The effect of ACC treatment during infection with *R. collo-cygni* was tested to investigate if ET plays a resistance-mediating role on RLS symptom development. Disease development of RLS was based on visual estimations of the percentage of green leaf area covered by symptoms. The first symptoms appeared as "pepper spots" at 7 dpi, before 10 mM ACC was applied. Typical reddish, brown lesions became apparent after 10 dpi and increased continuously until 28 dpi regardless of treatment as time had a significant effect on disease development (p-value: 0.0; Figure 4.2 A). The interaction between time and ACC treatment was significant (p-value: 7.86e-^{06}), with ACC treatment decreasing the amount of symptoms compared to the untreated control seen over time (Figure 4.2 A). Symptoms developed slower in ACC treated plants compared to untreated plants (Figure 4.2 A). For every unit of time, the untreated control increases symptom development by 0.7 ± 0.04 % compared to the ACC treatment that increases by 0.4 ± 0.09 %. The increase in symptom development correlated positively with a visual decrease in green leaf area. At the end of the experiment at 28 dpi, plants treated with ACC exhibited significantly (p-value: 0.005) less symptoms compared to the untreated plants (Figure 4.2 B, and Figure 4.2 C).

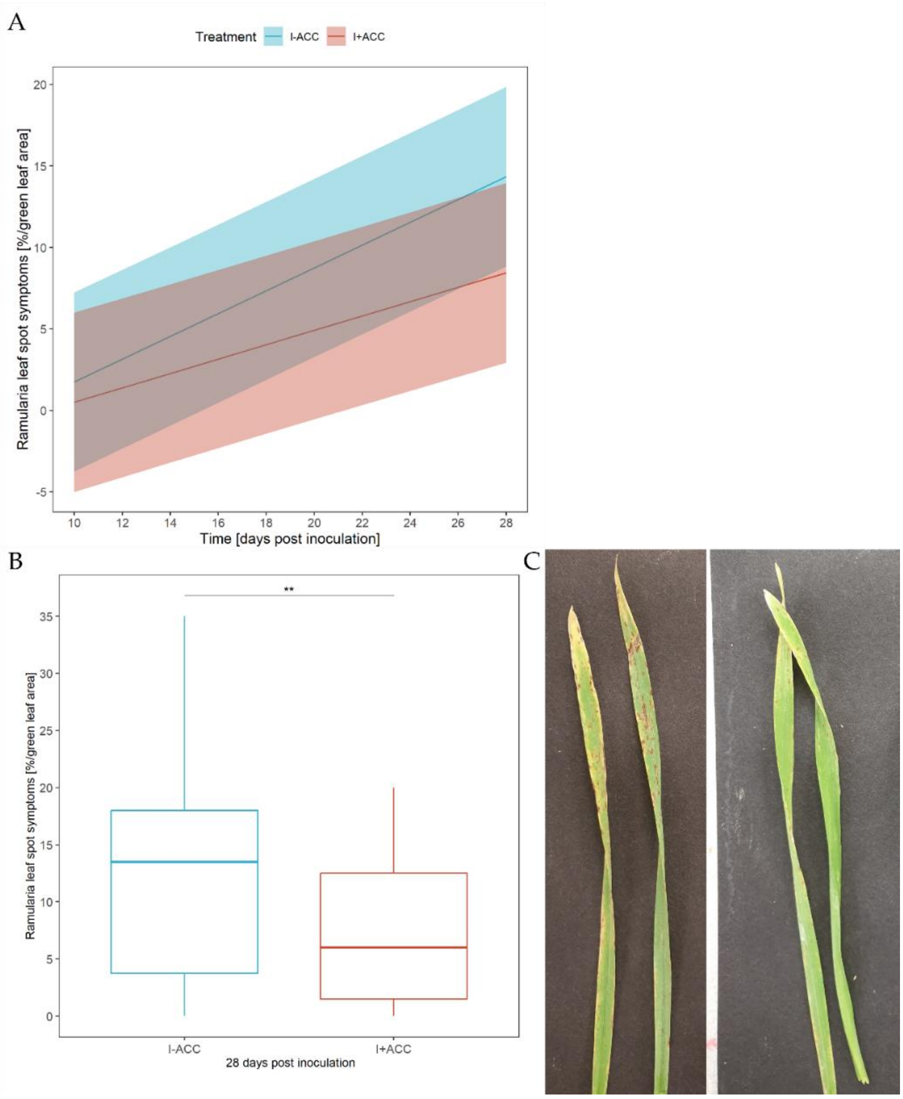

Figure 4.2: Ramularia leaf spot disease development after treatment with an ethylene precursor.

The evolution of RLS symptom development in *R. collo-cygni*-inoculated (I) treated 10 mM ACC (+ACC; red) compared to untreated (-ACC; blue) cv. Fairing plants. A: LMEM of symptom development from three independent inoculation experiments in which in total 36 prophyll leaves were assessed for disease development. Model was conducted with time and treatment as predicators on symptoms as the response variable and the independent inoculation experiments as the random effect. B: Boxplot shows RLS symptom expression at 28dpi from three independent inoculation experiments and significance p:value 0.005 was estimated using for pairwise comparison. C: Visual disease symptoms at 28 dpi of untreated (-ACC) and treated (+ACC) of *R. collo-cygni*-inoculated barley cv. Fairing leaves.

4.3.2 Exogenous application of 1-Aminocyclopropane-1-carboxylic acid decreases fungal development during infection with *R. collo-cygni*

During RLS disease development, *R. collo-cygni* DNA was quantified at six different time points to better understand the relationship between asymptomatic growth and necrotrophic disease development (Figure 4.3 A). Even though, no symptoms appeared before 10 dpi, *R. collo-cygni* DNA was quantified at 5 dpi (33.67 pg) suggesting the colonisation by the fungus. The amount of *R. collo-cygni* DNA increased until the end of the experiment slower in ACC treated plants compared to untreated plants (Figure 4.3 A), with time having a significant effect on symptom development in both treatments (p-value: $6.22e^{-11}$). The untreated plants gained per unit of time 8.26 ± 1.27 pg in *R. collo-cygni* DNA, whereas ACC treated plants increased by only 3.22 ± 3.05 pg. At the end of the experiment at 28 dpi, the amount of *R. collo-cygni* DNA differed significantly (p-value: 0.02) between ACC treated and untreated plants (Figure 4.3 B).

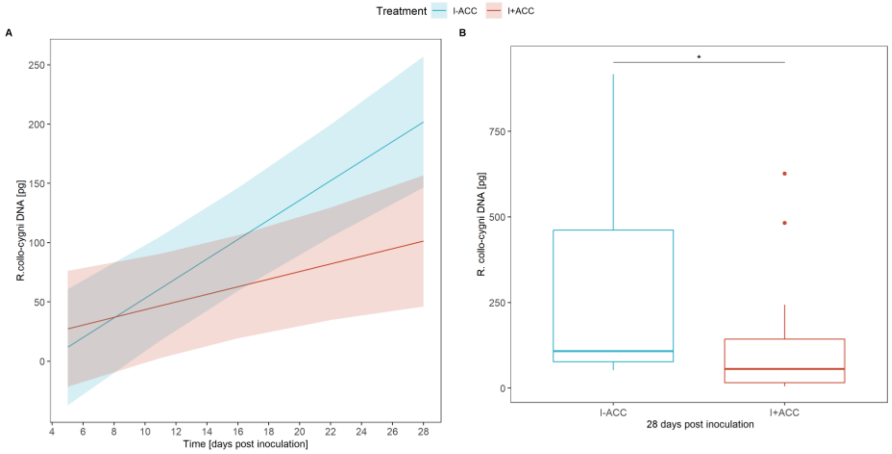

Figure 4.3: Colonisation by *R. collo-cygni* before and after treatment with Aminocyclopropane-1-carboxylic acid.
The evolution of fungal colonisation in *R. collo-cygni*-inoculated (I) treated 10 mM ACC (+ACC; red) compared to untreated (-ACC; blue) cv. Fairing plants. A: LMEM shows modelled data from three independent inoculation experiments in which in total 18 biological replicates, each consisting of two prophyll leaves were quantified for fungal DNA. Model was conducted with the interaction of time and treatment as the predictors on symptoms as the response variable. The independent experiments were accounted for as the random effect. B: Fungal colonisation at the end of the experiment at 28 dpi of I+ACC and I-ACC plants. A non-parametric Kruskal-Wallis test was performed to determine whether there is a statistical difference between the medians of fungal DNA of the different treatments. Following, a Dunn's Test to determine the significance between treatments.

4.3.3 Relative chlorophyll content post treatment with Aminocyclopropane-1-carboxylic acid

The decline of green leaf area was monitored during RLS development using SPAD readings that represent the relative chlorophyll content in leaves. SPAD readings were first taken on the day of ACC treatment and hence, time points are indicated as days post treatment (dpt). A significant difference between SPAD readings was observed between the non-inoculated (Mock) plants and the *R. collo-cygni*-inoculated (I) plants (Figure 4.4) at 0 dpt. The Mock plants exhibited SPAD values of 43.71 ± 3.01 (p-value: $1.29e^{-07}$) and 41.77 ± 3.01 (p-value: $8.77e^{-04}$) for ACC treated (Mock+ACC) and untreated (Mock-ACC) plants, respectively (Figure 4.4). *R. collo-cygni*-inoculated plants exhibited SPAD values at 0 dpt of 39.55 ± 3.01

and 38.44 ± 2.01 for ACC-treated (I+ACC) and untreated plant (I-ACC) respectively (Figure 4.4). No significant difference was observed between ACC treated and untreated plants inoculated with *R. collo-cygni* at 0dpt. In all treatments the relative chlorophyll content decreased over time, as time had a significant effect on SPAD values (p-value: 0). Regardless of treatment, both mock plants exhibited a slower decline in relative chlorophyll content compared to plants inoculated with *R. collo-cygni* (Figure 4.4). The fastest decline was observed in I-ACC plants with a decrease of -1.07 ± 0.06 per unit of SPAD (p-value: 0) compared to I+ACC with a decrease of -0.85 ± 1.06 per unit of SPAD (p-value: $9.85e^{-03}$). Overall, the relative chlorophyll content decreased faster in plants inoculated with *R. collo-cygni* compared to the mock-inoculated plants, with I-ACC observing the greatest decline.

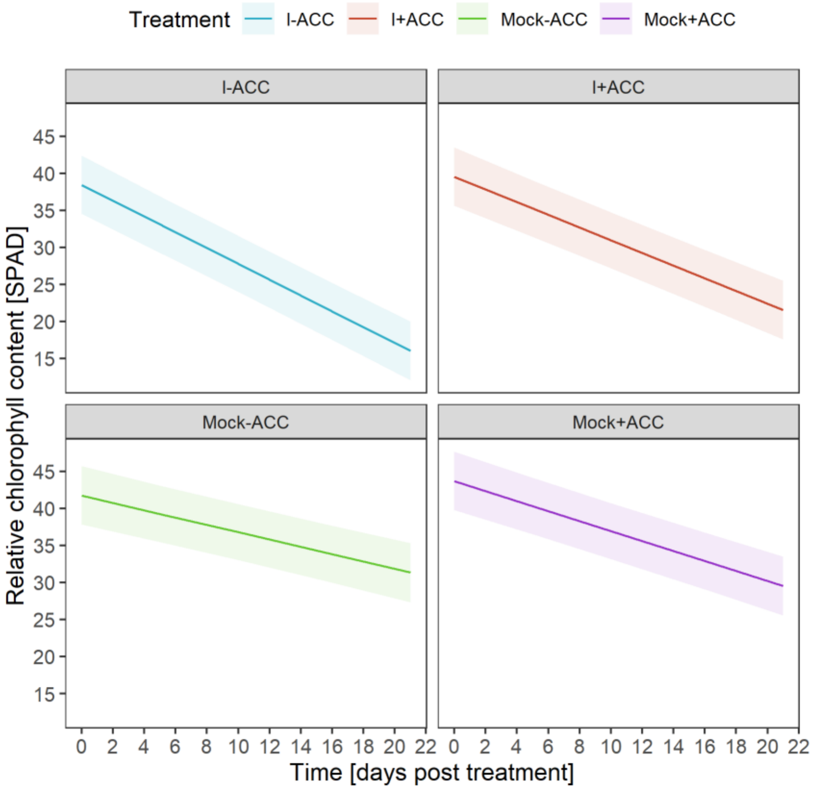

Figure 4.4: Relative chlorophyll content post Aminocyclopropane-1-carboxylic acid treatment.
The evolution of the decreasing relative chlorophyll content in cv. Fairing plants inoculated with *R. collo-cygni* (I) and non-inoculated (Mock) after treatment with 10 mM ACC (+ACC) or untreated (-ACC). LMEM shows the estimated mean ± standard error of the relative chlorophyll content assessed in three independent inoculation experiments, with 36 monitored prophyll leaves in total. Model was conducted with the interaction of time and treatment as the predictors on SPAD as the response variable.

4.3.4 Aminocyclopropane-1-carboxylic acid does not influence the leaf nitrogen content in barley leaves

To better understand the relationship between age-related nitrogen remobilisation and RLS development, the relative leaf N content was quantified using C&N analysis during disease development. During the asymptomatic phase of RLS development at 5 dpi, the leaf N content was significantly lower (p-value: 0) in untreated plants inoculated with *R. collo-*

cygni (I-ACC) compared to the other treatments. Whereas the non-inoculated plants treated with ACC (Mock+ACC) at 0 dpi exhibited significantly higher leaf N content of 5.14 ±0.58 % (p-value: $2.72e^{-02}$) compared to inoculated plants. Over time the leaf N content decreased in all treatments, and significantly decreased in I-ACC plants by -0.1% per unit of time (p-value: $1.87e^{14}$). However, there was no other significant interaction observed between the other treatments and time, suggesting that *R. collo-cygni* induced premature leaf senescence associated with increased symptom formation and increased fungal colonisation in untreated plants (Figure 4.2 and Figure 4.3)

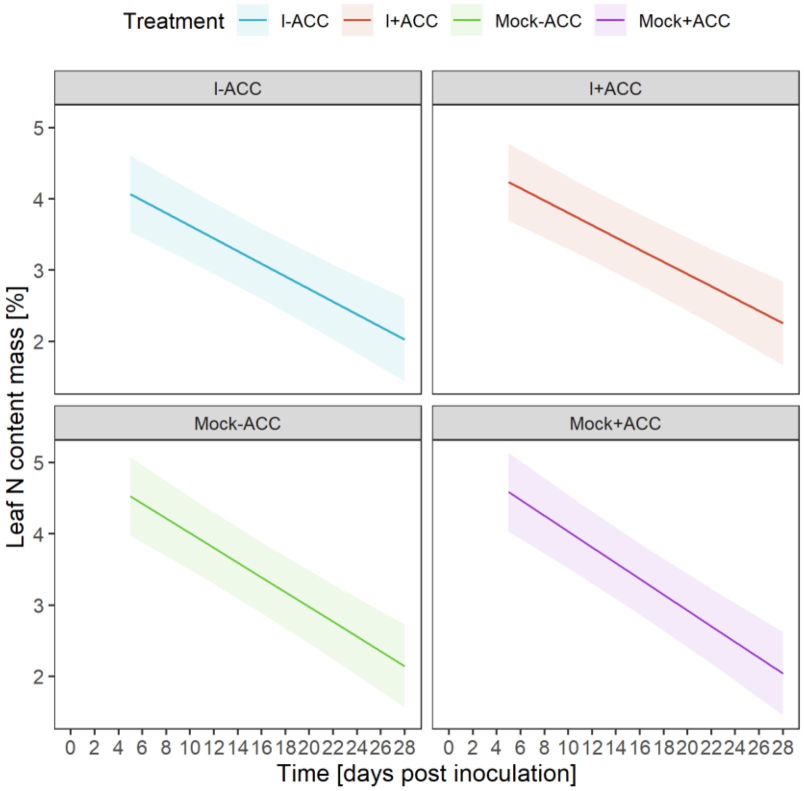

Figure 4.5: Evolution of leaf nitrogen contents in barley seedlings cv. Fairing following *R. collo-cygni* inoculation and Aminocyclopropane-1-carboxylic acid treatment. The decrease in leaf nitrogen inoculated with *R. collo-cygni* (I) and non-inoculated (Mock) in cv. Fairing plants that were treated with 10 mM ACC (+ACC) or untreated (-ACC). LMEM of three independent controlled inoculation experiments of in total six biological replicates, each consisting of two prophyll leaves.

4.3.4 The effect of Aminocyclopropane-1-carboxylic acid on *R. collo-cygni* growth *in vitro*

To test if ACC treatment has a putative growth stimulating or inhibiting effect on the fungus, the effect of ACC was tested on *in vitro* cultures of 14-days old *R. collo-cygni*. The growth of *R. collo-cygni* at 14 days post treatment was significantly (p-value: 0.04) smaller using 10 mM ACC compared to the control (Figure 4.6). No difference was found between 0.1 mM, 0.001 mM ACC respective and the control (Figure 4.6). *R. collo-cygni* plugs obtaining 0.001 mM ACC were significantly larger compared to plugs containing 10 mM ACC in the PDA media. Altogether, it appears that 10 mM ACC slightly inhibits the growth of *R. collo-cygni*, but this concentration is unlikely to reach the mesophyll cells of barley which *R. collo-cygni* colonises.

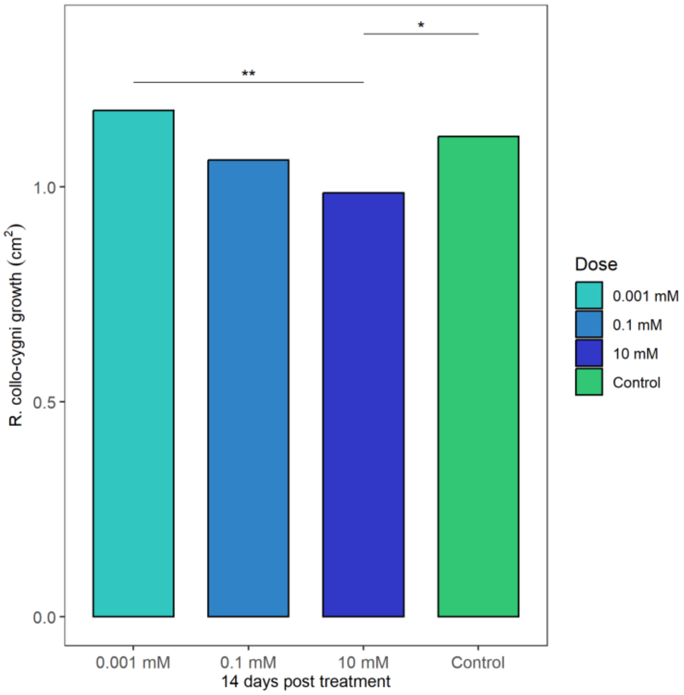

Figure 4.6: *In vitro R. collo-cygni* growth assay following media treatments of varying Aminocyclopropane-1-carboxylic acid concentrations.
Bar plot represents the mean ± standard error of three biological replicates of *R. collo*-cygni grown on PDA varying ACC concentrations. One-way anova and Tukey were performed to test for significant differences among the treatment groups.

4.3.5 Gene expression profiles of putative ethylene-responsive genes in barley

Transcript levels of three putative ERFs were quantified to test the response to ACC in different barley varieties that demonstrate differences in their susceptibility/resistance to RLS both at the adult and seedling stage. Overall, ACC-induced gene expression of *HvERF* genes appear to be only induced in *HvERF2.16* on chromosome 4H in all varieties (Figure 4.7 A). Throughout all time points *HvERf2.16* showed a large ACC-induced increase in the susceptible varieties Chieftain and Hydra, as well as the partially resistant variety Heather (Figure 4.7 A). By contrast, in Fairing, RGT Planet and Tocada the induction by ACC of *HvERf2.16* was observed at 12h, 24h and 48h respectively (Figure 4.7 A). Transcript levels of *HvERF3.8* on chromosome 2H seemed to be higher upon ACC treatments in Hydra 6h post treatment and then appearing to dampen off after 12h (Figure 4.7 B). A response of *HvERF3.8* post ACC treatment was also indicated in Tocada at 6h, 24h and 48h respectively. While in Chieftain and Fairing, *HvERF3.8* appears to be downregulated in response to ACC treatment compared to the control (Figure 4.7 B). Both *HvERFs* in RGT Planet showed very low responsiveness to either ACC or the control (Figure 4.7 A-B). The response to ACC of *HvERF5.2* on chromosome 5H appeared to follow a similar trend in all varieties apart from Tocada, as a downregulation compared to the control was indicated. To summarise, this study found one *HvERF* gene at the end of chromosome 4H that is ethylene-responsive across all six tested varieties, however, there is no clear observation showing that the susceptible/resistant varieties follow a different trend.

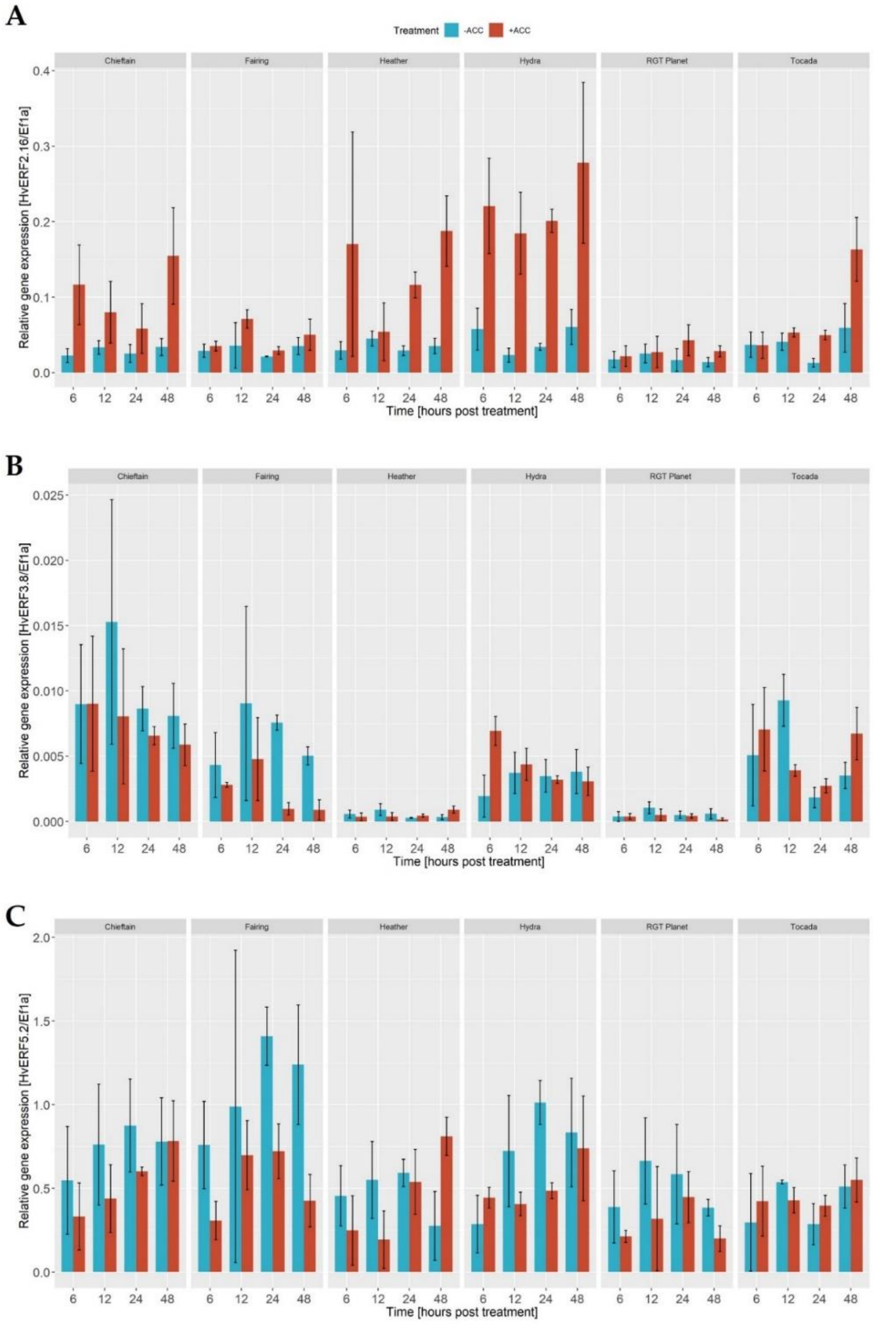

Figure 4.7: Relative gene expression of *Hordeum vulgare*-ethylene responsive factor (*Hv*ERF) post Aminocyclopropane-1-carboxylic acid treatment in a subset of barley varieties.

Bar plots represent mean ± standard error of relative gene expression of three *ethylene-responsive-factor* (HvERF) genes. Gene expression is shown in relation to transcript levels of the housekeeping gene EF1-α**.** **A:** *HvERF2.16*/EF1-α*;* **B:** *HvERF3.8*/EF1-α*;* **C:** *HvERF5.2*/EF1-α. Gene expression was quantified via RT-qPCR in two biological replicates. Treatments of Aminocyclopropane-1-carboxylic acid (+ACC; red) and control (-ACC; blue) were applied 21 days post sowing. Total RNA was extracted from six barley seedling varieties (Chieftain, Fairing, Heather, Hydra, RGT Planet, Tocada).

4.4 Discussion

One approach to develop plant resistance relies on the modulation of phytohormones with the aim to maintain genetic potential of the crop plant (Gupta et al., 2023). Phytohormones do not only orchestrate intrinsic developmental programmes, but also communicate environmental stimuli and drive adaptive responses to both abiotic and biotic stresses (De Vleesschauwer et al., 2014). ET and ET-releasing compounds are known to induce resistance to various pathogens due to ability to induce immune gene expression (Jasrotia and Jasrotia, 2022; Nakano et al., 2006; Ohme-Takagi and Shinshi, 1995).

This study presented here has shown that ACC treatment significantly reduced both RLS symptom development and *R. collo-cygni* growth in controlled inoculation experiments in cv. Fairing (Figure 4.2 and Figure 4.3), suggesting ET signalling induces an effective immune response against this pathogen. Manipulation of the ET-mediated host response, using exogenous application of ethephon on seedlings of habanero pepper, induced resistance against the oomycete *Phytophthora capsici,* the causative agent of Phytophthora blight (PB) (Núñez-Pastrana et al., 2011). The study also found an increase of transcript levels of *ACO*, *PR1* and *PDF1*.2 between 24- to 72h post ethephon treatment in *P. capsici* infected seedlings compared to untreated plants (Núñez-Pastrana et al., 2011). Resistance was neither observed in response to MeJA nor SA, suggesting that resistance was conferred due to an ET-mediated immune response (Núñez-Pastrana et al., 2011). Similarly, ethephon-induced resistance was reported against the Dothideomycete, *M. phaseolina,* causing charcoal rot in the legume *M. truncatula* (Gaige et al., 2010).

Since this study did not investigate the response of *PR* genes to *R. collo-cygni* infection and ACC treatment, differential expression of Hv*ERFs* genes in response to ACC, cannot support the hypothesis that ET-mediated immune responses might have led to increased resistance to RLS under controlled conditions. This study demonstrated that ACC induced activation of the transcription factor *HvERF2.16* in all six spring barley varieties, following a similar pattern of early induction at 6 h, dampening off between 12-24 h and a strong response at 48h post treatment (Figure 4.7). On the other hand, *HvERF3.8* and *HvERF5.2* appeared to be downregulated in most of the varieties in response to ACC treatment (Figure 4.7 B-C). All three transcription factors belong to the subfamily of in total 54 ERFs in barley that are characterised by their ability to bind to the GCC-box of ET/JA responsive defense genes (Gutterson and Reuber, 2004; Ohme-Takagi and Shinshi, 1995). Guo et al. (2016) showed that the expression profile appears to be tissue- and developmental specific for *HvERF2.16.* While *HvERF3.8* appeared to be constitutively expressed and *HvERF5.2* was highly expressed during the tilling at the six-leaf stage (Guo et al., 2016).

Transcriptomic analyses in barley during the response to *R. collo-cygni* infection under controlled seedling inoculations, previously showed the upregulation of ET biosynthesis genes as well as ERFs and *PR* genes, including *PR1* and chitinases (Lemcke et al., 2021; Sjokvist et al., 2019). Altogether, the presence of one ERF in the identified QTL on barley chromosome 4H (Chapter 3; Figure 3.2) and the results of this chapter,highlight the importance to investigate a putative role for ethylene in regulating the response to RLS disease development that needs to be exploited in transgenic ERF barley lines. The end of chromosome 4H contains three ERF transcription factors (Guo et al., 2016), of which *HvERF2.16* was inducible, but did not show a distinctive patten between susceptible/resistant barley varieties (Figure 4.7.A). This region might provide interesting candidate genes for future research to investigate the association between RLS resistance and host immune response, that could provide functional insights beneficial for barley breeding programmes.

The observation in the *ACC in vitro* assay on *R. collo-cygni* mycelium (Figure 4.6) to reduce 12% of the fungal growth as a result of exposure to 10 mM ACC compared to the control, indicates on one hand a putative toxic effect on the fungus. Similar growth inhibition was observed upon ethephon treatments *in vitro* on *P. capsici* to test toxicity, however, the results did not match their *in planta* results (Núñez-Pastrana et al., 2011). Here, the two lower concentrations did not show any inhibitory effect on fungal growth, and one could argue that even though, 10 mM ACC was applied, the same concentration must have not necessarily penetrated through the barley leaf. Moreover, the ACC treatment led to a decrease of 12% fungal growth, while the difference in symptom development amounted to 62% (Figure 4.2 B and Figure 4.4) and hence, the effect of ACC on reducing both RLS symptoms and *R. collo-cygni* DNA can be attributed to molecular responses to ACC, rather than its toxic effect.

This study also demonstrated that ACC treatment did not promote early senescence in non-inoculated plants (Figure 4.4) as seen in *A. thaliana* (Grbić and Bleecker, 1995). In contrast, *R. collo-cygni*-inoculated plants have shown lower relative chlorophyll contents regardless of treatments during the asymptomatic growth stage at 5 dpi (Figure 4.4), indicating that the fungus induced premature leaf senescence. This is supported by the observations of the significant decrease of leaf N levels in inoculated, untreated plants (I-ACC; Figure 4.5). With increasing RLS symptom development of the inoculated, untreated plants (I-ACC), the rate of senescence and leaf N declined faster compared to the infected ACC-treated plants (I+ACC; Figure 4.4 and Figure 4.5). In the closely related species, *Z. tritici,* it was also shown that fungal induced senescence plays part of an important component of its colonisation strategy (Ma et al., 2018). Similarly, the necrotrophic fungus *B. cinerea* has been suggested to induce premature leaf senescence in tomato as part of its infection strategy, as the promotors *SAG12* and *SAG13* were found to be induced upon *B. cinera* infection in tomato plants (Swartzberg et al., 2008). Moreover, expression of *isopentenyl transferase* (*IPT*) under the promotors *SAG12* and *SAG13* were shown to delay leaf senescence in tomato plants (Swartzberg et al., 2006) and led to increased resistance to *B. cinerea* infection (Swartzberg et al., 2008). A previous expression profile study of controlled inoculation experiments with *R. collo-cygni* has shown that upon fungal infection *SAGs* were upregulated compared to control plants and resembled genetic patterns of naturally senescing barley leaves (Kucharewicz et al., 2017; Sjokvist et al., 2019). Congruently, McGrann et al. (2015) has shown that the overexpression of barley *stress-responsive NAC* transcription factor (*HvSNAC1)* that is associated with senescence (Kim et al., 2014), led to increased resistance to RLS and reduced fungal DNA under controlled inoculation experiments. The results of the non-inoculated plants in this study, in which ACC did not alter the relative chlorophyll content in cv. Fairing plants, suggests that the observed ET-induced resistance to RLS may be promoted by other factors than senescence. However, the result of this study raises the question whether *R. collo-cygni* triggered senescence and in turn, allowed enhanced symptom development (Figure 4.2 A) or whether the RLS symptom development led to the decline in relative chlorophyll content and nutrient remobilisation

(Figure 4.4). The data here suggests that upon ACC treatment, the fungal colonisation appeared to be slower than in the untreated plants (Figure 4.3 A) and thus, points towards the latter explanation. Gene expression analysis in response to ACC treatment during infection with *R. collo-cygni* is needed to elucidate the molecular mechanisms that led to increased resistance to RLS and whether ACC treatment induced the expression of *PR* genes that explain enhanced resistance.

Chapter 5:

General Discussion

This study investigated the potential for genetic control of Ramularia leaf spot (RLS) in barley (*Hordeum vulgare*). *Ramularia collo-cygni*, the causative agent of RLS, has become increasingly concerning as a threat to barley production in the temperate regions worldwide over the past 40 years. The aim of this study was to gain a better understanding of the relationship between the host genetics and *R. collo-cygni* in order to elucidate whether the barley genome can be used as a source of resistance to RLS. Furthermore, this project investigated the role of physiological changes in barley in relation to RLS development by increasing our understanding of the role of senescence during disease development and fungal colonisation. This project used a combination of bioinformatics to examine associations between barley and RLS resistance, field- and controlled environment studies to validate those findings at the adult and seedling stage, and molecular biology to better understand the relationship between asymptomatic and symptomatic disease development.

In agriculture, yield losses can result from plant diseases caused by pathogenic fungi, oomycetes, bacteria, viruses, nematodes, and weeds. A global survey of over 139 pathogens and pests associated with plant diseases by Savary et al. (2019) showed that crop shortfall in the three most important cereal crops, wheat (*Triticum aestivum*), rice (*Oryza sativa*), and maize (*Zea mays*) amount to an average of 21.5%, 30% and 22.5%, respectively. Plants have evolved mechanisms to detect pathogen-associated molecular patterns (PAMPs) via cell surface localised pattern-recognition receptors (PRRs), which activate PAMP-triggered immunity (PTI) resulting for example in the closure of stomata to prevent pathogen entry (Mata-Pérez and Spoel, 2019; Spoel and Dong, 2012). The co-evolution of pathogens with plants, however, has enabled pathogens to overcome PTI by releasing pathogen avirulence (Avr) effector proteins that can suppress host immune signalling (Bent and Mackey, 2008). This in turn, activates effector-triggered immunity (ETI) in plants mainly via intracellular receptors known as resistance (R) proteins that recognise these effectors (Jones and Dangl, 2006). There are six classes of R genes, with the most common class encoding cell surface receptors such as nucleotide-binding domains and leucine-rich repeat (NB-LRR) receptors (NLR; Poland et al. 2009). For each *R* gene in plants there is often a corresponding pathogen Avr gene (Bent and Mackey 2008). Most R genes confer pathogen isolate resistance or race-specific resistance. Due to mutations and virulence shifts in pathogen populations that challenge the effectiveness of the resistance in the host, isolate resistance or race-specific resistance is prone to R gene breakdowns (Li et al., 2020). This type of resistance is often called qualitative resistance and refers to either a complete or high level of resistance (Pilet-Nayel et al., 2017). Plant breeders have long relied on the use of single dominant or recessive qualitative resistance due to their strong effect on the phenotype (Li et al., 2020). However, an example of breakdown of R genes is given by a new isolate of *Puccinia graminis f.sp. tritici, Ug99* in Africa for wheat stem rust that re-occurred in isolated outbreaks after being eradicated in Western Europe, and highlights the challenges plant growers, breeders and pathologists face (Ayliffe et al., 2008; Orton et al., 2019; Singh et al., 2011).

In contrast, the more durable quantitative resistance often confers partial resistance which is associated with genomic regions or quantitative trait loci (QTL; Pilet-Nayel et al., 2017). This is also referred to as polygenic traits due to the control of several to many genes with small and often additive effects, which are sensitive to environmental conditions, but usually effective against multiple strains of a pathogen (Michelmore et al., 2013). In many crops, economically relevant traits including yield, seed protein content, quality attributes and resistance to both abiotic and biotic stresses, are inherited quantitatively (St.Clair, 2010). Quantitative resistance is associated with increased durability, compared to qualitative resistance, as it is conferred by QTLs and exert a lower selection pressure on the pathogen population (Pilet-Nayel et al., 2017; Stuthman et al., 2007). The genes underlying resistance QTLs are less well-known than molecular mechanisms underlying R gene-mediated resistance (Michelmore et al., 2013; Poland et al., 2009). The cloning of loci associated with quantitative disease resistance (QDR) genes has been challenging due to the small phenotypic effect of many QDR loci and the challenge of consistent phenotyping disease traits across different environments (Poland et al., 2009). French et al. (2016) have reviewed putative functions for genes underlying resistance QTLs, which included genes involved in plant development or morphology, basal defense, detoxification, and transduction of defense signalling. One of the examples why investigating underlying genes of QTL is important, is highlighted by the wheat *leaf rust 34* (*Lr34)* QTL providing important implications of resistance for control of rust diseases in several cereals such as maize, rice, and barley (French et al., 2016). The LR34 protein resembles ATP-binding cassette (ABC) transporters, with alleles of *Lr34* gene conferring resistance or susceptibility, depending on three genetic polymorphism (Krattinger et al., 2009). The QTL *Lr34* confers resistance to wheat stripe rust caused by *Puccinia striiformis*, leaf rust caused by *Puccinia triticina*, stem rust caused by *Puccnia graminis*, but also to the powdery mildew agent *Blumeria graminis* (Krattinger et al., 2009).For over 50 years wheat QTL *Lr34* remained durable since its first documentation in Canada (French et al., 2016). The *Lr34* gene is expressed in the wheat flag leaf during the grain-filling stage (≥GS71), and pyramiding *Lr34* with other wheat resistance genes showed near-complete resistance to rust and powdery mildew (French et al., 2016; Krattinger et al., 2009; Young, 1996). Although the mechanism underlying *Lr34*-mediated resistance is not fully understood, some speculated that it may be the result of senescence-like processes (French et al., 2016). *Lr34* was since successfully introduced into other crop species to provide broad-spectrum resistance against several host-specific diseases such as rice blast, barley leaf rust, stem rust and barley powdery mildew caused by *Magnaporthe oryzae, Puccinia hordei, Puccinia graminis* f. sp. *tritici, B. graminis* f. sp. h*ordei*; respectively (Chauhan et al., 2015; Krattinger et al., 2016; Risk et al., 2013).

This study, aimed to contribute to better our understanding of the genetic basis of RLS disease resistance in barely. The GWAS results (Chapter 3) strongly support the hypothesis that RLS is a quantitative trait controlled by a number of genes of small effect (Havis et al., 2015). The QTL that was previously reported by Brown et al. (2015) at the end of chromosome 4H was confirmed here using the improved 50k Illumina Infinium iSelect genotyping array (Bayer et al., 2017). The QTL (JHI-Hv50k-2016-266331) was characterised as an A/G polymorphism (Chapter 3; Figure 3.3), with the A allele being associated with increased resistance to RLS. Furthermore, this was the first study to validate a GWAS associated to RLS resistance using field trials in Scotland and Germany between 2021 and 2022 (Chapter 3, Figure 3.4-6). Results from the field trials demonstrated that spring barley varieties carrying the resistant A allele exhibited reduced RLS levels and confirmed the marker-trait association (Chapter 3, Figure 3.6). Moreover, this study indicated the presence of three new MLO markers in addition to the three previously characterised MLO markers within the major QTL (Chapter 3; Figure 3.17; Piffanelli et al. 2002; McGrann et al. 2014). The presence of mutations at the *Mlo* locus controlling powdery mildew in barley and increased susceptibility to other pathogens is well known. In detached leaf assays using backcross *mlo-5* and *Mlo* wild-type in cv. Ingrid lines, Kumar et al. (2001) showed an increased susceptibility to *Bipolaris sorokiniana* causing spot blotch in wheat and barley. Similar observations were made with *Fusarium graminearum* and *M. oryzae* causing rice blast in barley (Jarosch et al., 1999; Makepeace et al., 2007). The link between *mlo* in spring barley cultivars and increased susceptibility to RLS was first studied at the beginning of the 21st century, however, there have been contradicting findings about the effect of *mlo*-alleles on the severity of RLS (Hofer et al., 2015; Makepeace et al., 2007). While most studies have shown that barley plants carrying *mlo*-alleles exhibit greater RLS symptoms (Brown and Makepeace, 2009; McGrann et al., 2014; Pinnschmidt and Sindberg, 2009), most studies have also indicated that the strength of the *mlo*-effect differs with the environment, location and genetic background of the plant (Havis et al., 2015). The molecular functions of the MLO protein that explain the pleiotropic effect of *mlo* on *M. oryzae, F. graminearum* and *R. collo-cygni* are not fully understood yet (Kusch and Panstruga, 2017). The MLO protein contains a seven transmembrane domain that binds calmodulin, and is a negative regulator of plant defence, which is required for conferring full susceptibility to powdery mildew in barley (Kim et al., 2002). It appears that wild-type *Mlo* function is linked to calcium (Ca^{2+}) homeostasis, with the ubiquitous Ca^{2+} sensor calmodulin binding to MLO (Kim et al., 2002). Transient ectopic expression of the calcium-dependent protein kinase (CDPK) genes in *mlo* barley leaf epidermal cells showed that *Hv*CDPKs can partially restore *mlo* resistance to powdery mildew. Constitutive activation of *HvCDPK3* and dominant negative inhibition via the junction domain of *Hv*CDPK4 led to partially increased colonisation of *B. graminis* f. sp. *hordei* (Freymark et al., 2007). Plant CDPKs are also known to be involved in responses to both abiotic and biotic stresses by acting as messengers that sense molecules such as reactive oxygen species (ROS), and phytohormones that are released in response to stresses (Cheng et al., 2002; Fichman and Mittler, 2020; Liang and Zhou, 2018).

Among the 308 genes underlying the identified QTL (JHI-Hv50k-2016-266331; Chapter 3), two CDPKs and two MLO proteins were found in this study. CDPK (HORVU.MOREX.r3.4HG0410700.1) has given 100% homology on NCBI BLAST to *HvCDPK7* (AKA249361.a) in barley that has been recently published and characterised by Yang et al. (2017). Moreover, Yang et al. (2017) have studied the response of *Hv*CDPKs to methyl jasmonate (MeJA) and found lower expression levels in the group of CDPKs that includes *HvCDPK7*. *HvCDPK7* was also down-regulated in barley seedlings in response to cold, salt and drought stress (Yang et al. 2017). CDPKs can also act as negative regulators of stress responses. According to Yang et al. (2017), *HvCDPK7* is 76% homologous to rice *OsCPK7* (*OsCDPK13*) which was induced after cold stress (Abbasi et al., 2004). The orthologue to *OsCPK7* in maize (*Zea mays*), *ZmCPK1* functions as a negative regulator of cold signalling. Ectopic overexpression in maize mesophyll cells revealed that the kinase active *ZmCPK1* suppresses the expression of the cold-induced maize marker gene, ethylene responsive factor *Zmerf* (Weckwerth et al., 2015). Deciphering the molecular function of genes that may comprise the barley *mlo*-mediated pleiotropic effect on other pathogens such as *M. oryzae* or *R. collo-cygni* could be the aim of future studies.

The results from this project will help to choose candidate genes such as *HvCDPK7* to study under controlled *R. collo-cygni*-inoculation studies to better understand the role in the plant-pathogen interaction. Before candidate genes are selected, current databases such as BARLEX and the exome capture dataset pose valuable tools to ascertain if a candidate gene is a capture target, which could reveal promising haplotypes underlying associated traits (Colmsee et al., 2015; Mascher et al., 2013). The results obtained from the controlled inoculation studies (Chapter 3; Figure 3.10-11) have supported the findings of the GWAS that varieties carrying the A allele are more resistant to RLS than those carrying the G allele. The varieties Chieftain and Tocada are respectively susceptible and resistant to RLS, both at the adult and seedling stage (Chapter 3). Preliminary findings showed that these varieties carry the two diagnostic marker for *mlo-11* (resistant to powdery mildew) and *Mlo-11* respectively, which makes them ideal varieties to examine the role of candidate genes on *mlo*-mediated effects in two different backgrounds by using for example double mutants: *cdpk7-mlo11, CDPK7-mlo11, cdpk7-Mlo11, CDPK7-Mlo11*. Studying the role of genes potentially affecting the pleiotropic effect of barley plants resistant to powdery mildew will help better understand the plant-pathogen interaction and potentially design diagnostic markers for marker assisted selection for barley breeders (Hasan et al., 2021). If barley *Hv*CDPKs can partially restore *mlo* resistance to powdery mildew, and CDPKs are known to play a role in abiotic and biotic stress responses they may be promising targets. (Freymark et al., 2007; Weckwerth et al., 2015; Yang et al., 2017).

To date, the molecular basis of *mlo*-based resistance to *B. graminis* f. sp. *hordei* and susceptibility to other pathogens has been the subject of several studies (Kusch and Panstruga, 2017). Two genetic loci that are *required for* *mlo-specified resistance 1* (*Ror1*) and *Ror2* to powdery mildew resistance have been studied in great detail (Freialdenhoven et al., 1996). Studies on the effect on RLS development showed that *ror1-2* mutants in barley near NILs with *mlo5* in the background, led to reduced RLS symptom formation, but not *R. collo-cygni* DNA-levels (McGrann et al., 2014). Furthermore, investigations of the *mlo*-mediated susceptibility to *M. oryzae* in barley identified two loss function mutations; *Enhanced Magnaporthe Resistance* (*emr1* and *emr2*) that led to partial resistance to *M. oryzae* without compromising on mildew resistance (Jansen et al., 2007). While under controlled inoculation studies using double mutants *emr1 mlo5* lines demonstrated similar resistance to RLS development, field trials using the same genotypes did not result in the same phenotype (McGrann et al., 2014, 2020). These results highlight the fact that other factors influence *mlo*-mediated susceptibility to necrotrophic or hemibiotrophic pathogens, and the importance of narrowing down the identified QTL region of this study to identify genes controlling the trait(Chapter 3).

Congruent with previous studies, the findings of this project support the hypothesis that susceptibility to RLS is highly dependent on environmental conditions. This was seen in the lack of consistent performance across barley genotypes in the field studies and the marker-trait association being not significant in 2021, but in 2022 (Chapter 3; Figure 3.6-.7; Brown and Makepeace 2009; McGrann et al. 2014). In contrast, inoculation studies carried out under controlled conditions gave consistent results over two independent repetitions (Chapter 3). Taken together, these results suggest that adverse environmental conditions play an important role in the transition from endophytic to necrotrophic lifestyle of *R. collo-cygni*

(McGrann and Havis, 2017). This was supported by studies showing that abiotic stresses can either promote RLS disease development such as waterlogging and high light intensity, or inhibit symptom development via putative drought-induced resistance (Hoheneder et al., 2021; McGrann and Brown, 2018). Wu and Von Tiedemann (2004) have also reported that barley cultivars carrying the *mlo* mutation were more sensitive to abiotic stress and more susceptible to form physiological leaf spots. Field trials in Scotland comparing wildtype *MLO* to *mlo* cultivars show contradicting results (Makepeace et al., 2007; McGrann et al., 2014), which further support the hypothesis that RLS development is strongly dependent on the environment and that *mlo* might be modulated by abiotic stress responsive genes. Seedling studies carried out under controlled conditions from the late 20th century have indicated that resistance to powdery mildew in barley cv. Atem, which carries the *mlo11* allele, breaks down in response to drought stress (Newton and Young, 1996). Moreover, the genetic background of plants carrying the *mlo* gene affected the extend of resistance breakdown in experiments with drought stress following water relief (Newton and Young, 1996). Furthermore, leaf segments of barley plants carrying *mlo* mutations exposed to heat treatment prior mildew infection *in vitro*, exhibit higher rates of powdery mildew infection compared to varieties without the resistant *mlo* alleles (Schwarzbach, 2001). Considering the role of the *mlo* mutation as a susceptibility factor to RLS, as shown by several studies, it is important to untangle which environmental factors can shape the role of *Mlo/mlo* in the interaction between RLS and barley. Plants respond to environmental changes through phenotypic plasticity, which is defined as the capacity of a single genotype to produce multiple phenotypes (Schlichting, 1986). Plasticity can lead to phenotypic shifts that are adaptive, neutral or reduce fitness (Napier et al., 2023). The degree of underlying genetic variation in populations determines the ability of plasticity to evolve (Via, 1987). Many studies have therefore studied the detection of genotype-by-environment (G x E) interactions in plants (Van Kleunen and Fischer, 2005). The validation of specific genes that are functionally linked to the variation in plasticity will help to use population genomic tools to study the dynamic of this adaptive evolution (Lee et al., 2014). Multi environment field trials such as used in this study (Chapter 3) have a long history in plant breeding to evaluate G x E interactions and usually focus on the performance of specific genotypes at representative production sites. However, many statistical frameworks used to study these G x E interactions in plant breeding neglect all environmental and local factors such as weather and soil types and usually summarise them into a single categorical "site" effect variable (Napier et al., 2023). For example, a preliminary GWAS performed in this study that did not segregate the phenotypes by "site" did not allow the identification of the QTL at the end of chromosome 4H. The GWAS performed in this study (Figure 3.2) and the identification of the QTL at the Scottish site, but not in Germany or Norway, highlight the importance of studying G x E effects in detail, and future studies could aim to use new G x E algorithms to examine these effects. While traditional approaches were not designed to identify specific environmental drivers of plastic responses, several modern approaches now exist to quantify the environmental aspects of G x E interactions (Napier et al., 2023). One of these approaches uses an algorithm to examine environmental drivers underlying G x E interactions in large-scale multi environment data of rice flowering time genes that differentially responded to temperature at the early growth stage (Guo et al., 2020). This approach investigated how genetic effects vary in size and direction along environmental gradients such as temperature, and whether crop growth stages over which these gradient occur are important (Napier et al., 2023). Similarly, Onogi et al. (2021) used an Environmental Covariate Search Affecting Genetic Correlations (ECGC) approach to investigate meteorological factors that affect soybean (*Glycine max*) traits such as yield. The association study found a link between hours of sunshine at maturity as well as precipitation around the date of sowing and yield (Onogi et al., 2021). As these algorithms have been only recently established as common tools to integrate in GWAS studies, it might explain why few studies focussed on G x E interaction during fungal disease development (Nehe et al., 2023). Adding meteorological data such as rainfall patterns, along with data on growth stages to understand the drivers of G x E interaction will help to identify key genetic interactions that shape the response to environmental stimuli based on underlying genetic variation (Napier et al., 2023). Future studies could re-assess already collected RLS disease datasets, as in this study, by using available meteorological datasets. This would help to elucidate association of weather patterns, barley genotypes and the observations late in the season outbreaks of RLS in the field.

The appearance of RLS disease symptoms in the field is often associated with the onset of leaf senescence in the host, but it remains difficult to ascertain whether there is a causal relationship between senescence and RLS (McGrann and Brown, 2018). Physiologically, monocarpic senescence is accompanied by the loss of antioxidative capacity, and this in turn leads to the production of ROS (Zimmermann and Zentgraf, 2004). Antioxidants, that are produced by plants protect themselves from oxidative damage, include ascorbic acid and glutathione (GSH) or ROS-scavenging enzymes such as ascorbate peroxidase (APX), catalase (CAT) and GSH-peroxidase (Mittler et al., 2004). Field studies of winter barley undergoing monocarpic senescence demonstrated that first visible symptoms of RLS correspond to the breakdown of the GSH pool, which supported the hypothesis that barley susceptibility to RLS may be affected by the decline of the antioxidative system in the host (Schützendübel et al., 2008). Transcriptome studies have indicated that *R. collo-cygni* induces premature leaf senescence in barley (Sjokvist et al., 2019). Triggering host senescence as part of the pathogen's infection strategy also happens in the closely related species *Zymoseptoria tritici* causing Septoria blotch of wheat (Ma et al., 2018). Similarly, the necrotrophic pathogen *Botrytis cinera* induces host senescence as part of its infection strategy in tomato (*Solanum lycopersicum*) and *A. thaliana* (Swartzberg et al., 2008). In *A. thaliana*, the expression of *SAG12,* the promotor of a senescence associated gene, was induced upon *B. cinera* infection. The expression of an *isopentenyl transferase* (*IPT)* under *SAG12* and *SAG13* promotors delayed senescence and reduced the severity of disease symptoms caused by *B. cinera* (Swartzberg et al., 2008, 2006). Premature leaf senescence is devastating for agricultural crops, as the declining rate of photosynthesis results in major yield losses. In rice, the delay of flag leaf senescence by one day can lead to yield increases of up to 2%, highlighting the importance of understanding senescence in agriculture (Li et al., 2021). However, the induction and progression of leaf senescence is influenced by internal as well as external factors (Guo et al., 2021). Senescence can be triggered by abiotic stresses such as drought, high temperatures and salinity, but also by biotic stresses as mentioned above (Tan et al., 2023).

The results of this project contribute to better our understanding of the relationship between senescence and RLS development. All controlled condition inoculation studies with *R. collo-cygni* inoculation showed that the fungus significantly reduced the relative chlorophyll content in the host during RLS infection compared to non-inoculated plants and that rate of senescence increased as RLS progressed (Chapter 2 to 4; Evolution of the relative chlorophyll content during RLS disease development). Considering that these observations were also true for genotypes that are less susceptible to RLS (Chapter 2), these results suggest that inducing senescence is not the only factor contributing to symptom expression in barley. Indeed, this study showed that delaying leaf senescence in barley leads to increased susceptibility to RLS, which contradicts the hypothesis that delaying leaf senescence will result in enhanced resistance to *R. collo-cygni*. Furthermore, inducing leaf senescence through nitrogen starvation produced a consistent increased resistance to RLS (Chapter 2). The second part of the lifestyle of *R. collo-cygni* is regarded as necrotrophic, but the factors triggering the transition between endophytic to necrotrophic lifestyle are at present not fully understood (Dussart et al. 2020). Generally, necrotrophic pathogens have evolved strategies to induce senescence by manipulating signalling pathways in the host and consequently, defense strategies against those necrotrophic pathogens would imply the prevention of senescence-like processes by the host plant (Häffner et al., 2015; Mengiste, 2012). In contrast, biotrophic pathogens benefit from living cells and usually change signalling processes to delay senescence, and defense responses in the host include induction of cell death during the hypersensitive response to starve the pathogen of nutrients (Häffner et al., 2015). Although this study has contributed to a better understanding of the fungus lifestyle by highlighting that the delay in leaf senescence has led to increased RLS symptom development as expected for biotrophic pathogens, elucidating which molecular processes in the host triggered the fungus to become necrotrophic and how this knowledge can be applied to the field, needs to be ascertained to better understand the epidemiology of the fungus. Future studies could investigate barley mutants that show a delayed senescence and/or induced senescence phenotype of a resistant and susceptible genotype respectively to study the effect under controlled inoculation studies with *R. collo-cygni*. Moreover, those studies could monitor; 1) the ROS burst in those mutants, 2) the gene expression of *PR* genes, 3) gene expression of phytohormones involved in senescence such as cytokinin (CYT), ethylene (ET) and abscisic acid (ABA) and 4) their stress-responsive marker genes in response to infection to understand the molecular processes involved in disease susceptibility/resistance to *R. collo-cygni*.

Phytohormones such as ET, jasmonic acid (JA), salicylic acid (SA) and ABA are among the most important players involved in inducing senescence and pathogen defense. In contrast, CYT, auxins and gibberellins are known as phytohormones that delay senescence (Häffner et al., 2015). ET is a gaseous phytohormone that acts as an endogenous modulator of leaf senescence and fruit ripening (Abeles et al., 1992). While the exogenous application of ET has accelerates leaf senescence, the application of ET biosynthesis inhibitors results in delayed senescence in *A. thaliana* (Wang et al., 2001). Jing et al. (2005) has supported previous findings of the effect of ethylene being under the influence of leaf age in *A. thaliana*. The central transcription factors *ETYLENE-INSENSITIVE* (*EIN*) *EIN2* and *EIN3* are involved in senescence gene regulation. *EIN2* partially controls senescence through the regulation of the expression of microRNA (*miR164)* and its target gene product ORESARA1/*ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 92* (*ORE1*)/A*NAC 92*/*AtNAC2*. Indeed, knockout of *ORE1*/*ANAC92* results in increased leaf longevity (Kim et al., 2009). *EIN3* is known to act as a positive regulator of leaf senescence (Z. Li et al., 2013). In *A. thaliana, ANAC92/ORE1* are also involved in salt-induced senescence by controlling genes in response to salt stress (Balazadeh et al., 2010). The activation of plant defense responses against necrotrophic pathogens, which involves the production of PLANT DEFENSIN1.2 (PDF1.2), similarly requires the phytohormones ET and JA (Pieterse et al., 2012; Pré et al., 2008). ET and JA induce transcriptional activation of the ET responsive factor ERF1 and OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF59 (ORA59) via EIN3 and EIN3-LIKE 1 (EIL1; Shigenaga and Argueso 2016). In this study, the exogenous application of ACC, a precursor of ET, did not induce premature senescence in seedlings of spring barley cv. Fairing. Interestingly, ACC-treated plants exhibited reduced RLS symptoms when challenged with *R. collo-cygni* (Chapter 4; Figure 4.2 and 4.4). Moreover, the colonisation of *R. collo-cygni* was reduced in ACC-treated plants compared to control plants as demonstrated by reduced fungal DNA in the host (Chapter 4; Figure 4.3). The expression of two ERF transcription factors, *HvERF3.8* and *HvERF5.2* was strongly downregulated in cv Fairing, whereas that of *HvERF2.16* was slightly upregulated after 12h post treatment, indicating different responses to ET signalling (Chapter 4; Figure 4.7). Son et al. (2012) showed that *A. thaliana AtERF5* appears to negatively regulate resistance to the necrotrophic pathogen *Alternaria brassicola* via the repression of chitin-mediated defense responses. More research is needed in barley to ascertain which genes are responsible for the ET-mediated resistance observed in this study. The results of the study presented here are not sufficient to draw conclusions whether senescence-mediated molecular processes or defense-mediated processes as described above led to enhanced resistance to RLS under controlled conditions (Chapter 2, 4). Altogther this study showed that nitrogen starvation-induced senescence and exogenous applied ET can lead to reduced RLS symptom development and also showed that delayed leaf senescence leads to increased susceptibility to RLS (Chapter 2).Highlighting independently that senescence involved processes modulate the development of RLS under controlled conditions. Even though, exogenously applied ACC did not lead to phenotypic premature leaf senescence during the time course of these experiments, it does not imply that molecular processes involved in senescence were not activated and hence, involved in the decrease of RLS symptom development (Chapter 4; Figure 4.2, 4.4). In order to elucidate which molecular processes are responsible for these phenotypes, more research has to be conducted. The hormonal crosstalk existing in plants, makes it difficult to ascertain the results in this study solely on defense responses or the impact on senescence processes (Pieterse et al., 2012). Therefore, future studies could comprise of repeating *R. collo-cygni*-inoculation studies using exogenously applied ACC treatments as used in this study, but including 1) different barley genotypes differing in their susceptibility to RLS to test if the response to ACC differs, 2) gene expression analysis of ET-biosynthesis genes to investigate the response rate in different genotypes, 3) gene expression analysis of host immune defence-genes to examine the response to ACC and the fungus and 4) gene expression analysis of barley-senescence marker genes to investigate whether an upregulation of those were involved in the observed resistant phenotype. Furthermore, these studies could also compare the results to exogenously applied Aminoethoxyvinylglycine (AVG), which inhibits ACC synthase activity (Akagi and Stotz, 2007). This could be used in controlled condition inoculation experiments with *R. collo-cygni*, to examine whether increased susceptibility to the fungus would be achieved.

Altogether, the findings from Chapter 2 and 4 together with the findings from the GWAS analysis (Chapter 3) which identified transcription factors such as WRKY, NAC and ERF associated both with abiotic and biotic stress responses to be underlying the major QTL on barley chromosome 4H, indicates a potential role for these transcription factors in the interaction between senescence, barley genetics and RLS development. Identifying candidate genes for future studies is essential to elucidate how these are involved in contributing to the variability in resistance/susceptibility to RLS development and whether the association of abiotic stress leading to enhanced susceptibility to RLS is due to the activation of transcription factors which are integration points of both stress responses (Häffner et al., 2015). WRKY transcription factors are involved in senescence regulation as well as responses to abiotic and biotic stresses (Kumar et al., 2018; Li et al., 2021; McGrann et al., 2015a; Puranik et al., 2012). At least nine WRKY transcription factors are known to either positively or negatively regulate the response of rice plants to *M. oryzae* infection (Li et al., 2021). Resistance to *M. oryzae* in rice is enhanced by overexpression of Os*WRKY67* (Vo et al., 2018), whereas for *OsWRKY42* appears to negatively regulate the response to the blast fungus by suppressing JA signalling-related genes (Cheng et al., 2015). Overexpression of rice *OsWRKY23* in transgenic *A. thaliana* led to increased expression of pathogenesis-related (*PR*) genes and increased resistance to *Pseudomonas syringae* (Jing et al., 2009; Sade et al., 2018). Moreover, *WRKY23* was induced upon dark induced senescence (Jing et al., 2009). The association of transcription factors such as NAC and WRKY that are involved in both abiotic and biotic stress was shown to be upregulated during fungal colonisation of the apoplast by *R. collo-cygni* (Sjokvist et al., 2019). McGrann et al. (2015) showed that the overexpression of barley salt and drought responsive *NAC* (*HvSNAC1*) transcription factor leads to reduced RLS symptom formation and fungal DNA. The authors speculated whether increased resistance to RLS was mediated by mechanisms controlled by *HvSNAC1* that are involved in tolerance to abiotic stress and inhibition of senescence. Congruently, Hoheneder et al. (2021) showed increased resistance to RLS under field conditions in partially resistant spring barley genotypes. Taken together, these results support the hypothesis that senescence-mediated processes, host genetics and environmental factors that can affect both senescence and genetic responses, may lead to either resistance or susceptibility to RLS depending on the stress response in the host (Schützendübel et al. 2008; Dussart et al. 2020; Röhrig and Dussart 2022).

This study highlights how senescence can affect resistance to RLS (Chapter 2), which is highly relevant as fertilisers that contain nitrogen may affect the lifespan of the crop (Bingham et al., 2019). Furthermore, cultivars can differ in their maturity and might exhibit delayed leaf senescence that in turn, may results in the transition of *R. collo-cygni* becoming necrotrophic. The study has not only validated the hypothesis of RLS being quantitatively controlled, but also provided a list of genes underlying the genetic resistance to RLS, which indicate that key senescence transcription factors that are involved in abiotic and biotic stress responses (Chapter 3). Moreover, the investigation whether ethylene can modulate the host response and lead to increased resistance to RLS, can be used in future studies to untangle which molecular processes are responsible for this phenotype and if this can used in plant breeding.

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Thesis outputs

- **Publications:**

Röhrig, L., & Dussart, F. (2022). Does Abiotic Host Stress Favour Dothideomycete-Induced Disease Development?. Plants, 11(12), 1615.

- **Planned Publications:**

Authors: **Röhrig, L**., Hall, A., Piatti, F., Balducci, E., Bingham, I., Havis, N., Dussart, F.

Proposed title: The role of barley leaf senescence in the host/pathogen interaction with *Ramularia collo-cygni*.

Authors: **Röhrig, L.,** Dussart, F., Loosely, M., Ramsay, L., Houston, K., Russell, J., Spoel, S., Havis., N.

Proposed title: Genome Wide Association Scan reveals potential for genetic control for Ramularia leaf spot in barley.

- **Conference posters:**

Authors: **Röhrig, L.,** Dussart, F., Loosely, M., Russell, J., Spoel, S., Havis., N.

Title: Understanding the genetic basis of Ramularia disease resistance in barley.

Conference: Barley Away Days, February 2020, Dunkeld, UK

Authors: **Röhrig, L.,** Dussart, F., Houston, K., M., Russell, J., Brosnan, J., Spoel, S., Havis., N.

Title: Is there a potential for a genetic control of the barley disease, Ramularia leaf spot?

Conference: 12th International Congress for Plant Pathology, August 2023, Lyon, France

Appendix

A.1: Genes underlying QTL JHI-Hv50k-2016-266331 obtaining high confidence.

Chromosome	*AGP Start*	*AGP End*	*ID*	*Confidence*	*Description*
chr4H	587291458	587296384	HORVU.MOREX.r3.4HG0409850.1	high	4-coumarate--CoA ligase-like 4
chr4H	587325235	587327404	HORVU.MOREX.r3.4HG0409870.1	high	Expansin-like protein
chr4H	587354582	587357099	HORVU.MOREX.r3.4HG0409880.1	high	Metal tolerance protein
chr4H	587406525	587409379	HORVU.MOREX.r3.4HG0409890.1	high	Calcium-dependent protein kinase
chr4H	587410564	587416298	HORVU.MOREX.r3.4HG0409900.1	high	Multifunctional CCA protein
chr4H	587466994	587476092	HORVU.MOREX.r3.4HG0409910.1	high	Ubiquitin family protein, expressed
chr4H	587469528	587476092	HORVU.MOREX.r3.4HG0409910.2	high	Ubiquitin-like protein
chr4H	587479048	587482147	HORVU.MOREX.r3.4HG0409940.1	high	Catalase
chr4H	587479048	587482147	HORVU.MOREX.r3.4HG0409940.2	high	Catalase
chr4H	587510757	587511530	HORVU.MOREX.r3.4HG0409950.1	high	AP2/B3 transcription factor family protein
chr4H	587515091	587515625	HORVU.MOREX.r3.4HG0409970.1	high	Alpha-1,4-glucan-protein synthase [UDP-forming] 1
chr4H	587645439	587645813	HORVU.MOREX.r3.4HG0410000.1	high	RECQ helicase SIM
chr4H	587746642	587747749	HORVU.MOREX.r3.4HG0410010.1	high	AP2/B3 transcription factor family protein
chr4H	587888698	587894052	HORVU.MOREX.r3.4HG0410020.1	high	Plant regulator RWP-RK family protein, putative
chr4H	587894387	587898091	HORVU.MOREX.r3.4HG0410030.1	high	Protein kinase
chr4H	587960178	587965968	HORVU.MOREX.r3.4HG0410040.1	high	Protein kinase family protein
chr4H	587960329	587965408	HORVU.MOREX.r3.4HG0410040.2	high	Protein kinase family protein
chr4H	588052744	588056525	HORVU.MOREX.r3.4HG0410050.1	high	Bromodomain containing protein, expressed
chr4H	588125683	588126091	HORVU.MOREX.r3.4HG0410080.1	high	KED-like protein
chr4H	588126591	588131540	HORVU.MOREX.r3.4HG0410090.1	high	Hypersensitive-induced response protein 1
chr4H	588160574	588161181	HORVU.MOREX.r3.4HG0410110.1	high	Cysteine proteinase

chr4H	588164177	588166512	HORVU.MOREX.r3.4HG0410120.1	high	Nucleoside-triphosphatase THEP1
chr4H	588171624	588175204	HORVU.MOREX.r3.4HG0410140.1	high	RB1-inducible coiled-coil protein 1, putative isoform 2
chr4H	588192127	588192729	HORVU.MOREX.r3.4HG0410160.1	high	Pectinesterase inhibitor
chr4H	588281116	588285643	HORVU.MOREX.r3.4HG0410170.1	high	ATP-dependent RNA helicase-like protein
chr4H	588394377	588395616	HORVU.MOREX.r3.4HG0410180.1	high	Werner Syndrome-like exonuclease
chr4H	588396936	588404092	HORVU.MOREX.r3.4HG0410190.1	high	Calcium-binding EF hand protein
chr4H	588481908	588484501	HORVU.MOREX.r3.4HG0410200.1	high	Adenylate kinase
chr4H	588547699	588556433	HORVU.MOREX.r3.4HG0410210.1	high	Protein O-linked-mannose beta-1,4-N-acetylglucosaminyltransferase 2
chr4H	588588083	588588352	HORVU.MOREX.r3.4HG0410270.1	high	Leucine--tRNA ligase
chr4H	588589646	588590094	HORVU.MOREX.r3.4HG0410290.1	high	Defensin
chr4H	588596497	588597314	HORVU.MOREX.r3.4HG0410300.1	high	Defensin
chr4H	588701434	588703182	HORVU.MOREX.r3.4HG0410340.1	high	CoA ligase
chr4H	588841541	588841921	HORVU.MOREX.r3.4HG0410360.1	high	B3 domain-containing protein
chr4H	588875809	588882160	HORVU.MOREX.r3.4HG0410390.1	high	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7
chr4H	588889254	588893529	HORVU.MOREX.r3.4HG0410400.1	high	MYB transcription factor-like
chr4H	588896608	588897882	HORVU.MOREX.r3.4HG0410410.1	high	WRKY transcription factor
chr4H	588921104	588923570	HORVU.MOREX.r3.4HG0410440.1	high	Glycosyltransferase
chr4H	588937838	588938086	HORVU.MOREX.r3.4HG0410450.1	high	Clavata3/ESR (CLE) gene family member
chr4H	589053514	589054618	HORVU.MOREX.r3.4HG0410500.1	high	Myb transcription factor
chr4H	589066901	589067539	HORVU.MOREX.r3.4HG0410510.1	high	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin-l
chr4H	589167817	589168744	HORVU.MOREX.r3.4HG0410540.1	high	P-glycoprotein 5
chr4H	589198132	589200473	HORVU.MOREX.r3.4HG0410550.1	high	Glyceraldehyde-3-phosphate dehydrogenase
chr4H	589212039	589221337	HORVU.MOREX.r3.4HG0410560.1	high	DIS3-like exonuclease 2
chr4H	589233591	589234296	HORVU.MOREX.r3.4HG0410610.1	high	Urease accessory protein UreG 2
chr4H	589324720	589327859	HORVU.MOREX.r3.4HG0410620.1	high	MLO-like protein
chr4H	589327883	589330672	HORVU.MOREX.r3.4HG0410630.1	high	Phosphatidylinositol-glycan biosynthesis class F protein
chr4H	589330753	589336128	HORVU.MOREX.r3.4HG0410640.1	high	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subu

chr4H	589343850	589345354	HORVU.MOREX.r3.4HG0410650.1	high	Ring finger protein
chr4H	589355937	589357886	HORVU.MOREX.r3.4HG0410660.1	high	MLO-like protein
chr4H	589379941	589383926	HORVU.MOREX.r3.4HG0410670.1	high	Monosaccharide-sensing protein 2
chr4H	589386314	589386977	HORVU.MOREX.r3.4HG0410680.1	high	B3 domain-containing protein
chr4H	589396076	589399312	HORVU.MOREX.r3.4HG0410690.1	high	Glycerol kinase
chr4H	589396076	589399312	HORVU.MOREX.r3.4HG0410690.2	high	Glycerol kinase
chr4H	589397759	589402492	HORVU.MOREX.r3.4HG0410700.1	high	Calcium dependent protein kinase
chr4H	589416090	589420038	HORVU.MOREX.r3.4HG0410710.1	high	DNA polymerase delta small subunit
chr4H	589416364	589420139	HORVU.MOREX.r3.4HG0410710.2	high	DNA polymerase delta small subunit
chr4H	589420534	589422422	HORVU.MOREX.r3.4HG0410720.1	high	Alpha-2C adrenergic receptor
chr4H	589425362	589425981	HORVU.MOREX.r3.4HG0410730.1	high	auxin transport protein (BIG)
chr4H	589431057	589460084	HORVU.MOREX.r3.4HG0410740.1	high	Callose synthase
chr4H	589460906	589462608	HORVU.MOREX.r3.4HG0410750.1	high	Fasciclin-like arabinogalactan protein
chr4H	589467784	589471121	HORVU.MOREX.r3.4HG0410760.1	high	Proteasome maturation factor UMP1 family protein
chr4H	589500044	589500874	HORVU.MOREX.r3.4HG0410780.1	high	Glutathione S-transferase
chr4H	589518078	589525806	HORVU.MOREX.r3.4HG0410790.1	high	Potassium efflux antiporter
chr4H	589604262	589605474	HORVU.MOREX.r3.4HG0410800.1	high	Hydroxyproline-rich glycoprotein family protein, putative
chr4H	589605804	589608885	HORVU.MOREX.r3.4HG0410810.1	high	Receptor-like kinase
chr4H	589620678	589626320	HORVU.MOREX.r3.4HG0410820.1	high	SMAD/FHA domain-containing family protein
chr4H	589627169	589629308	HORVU.MOREX.r3.4HG0410830.1	high	Transcription factor
chr4H	589757536	589758030	HORVU.MOREX.r3.4HG0410870.1	high	myosin heavy chain, embryonic smooth protein
chr4H	589770425	589773203	HORVU.MOREX.r3.4HG0410880.1	high	NAC domain-containing protein
chr4H	589822163	589823702	HORVU.MOREX.r3.4HG0410900.1	high	Cell growth defect factor 2
chr4H	589831093	589832633	HORVU.MOREX.r3.4HG0410920.1	high	Guanylate-binding protein 1
chr4H	589857847	589862339	HORVU.MOREX.r3.4HG0410930.1	high	Non-specific serine/threonine protein kinase
chr4H	589985517	589987124	HORVU.MOREX.r3.4HG0410950.1	high	Heavy metal transport/detoxification superfamily protein
chr4H	590314242	590314949	HORVU.MOREX.r3.4HG0410980.1	high	transcription repressor

chr4H	590338448	590339701	HORVU.MOREX.r3.4HG0410990.1	high	RNA exonuclease 4
chr4H	590343414	590343827	HORVU.MOREX.r3.4HG0411000.1	high	Zinc finger protein
chr4H	590432943	590433380	HORVU.MOREX.r3.4HG0411010.1	high	Zinc finger family protein
chr4H	590454652	590458850	HORVU.MOREX.r3.4HG0411020.1	high	S-adenosyl-L-methionine-dependent methyltransferase domain-conta
chr4H	590461271	590467037	HORVU.MOREX.r3.4HG0411030.1	high	S-adenosylmethionine-dependent methyltransferase, putative
chr4H	590469930	590473467	HORVU.MOREX.r3.4HG0411040.1	high	Chaperone protein dnaJ
chr4H	590479539	590483279	HORVU.MOREX.r3.4HG0411050.1	high	C-terminal binding protein
chr4H	590511565	590511876	HORVU.MOREX.r3.4HG0411090.1	high	NAM-like protein
chr4H	590512491	590518301	HORVU.MOREX.r3.4HG0411100.1	high	Anthranilate phosphoribosyltransferase
chr4H	590521616	590523782	HORVU.MOREX.r3.4HG0411110.1	high	Galactose mutarotase-like superfamily protein
chr4H	590535382	590540839	HORVU.MOREX.r3.4HG0411120.1	high	DUF21 domain-containing protein
chr4H	590704298	590704927	HORVU.MOREX.r3.4HG0411150.1	high	Ethylene-responsive transcription factor
chr4H	590806731	590810400	HORVU.MOREX.r3.4HG0411160.1	high	DET1-and DDB1-associated protein 1
chr4H	590863912	590867759	HORVU.MOREX.r3.4HG0411170.1	high	Kinase family protein
chr4H	590878179	590881672	HORVU.MOREX.r3.4HG0411180.1	high	Diacylglycerol kinase family protein
chr4H	590922714	590927222	HORVU.MOREX.r3.4HG0411190.1	high	G patch domain-containing protein 1
chr4H	590939944	590942021	HORVU.MOREX.r3.4HG0411210.1	high	Beta-carotene hydroxylase, putative, expressed
chr4H	590984198	590987596	HORVU.MOREX.r3.4HG0411230.1	high	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
chr4H	591039017	591045423	HORVU.MOREX.r3.4HG0411240.1	high	WD-repeat protein, putative
chr4H	591039017	591045423	HORVU.MOREX.r3.4HG0411240.2	high	WD-repeat protein, putative
chr4H	591426913	591427596	HORVU.MOREX.r3.4HG0411270.1	high	N-acetyltransferase
chr4H	591428454	591428915	HORVU.MOREX.r3.4HG0411290.1	high	Late embryogenesis abundant protein At3g53040
chr4H	591429523	591430012	HORVU.MOREX.r3.4HG0411300.1	high	dipeptide transport ATP-binding protein
chr4H	591430784	591439304	HORVU.MOREX.r3.4HG0411310.1	high	Zinc finger protein
chr4H	591511165	591516845	HORVU.MOREX.r3.4HG0411320.1	high	Appr-1-p processing enzyme family protein
chr4H	591522926	591524760	HORVU.MOREX.r3.4HG0411330.1	high	Phosphate transporter
chr4H	591581148	591585872	HORVU.MOREX.r3.4HG0411360.1	high	Splicing factor u2af large subunit, putative

chr4H	591585957	591589189	HORVU.MOREX.r3.4HG0411370.1	high	Splicing factor u2af large subunit, putative
chr4H	591595383	591598620	HORVU.MOREX.r3.4HG0411380.1	high	Serine/threonine-protein kinase
chr4H	591614756	591616033	HORVU.MOREX.r3.4HG0411400.1	high	eisosome protein
chr4H	591619986	591623433	HORVU.MOREX.r3.4HG0411420.1	high	Mammalian uncoordinated homology 13, domain 2
chr4H	591638354	591638849	HORVU.MOREX.r3.4HG0411430.1	high	cell cycle checkpoint control protein family
chr4H	591669251	591671554	HORVU.MOREX.r3.4HG0411440.1	high	Superoxide dismutase [Cu-Zn]
chr4H	591696306	591702861	HORVU.MOREX.r3.4HG0411460.1	high	Peptide transporter
chr4H	591720785	591722216	HORVU.MOREX.r3.4HG0411500.1	high	Werner Syndrome-like exonuclease
chr4H	591809155	591810425	HORVU.MOREX.r3.4HG0411520.1	high	Transcription factor
chr4H	591946399	591946668	HORVU.MOREX.r3.4HG0411530.1	high	Homeobox-leucine zipper family protein
chr4H	592020483	592024224	HORVU.MOREX.r3.4HG0411550.1	high	transducin family protein / WD-40 repeat family protein
chr4H	592079043	592085310	HORVU.MOREX.r3.4HG0411570.1	high	transducin family protein / WD-40 repeat family protein
chr4H	592092158	592093445	HORVU.MOREX.r3.4HG0411580.1	high	golgin family A protein
chr4H	592157188	592159188	HORVU.MOREX.r3.4HG0411590.1	high	GDSL esterase/lipase
chr4H	592212851	592220687	HORVU.MOREX.r3.4HG0411620.1	high	Thyroid adenoma-associated protein-like protein
chr4H	592221164	592223005	HORVU.MOREX.r3.4HG0411630.1	high	DUF3527 domain protein
chr4H	592288468	592291342	HORVU.MOREX.r3.4HG0411680.1	high	Zinc finger protein CONSTANS
chr4H	592302989	592309703	HORVU.MOREX.r3.4HG0411700.1	high	Methyltransferase
chr4H	592302989	592309703	HORVU.MOREX.r3.4HG0411700.2	high	Methyltransferase
chr4H	592337682	592341483	HORVU.MOREX.r3.4HG0411730.1	high	Mechanosensitive ion channel
chr4H	592350525	592360186	HORVU.MOREX.r3.4HG0411740.1	high	ATP-dependent RNA helicase
chr4H	592362028	592367783	HORVU.MOREX.r3.4HG0411750.1	high	WD40 repeat-like protein
chr4H	592369159	592374327	HORVU.MOREX.r3.4HG0411760.1	high	Serine carboxypeptidase, putative
chr4H	592518257	592519288	HORVU.MOREX.r3.4HG0411810.1	high	Zinc finger protein, putative
chr4H	592587788	592588357	HORVU.MOREX.r3.4HG0411820.1	high	Zinc finger protein, putative
chr4H	592690853	592691517	HORVU.MOREX.r3.4HG0411830.1	high	Leucine-rich repeat (LRR) family protein
chr4H	592784585	592785722	HORVU.MOREX.r3.4HG0411840.1	high	Zinc finger protein, putative

chr4H	592846881	592849408	HORVU.MOREX.r3.4HG0411870.1	high	Cysteine-rich repeat secretory protein
chr4H	592885851	592886450	HORVU.MOREX.r3.4HG0411890.1	high	DNA topoisomerase
chr4H	592889043	592890773	HORVU.MOREX.r3.4HG0411900.1	high	Heat shock 70 kDa protein
chr4H	592894914	592896053	HORVU.MOREX.r3.4HG0411910.1	high	Eukaryotic aspartyl protease family protein, expressed
chr4H	593001339	593002985	HORVU.MOREX.r3.4HG0411930.1	high	Heat shock 70 kDa protein
chr4H	593025153	593025944	HORVU.MOREX.r3.4HG0411940.1	high	Receptor-like kinase
chr4H	593059274	593085900	HORVU.MOREX.r3.4HG0411950.1	high	Eukaryotic aspartyl protease family protein, expressed
chr4H	593090430	593091776	HORVU.MOREX.r3.4HG0411960.1	high	Tetratricopeptide repeat (TPR)-like superfamily protein
chr4H	593096907	593099566	HORVU.MOREX.r3.4HG0411980.1	high	Cation/H(+) antiporter
chr4H	593196096	593198883	HORVU.MOREX.r3.4HG0412030.1	high	vacuolar sorting-associated protein (DUF946)
chr4H	593203074	593210307	HORVU.MOREX.r3.4HG0412040.1	high	Multidrug resistance protein ABC transporter family protein
chr4H	593258619	593260031	HORVU.MOREX.r3.4HG0412050.1	high	Werner Syndrome-like exonuclease
chr4H	593375790	593376728	HORVU.MOREX.r3.4HG0412080.1	high	Myb transcription factor
chr4H	593378369	593384130	HORVU.MOREX.r3.4HG0412090.1	high	C3HC4-type RING finger-containing protein
chr4H	593434745	593437167	HORVU.MOREX.r3.4HG0412120.1	high	Zinc finger (C3HC4-type RING finger) family protein
chr4H	593485485	593487149	HORVU.MOREX.r3.4HG0412140.1	high	Zinc finger (C3HC4-type RING finger) protein family-like
chr4H	593582459	593585506	HORVU.MOREX.r3.4HG0412150.1	high	Beta-amylase
chr4H	593676681	593681221	HORVU.MOREX.r3.4HG0412180.1	high	Methyltransferase-like protein
chr4H	593682738	593684920	HORVU.MOREX.r3.4HG0412200.1	high	Pectin lyase-like superfamily protein
chr4H	593682738	593685799	HORVU.MOREX.r3.4HG0412200.2	high	Pectin lyase-like superfamily protein
chr4H	594058659	594059318	HORVU.MOREX.r3.4HG0412230.1	high	Translation initiation factor IF-2
chr4H	594061712	594062899	HORVU.MOREX.r3.4HG0412240.1	high	Serine/threonine-protein phosphatase 7 long form-like protein
chr4H	594069841	594070692	HORVU.MOREX.r3.4HG0412250.1	high	GTPase-activating protein BEM2/IPL2
chr4H	594084110	594085345	HORVU.MOREX.r3.4HG0412260.1	high	Membrane-associated kinase regulator-like protein, putative
chr4H	594094042	594095785	HORVU.MOREX.r3.4HG0412270.1	high	receptor-like kinase
chr4H	594132059	594132863	HORVU.MOREX.r3.4HG0412290.1	high	F-box protein
chr4H	594133390	594136381	HORVU.MOREX.r3.4HG0412300.1	high	Receptor-like protein kinase, putative

chr4H	594143333	594145032	HORVU.MOREX.r3.4HG0412310.1	high	Beta-1,3-N-acetylglucosaminyltransferase lunatic fringe
chr4H	594170946	594171653	HORVU.MOREX.r3.4HG0412320.1	high	PDZ and LIM domain protein 2
chr4H	594228348	594233452	HORVU.MOREX.r3.4HG0412330.1	high	Homeobox protein BEL1-like protein
chr4H	594228685	594233452	HORVU.MOREX.r3.4HG0412330.2	high	Homeobox protein BEL1-like protein
chr4H	594228685	594233452	HORVU.MOREX.r3.4HG0412330.3	high	Homeobox protein BEL1-like protein
chr4H	594329309	594331634	HORVU.MOREX.r3.4HG0412340.1	high	Alpha/beta hydrolase related protein
chr4H	594360071	594364210	HORVU.MOREX.r3.4HG0412360.1	high	PLAC8 family protein
chr4H	594364765	594369936	HORVU.MOREX.r3.4HG0412370.1	high	Homeobox protein knotted-1, putative
chr4H	594425001	594429302	HORVU.MOREX.r3.4HG0412380.1	high	WD-repeat protein, putative
chr4H	594437789	594439683	HORVU.MOREX.r3.4HG0412420.1	high	RNA binding protein
chr4H	594450010	594451855	HORVU.MOREX.r3.4HG0412430.1	high	Sugar transporter, putative
chr4H	594479549	594482003	HORVU.MOREX.r3.4HG0412450.1	high	E2 ubiquitin-conjugating-like enzyme
chr4H	594538879	594559903	HORVU.MOREX.r3.4HG0412460.1	high	MADS-box transcription factor
chr4H	594615293	594615652	HORVU.MOREX.r3.4HG0412480.1	high	Glutathione S-transferase T3
chr4H	594617172	594621229	HORVU.MOREX.r3.4HG0412490.1	high	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily pr
chr4H	594635903	594636987	HORVU.MOREX.r3.4HG0412500.1	high	ArfGap/RecO-like zinc finger domain-containing protein
chr4H	594928758	594929288	HORVU.MOREX.r3.4HG0412540.1	high	GRF zinc finger family protein
chr4H	594964035	594964520	HORVU.MOREX.r3.4HG0412550.1	high	Amino acid dehydrogenase family protein
chr4H	594964865	594967720	HORVU.MOREX.r3.4HG0412560.1	high	Basic helix-loop-helix transcription factor
chr4H	594978685	594985599	HORVU.MOREX.r3.4HG0412570.1	high	Kinase family protein
chr4H	595124490	595134488	HORVU.MOREX.r3.4HG0412600.1	high	Endoribonuclease Dicer-like protein 3
chr4H	595134588	595135935	HORVU.MOREX.r3.4HG0412610.1	high	Nascent polypeptide-associated complex alpha subunit, putative, expr
chr4H	595184235	595185845	HORVU.MOREX.r3.4HG0412620.1	high	Syntaxin, putative
chr4H	595214137	595215389	HORVU.MOREX.r3.4HG0412630.1	high	Peroxidase
chr4H	595218235	595222497	HORVU.MOREX.r3.4HG0412640.1	high	Peroxidase
chr4H	595230536	595235207	HORVU.MOREX.r3.4HG0412650.1	high	TVP38/TMEM64 family membrane protein
chr4H	595388187	595389934	HORVU.MOREX.r3.4HG0412680.1	high	B3 domain-containing protein

chr4H	595518699	595520360	HORVU.MOREX.r3.4HG0412720.1	high	Gag polyprotein
chr4H	595533189	595537976	HORVU.MOREX.r3.4HG0412730.1	high	Purple acid phosphatase
chr4H	595539888	595540403	HORVU.MOREX.r3.4HG0412740.1	high	Non-specific lipid-transfer protein
chr4H	595540806	595546418	HORVU.MOREX.r3.4HG0412750.1	high	Basic-leucine zipper (bZIP) transcription factor family protein
chr4H	595558030	595560041	HORVU.MOREX.r3.4HG0412760.1	high	Heavy metal transport/detoxification superfamily protein
chr4H	595583032	595591359	HORVU.MOREX.r3.4HG0412770.1	high	Dihydroflavonol-4-reductase
chr4H	595598659	595599294	HORVU.MOREX.r3.4HG0412780.1	high	DUF538 family protein (Protein of unknown function, DUF538)
chr4H	595626779	595630322	HORVU.MOREX.r3.4HG0412790.1	high	Remorin
chr4H	595630950	595631210	HORVU.MOREX.r3.4HG0412800.1	high	SWAP (Suppressor-of-White-APricot)/surp domain-containing protei

A.2 Primer Design:

>HvEF1a_L
ATGATTCCCACCAAGCCCAT
>HvEF1a_R
ACACCAACAGCCACGTTTGC

>HvGAPDH_L
CCTTCCGTGTTCCCACTGTTG
>HvGAPDH_R
ATGCCCTTGAGGTTTCCCTC

>HvTUBA_L
AGTGTCCTGTCCACCCACTC
>HvTUBA_R
AGCATGAAGTGGATCCTTGG

HvERF3.8_5
>Forward(5′-3′)
AGACAACCTGAATACTGCTCGT
>Reverse (5′-3′)
ACTCCTTCTAACCATGTCCAGC

HvERF5.2_4
> Forward (5′-3′)
GGAGAGGTCAAGGTGGAGGA
>Reverse (5′-3′)
CTGACCGCTTCTTTCGCTTC

HvERF2.16_5
>Forward(5′-3′)
GTTCCTTGTAAATGCCCGCG
>Reverse (5′-3′)
CAGCAACCCTTCCTTTCGTTC