

**Quantitative assessment and genetics of the interaction  
between the light leaf spot pathogen *Pyrenopeziza  
brassicae* and its oilseed rape host *Brassica napus***

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Submitted to the University of Hertfordshire in partial fulfilment of the  
requirements of the degree of Doctor of Philosophy

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February 2026

## Abstract

*Pyrenopeziza brassicae* is a pathogenic fungus responsible for light leaf spot disease, one of the most important diseases affecting winter oilseed rape (*Brassica napus*) crops across the UK and Europe, currently considered the most economically damaging disease affecting the UK crop. Current cultural and chemical control practices are insufficient to manage epidemics of the disease, therefore genetic host resistance against *P. brassicae* has become of increasing importance as an effective control strategy. The aim of this project was to expand the existing knowledge on *B. napus*-*P. brassicae* pathosystem and explore the genetic basis of host resistance, focusing on quantitative disease resistance (QDR).

To study the interaction of the *B. napus* - *P. brassicae* pathosystem, it is fundamental to establish a reliable method to detect and quantify the phenotypical changes related to the colonisation and determine the severity of the disease. Currently, the strategies used to assess this are based on the presence of acervuli on the surface of the leaves, visually measuring the percentage of area covered by the sporulating structures, classifying the disease severity on a 1-6 scale. Visual assessment, however, can lead to error; therefore, four different quantitative phenotyping strategies were tested and compared: acervuli quantification, spore counting, WAC assay and qPCR. Different *B. napus* cultivars that differed in their resistance response to *P. brassicae* were grown in controlled environment conditions and inoculated with a fungal pathogen population. Harvested samples were assessed using visual assessment and the four quantitative phenotyping techniques, and the results statistically compared. Quantitative techniques yielded better results than visual assessment, showing higher accuracy and an improved ability to distinguish between resistant phenotypes. Quantitative PCR produced the most significant results, followed by WAC assay, spore counting and acervuli quantification, in that order.

A previous study identified the BAHD acyltransferase *BnHAT1* gene as potentially involved in susceptibility to *P. brassicae* (Fell *et al.*, 2023). This current study aims to characterise the function of this candidate gene in relation to host resistance. To test this, TILLING mutant line (D167N) with SNP mutation in the gene of interest and wild type (WT) individuals were grown under controlled environment conditions, inoculated with a *P. brassicae* population and phenotyped. A similar experiment was performed using a segregating TILLING population with homozygous individuals that carried a SNP mutation, homozygous individuals with the WT allele and heterozygous individuals with both alleles. The results of these experiments concurred at showing that mutated individuals, in which the *BnHAT1* gene had been disrupted, were significantly more resistant, supporting the hypothesis that the *BnHAT1* gene is associated with susceptibility to the pathogen. This was complemented with the RNA-seq analysis of a gene expression experiment that showed that the expression of *BnHAT1* was significantly higher in susceptible cultivars than in resistant lines. The RNA-seq analysis also revealed differentially expressed genes in the transcriptome that could potentially be associated with resistance or susceptibility to the pathogen, and that are worth further study.

To study the genetics of QDR in order to identify QTLs potentially associated with resistance or susceptibility to *P. brassicae*, a bulked segregant analysis was done. A cross between a resistant and a susceptible cultivar, Lipid and Abukuma, respectively, resulted in an  $F_1$  hybrid that was selfed to produce an  $F_2$  population. The individuals from these populations were grown under glasshouse conditions in batches and inoculated with a fungal population of *P. brassicae*. The response of  $F_1$  to the pathogen was closer to that of the susceptible parent Abukuma, therefore it could be inferred that susceptibility to the pathogen is inherited as a dominant trait. The screening of the  $F_2$  individuals showed a continuous distribution of the disease severity levels, which did not fit Mendelian ratios, proving that QDR was associated with resistance, rather than *R*-genes. Finally, the extreme phenotypes of the  $F_2$  individuals were selected and pooled for a bulked segregant analysis, that revealed two QTL regions on chromosomes A01 and A06, associated with resistance to *P. brassicae*, and a QTL region on chromosome A07, associated with susceptibility, which had never been reported and could be a novel source of susceptibility to the pathogen.

Overall, this research discovered different sources of resistance and susceptibility to *P. brassicae* in the *B. napus* genome, all linked to QDR rather than to *R*-mediated resistance. These could be used to control the spread of light leaf spot epidemics by including them in breeding or bioengineering programs to produce resistant cultivars, which would be highly beneficial for farmers and breeders, as well as reduce the environmental impact.

## Acknowledgements

First and foremost, I would like to thank my principal supervisor Dr Henrik Stotz for giving me this opportunity, for his guidance and patience throughout this project, and for his invaluable input that always pushed me and my research in the right direction. I would also like to thank my supervisors Dr Rachel Wells and Dr Cristina Barrero-Sicilia for their constant support and encouragement. I have learnt so much working with them.

I am deeply grateful to the University of Hertfordshire and to the Perry Foundation for funding my research project, and especially grateful to Profesor Bruce Fitt for all his advice. Thank you very much to our industrial partner KWS and to the John Innes Centre for their collaboration in this project.

A huge thank you to Ajisa Muthayil-Ali, whose work preceded mine, for her brilliant research. I would like to extend my gratitude to all my friends and colleagues in the labs and in the Postgrad office, to the technical staff at the University of Hertfordshire for all their help with my work and their support. Special thanks to Heather Fell, who guided me on my first year, always answered my endless questions and made the countless hours of screening plants so pleasant.

I am also incredibly grateful to our colleagues from the John Innes Centre, I learnt so much and felt so inspired and motivated every time I visited. I would like to thank Catherine Chinoy and Saleha Bakht for their help with the TILLING mutants, Roland Wouters for teaching me the best way to extract DNA from my F<sub>2</sub> population, and to Burkhard Steuernagel and Aileen Magilin for their thorough help with the bioinformatics analysis of the bulked segregant analysis. I am incredibly grateful to Aileen for helping me understand the complex pipeline and for her constant availability when I was utterly confused.

A huge thank you goes to Zedi Gao who has helped me throughout the whole project every time I had a bioinformatics question. Thank you especially for the work with the RNA-seq counts table. In that regard, I would also like to thank Ivan Wolf and Gui Valente for their help with the analysis.

I am forever grateful to my family, especially to my grandparents Juan and Pilar, and to my parents Ricardo and Teresa, for making this possible, for always believing in me. Without their unconditional support I doubt I would have gotten this far. And to my sister Sonia, who has supported me in more ways she can imagine. Huge thanks to all the friends I made in Hatfield during the project, especially to Nat, Norbi, Yasmin and Caleb, and to Javi, the best and most supportive friend imaginable, being housemates was the best idea in the world and the most fun I had since I moved to Hatfield. Massive thanks to all the friends from Hastings who made me feel at home from the start. And thank you to my friends in Valencia who are a thousand miles away but make me feel like I never left in every phone call and every visit. And last but not least, thank you, Jacob, for all your love, support and understanding, and for encouraging me to take breaks that kept me sane and motivated during these last months of writing. You make life so exciting.

No thanks go to the powdery mildew that continuously destroyed multiple batches of my F<sub>2</sub> plants in the CE cabinets and in the glasshouse and set my progress back for months.

## List of conference proceedings

1. **Gimenez Molina, L.**, Fell, H. & Stotz, H. (July 2022). Detecting light leaf spot in oilseed rape: study of quantitative phenotyping strategies. Society of Spanish Researchers (SRUK) symposium, University of Oxford Brookes, Oxford, UK. Poster presentation. Appendix 10.1.
2. **Gimenez Molina, L.**, Fell, H., Muthayil-Ali, A. & Stotz, H. (September 2022). Detection of light leaf spot disease in oilseed rape: study of quantitative phenotyping strategies. The British Society for Plant Pathology Conference, University of Newcastle, Newcastle upon Tyne, UK. Poster presentation. Appendix 10.2.
3. **Gimenez Molina, L.**, Fell, H. & Stotz, H. (October 2022). Assessment of the resistance of *Brassica napus* TILLING mutants against *Pyrenopeziza brassicae*. UK-BRC OREGIN meeting, John Innes Centre, Norwich, UK. Oral presentation.
4. **Gimenez Molina, L.**, Muthayil-Ali, A., Fell, H., Chinoy, C., Wells, R. & Stotz, H. (May 2023). Resistance of oilseed rape against the light leaf spot disease. Society of Spanish Researchers (SRUK) symposium, London, UK. Poster presentation. Appendix 10.3.
5. **Gimenez Molina, L.**, Muthayil-Ali, A., Fell, H., Chinoy, C., Wells, R. & Stotz, H. (June 2023). Assessment of the resistance against *Pyrenopeziza brassicae* in *Brassica napus* TILLING mutants. School of Life and Medical Sciences Research Conference, University of Hertfordshire, Hatfield, UK. Poster presentation. Appendix 10.4.
6. **Gimenez Molina, L.**, Muthayil-Ali, A., Fell, H., Chinoy, C., Wells, R. & Stotz, H. (August 2023). Assessment of the resistance of *Brassica napus* TILLING mutants against *Pyrenopeziza brassicae*. International Conference of Plant Pathology, Lyon, France. Poster presentation. Appendix 10.5.
7. **Gimenez Molina, L.**, Muthayil-Ali, A., Fell, H., Chinoy, C., Wells, R. & Stotz, H. (November 2023). Assessment of the resistance of *Brassica napus* TILLING mutants against *Pyrenopeziza brassicae*. Agricultural Universities Council, Future Agricultural Researchers PhD Conference, Exeter, UK. Poster presentation. Appendix 10.5.
8. **Gimenez Molina, L.** & Stotz, H. (March 2024). Characterisation of durable quantitative resistance of *Brassica napus* (oilseed rape) against the light leaf spot pathogen *Pyrenopeziza brassicae*. The AgriFood Charities Partnership, NIAB farm, Cambridge. Oral presentation.
9. **Gimenez Molina, L.** & Stotz, H. (June 2024). Characterisation of durable quantitative resistance of *Brassica napus* (oilseed rape) against the light leaf spot pathogen *Pyrenopeziza brassicae*. School of Life and Medical Sciences Research Conference, University of Hertfordshire, Hatfield, UK. Oral presentation.

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## List of acronyms

|          |                                                         |
|----------|---------------------------------------------------------|
| AHCT     | Anthocyanin O-hydroxycinnamoyltransferase               |
| AHDB     | Agriculture and Horticulture Development Board          |
| ANOVA    | Analysis of variance                                    |
| AO       | Alternate allele observations                           |
| BBSRC    | Biotechnology and Biological Sciences Research Council  |
| BEAT     | Benzyl alcohol O-acyltransferase                        |
| BIC      | Bayesian Information Criterion                          |
| CDS      | Coding sequence                                         |
| CE       | Controlled environment                                  |
| CoA      | Coenzyme A                                              |
| Ct       | Quantification cycles                                   |
| cv.      | Cultivar                                                |
| cvs      | Cultivars                                               |
| °C       | Degree Celsius                                          |
| DAT      | 4-O-acetyltransferase                                   |
| DEFRA    | Department for Environment, Food & Rural Affairs        |
| DH       | Doubled haploid                                         |
| DP       | Total read depth                                        |
| dpi      | Days post inoculation                                   |
| DTT      | Dithiothreitol                                          |
| EFSA     | European Food and Safety Authority                      |
| EMS      | Ethylmethanesulphonate                                  |
| EPS1     | Enhanced Pseudomonas Susceptibility 1                   |
| FAM      | Fluorescein amidite                                     |
| FAO/STAT | Food and Agriculture Organisation of the United Nations |
| FDA      | Food and Drug Administration                            |
| FITC     | Fluorescein isothiocyanate                              |
| FRET     | Förster Resonance Energy Transfer                       |
| gDNA     | Genomic DNA                                             |
| GEM      | Gene Expression Marker                                  |
| GlcNAc   | N-Acetylglucosamine                                     |
| GMOs     | Genetically Modified Organisms                          |
| HCBT     | N-hydroxycinnamoyl/benzoyltransferase                   |
| HEAR     | High Erucic Acid Rapeseed                               |
| HEX      | Hexachlorofluorescein                                   |
| HSD      | Honestly Significant Difference                         |
| IPM      | Integrated pest management                              |
| ipTM     | Interface Predicted Template modelling                  |
| ITS      | Internal transcribed spacer                             |
| JIC      | John Innes Centre                                       |
| kD       | Kilodalton                                              |
| LEAR     | Low Erucic Acid Rapeseed                                |
| LLS      | Light leaf spot disease                                 |

|         |                                                          |
|---------|----------------------------------------------------------|
| MEA     | MEA Malt-agar extract                                    |
| mRNA    | Messenger RNA                                            |
| μL      | Microlitre                                               |
| NGS     | Next-Generation Sequencing                               |
| NTC     | Non-template control                                     |
| OSR     | Oilseed rape                                             |
| PCR     | Polymerase chain reaction                                |
| PDF 1.2 | Plant Defensin 1.2                                       |
| pLDDT   | predicted Local Distance Difference Test                 |
| pTM     | Predicted Template Modeling                              |
| QDR     | Quantitative disease resistance                          |
| qPCR    | Quantitative polymerase chain reaction                   |
| QTL     | Quantitative trait loci                                  |
| rDNA    | Ribosomal DNA                                            |
| RINe    | RNA Integrity Number Equivalent                          |
| rRNA    | Ribosomal RNA                                            |
| RT-qPCR | Real-Time quantitative polymerase chain reaction         |
| RFU     | Relative Fluorescence Units                              |
| ROS     | Reactive oxygen species                                  |
| ROX     | Carboxy-X-rhodamine                                      |
| SAP     | Shrimp alkaline phosphatase                              |
| SGR     | “Stay-green”                                             |
| SDW     | Sterile distilled water                                  |
| SNP     | Single Nucleotide Polymorphism                           |
| ssp.    | Subspecies                                               |
| TILLING | Targeting Induced Local Lesions in Genomes               |
| $T_m$   | Melting temperature                                      |
| USP     | Universal Stress Protein                                 |
| UK      | The United Kingdom of Great Britain and Northern Ireland |
| UTR     | Untranslated Regions                                     |
| USDA    | United States Department of Agriculture                  |
| vs      | Versus                                                   |
| Vst     | Variance stabilizing transformation                      |
| WAC     | Wheat-germ agglutinin chitin                             |
| WGA     | Wheat germ agglutinin                                    |
| WT      | Wild type                                                |
| w/v     | Weight per volume                                        |

# Chapter 1: General introduction

## 1.1 Fungal disease in crops

Plant pathogens have a severe impact on the agricultural economy, causing 16% of world-wide annual crop losses, of which 70-80% are caused by fungal pathogens, with over 20,000 fungi species identified as the cause of diseases in crop plants worldwide (Evans *et al.*, 2010; Moore *et al.*, 2011). It has been reported that every year, despite the widespread use of disease control strategies, growers worldwide lose between 10% and 23% of their crops to fungal disease, and an additional 10-20% of the crops post-harvest (Steinberg & Gurr, 2020). The total amount of these losses could provide 2,000 calories worth of food to 600 million to 4,000 million people daily for a year (Stukenbrock & Gurr, 2023). However, the impact of fungal diseases on agriculture is not reduced just to the direct damage to crops and yield loss. The economic impact includes the reduced marketability of affected produce and the expenses of disease management, consisting primarily of fungicide treatments and the labour associated with application. Furthermore, fungal diseases also have an ecological impact, since they can result in the decline of crop species and increase the risk of biodiversity loss, which can significantly impact local wildlife, altering ecosystems (Fisher *et al.*, 2012).

Fungal pathogens are responsible for a wide range of plant diseases; they are a severe threat to crops for numerous reasons. The millions of microscopic spores they release are very resistant and some of them can remain viable up to decades. They can spread over long distances by wind and water splash, and even via insects and other invertebrates, expanding their reach and aggravating disease epidemics (Stukenbrock & Gurr, 2023). Fungi also exhibit an extensive genetic diversity and plasticity, resulting from multiple mechanisms. These include both sexual and asexual recombination, mutations due to transposable elements inside the genome, and horizontal transfer of genetic material between fungal species or even between fungi and bacteria or plants (Möller & Stukenbrock, 2017).

Since the interactions between plant hosts and the pathogens that affect them are influenced by environmental conditions, it has been predicted that global warming is likely to affect fungal diseases on crops. It has been predicted that fungal epidemics will only be intensified by climate change (Bebber, 2015). There has been a latitude shift in species distribution, disease outbreaks change in space and time as pests and pathogens track optimal conditions, changing geographical regions, ecological niches and periods of the year in which they proliferate. Consequently, this also alters the disease management strategies farmers and growers need to adopt for efficient control (Chaloner *et al.*, 2021). It has been reported that, since 1990, fungal plant diseases common in the tropics have moved into more temperate latitudes, spread seven kilometres polewards per year (Bebber *et al.*, 2013). For instance, wheat stem rust infections that typically occur in the tropics, have been reported in Ireland and England (Stukenbrock & Gurr, 2023).

Changes to the climate influence the interactions between crops and their associated microbiomes in many ways. The rise on temperature is likely to create more suitable conditions for disease epidemics, drought weakens the crops allowing for disease to settle and efficiently infect the host, and extreme precipitation and flooding increases humidity creating optimal conditions for fungi (Zhou, J. *et al.*, 2024). Diverse environmental stresses could potentially result

in changes in molecular processes that lead to harmless endophytic fungi that are in a symbiotic relationship with the plant to evolve into pathogenic organisms. This has been studied in *Arabidopsis thaliana* models (Mesny *et al.*, 2021).

This rising global threat to food security in a warming world with an increasing population requires a better understanding of the fungal pathogens responsible for the diseases affecting crops. There needs to be a thorough study of their life cycles, dispersal mechanisms and pathogenicity, as well as developing reliable identification methods and scoring systems to assess disease severity (Strange & Scott, 2005).

### **1.1.1 Types of fungal pathogens according to their lifestyle**

Some of the most common and significant fungal diseases affecting major crops worldwide are rusts (*Puccinia*), wilts (*Fusarium*), rots (*Sclerotinia*, *Pythium*, *Botrytis*), leaf spots (*Alternaria*, *Septoria*), mildews and blights (Zhou, J. *et al.*, 2024). Some fungal diseases affect a wide range of crops, such as *Fusarium* and *Sclerotinia* rots. Downy and powdery mildews also affect a wide variety of vegetables, although individual species infect particular hosts. Other fungal diseases are specific to particular crops, such as clubroot and light leaf spot (LLS) disease affecting *Brassicaceae*. Understanding the nature of these fungal pathogens is crucial for disease management and minimising the yield loss.

Besides taxonomical groups, fungi can also be classified according to their pathogenic lifestyle and how they obtain nutrients from their hosts. They can be divided into biotrophic, necrotrophic and hemibiotrophic pathogens. Pathogens that live cooperatively with the organism they have infected are abundant, only some of them have severe detrimental effect on the host and they thrive in at the expense of an ill but living host, known as a biotrophic interactions.

Biotrophic pathogens depend on their host as an energy source, withdrawing essential nutrients from the living plant cells. These fungi have evolved to match the life cycle of their host plants, they do not normally kill the host, rather they establish a parasitic relationship, suppressing its defences and creating nutrient sinks they can use. Although the plants typically remain alive, their growth might be stunted, and they might show signs of infection. Some of the best-known pathogenic fungi that fall into this category are rusts and powdery and downy mildews (Doehlenhamm *et al.*, 2025). Many parasitic biotrophs have lost some of their biosynthetic activities by losing genes that were fundamental in metabolic pathways such as the nitrogen or sulphur assimilation pathways, making them obligate biotrophs, fully dependant on the substrates provided by the host as energy source (Kemen & Jones, 2012).

Conversely, necrotrophic pathogens cause cell death and eventually kill their infected hosts, extracting nutrients from the dead tissue. They amount for some of the most economically damaging pathogens, such as many species in the genus *Alternaria*. The early stages of infection, whilst the plant is still alive, are critical for pathogen success. The pathogen creates an area of necrotic tissue that serves as a refuge where it can feed and, at the same time, release effectors. These effectors are proteins produced by the pathogen that can enhance virulence to avoid or suppress plant immunity (Doehlenhamm *et al.*, 2025). Necrotrophs secrete massive amounts of hydrolytic enzymes that, in combination with other mechanisms, are used to degrade plant polymers for nutrient acquisition. Genomic studies have identified numerous genes coding for

cell-wall-degrading enzymes, phytotoxins and other types of hydrolytic enzymes in multiple necrotrophic fungi, expressed at different stages of the infection process (Amselem *et al.*, 2011; Kim *et al.*, 2016). Additionally, recent studies have shown that necrotrophic fungi target cell-death pathways to expand the necrosis throughout the host. They secrete different types of effectors during different stages of the infection to induce local necrosis, such as NEP (Necrosis and Ethylene-Inducing Peptide-like proteins) effectors and certain glycosyl hydrolases at early stages (Mengiste, 2012) and oxalic acid in later stages, identified in *Sclerotinia sclerotiorum*, which not only induces programmed cell death but also suppresses autophagy (Kabbage *et al.*, 2013). Another method of action of necrotrophs is the production of reactive oxygen species (ROS) that are likely to be involved in multiple processes and having multiple effects. They potentially serve as virulence factors (Heller & Tudzynski, 2011). For example, *S. sclerotiorum* and *Botrytis cinerea* are able to modulate the redox status of their hosts, exploiting the oxidative burst associated with the plant's immune system and producing additional ROS, which appears to be crucial for the development of the diseases they cause a broad range of crops (Govrin & Levine, 2000; Kim *et al.*, 2008; Shlezinger *et al.*, 2011; Williams *et al.*, 2011). Similar reactions have been identified in necrotrophic pathogen *Leptosphaeria maculans*, a significant pathogen affecting *Brassica napus* (Li *et al.*, 2008).

There is a third category known as hemibiotrophic pathogens, formed by those species that combine the biotrophic and necrotrophic lifestyles. These fungi often start their life cycle as biotrophs, infecting and colonising the host whilst drawing nutrients from its living cells. This phase is typically asymptomatic; the pathogen needs to suppress the plant defence system and does not show any detrimental signs on the plant. Later in their life cycle, the hemibiotrophic fungi will switch to a necrotrophic lifestyle and proceed to kill the host, feeding off the dead tissue (Doehlenhamm *et al.*, 2025). Some examples of hemibiotrophic pathogens are *Fusarium*, the apple scab fungus *Venturia inaequalis* and the light leaf spot (LLS) fungus *Pyrenopeziza brassicae*. They have evolved highly infectious strategies that make them very aggressive crop pathogens.

### 1.1.2 Mechanisms of host defence

Different fungal pathogens have different strategies for infection once they have landed on the foliar surface of the host. Some pathogens are able to directly penetrate the cuticle, aided by enzymes, whereas others use natural openings such as stomata or even exploit existing wounds. The space that they colonise after entry allows for the differentiation between pathogens that penetrate the hosts' cells and the pathogens that occupy the extracellular apoplast. Cell-penetrating fungi are predominantly biotrophs, they develop a specialised structure known as haustoria that acts as a "feeding tube", that physically penetrate the cell wall of plant cells. It does not penetrate the host cell membrane, which invaginates around it creating a structure known as extrahaustorial membrane, remaining outside of the cytoplasm. This structure allows the pathogen to extract nutrients from the cells (Mapuranga *et al.*, 2022). This structure is also used to deliver effector proteins into the hosts cytoplasm to suppress the host immune response (Jones & Dangl, 2006).

Apoplastic pathogens, on the other hand, colonise and thrive in between cells, in the host apoplast. These are all ascomycetes, and they include fungal pathogens that penetrate the foliar

cuticle and colonise the niche underneath, between the cuticle and the epidermal cells of the leaf, such as *P. brassicae*, responsible for light leaf spot on oilseed rape; *Rhynchosporium commune*, barley leaf blotch; and *Venturia inaequalis*, also known as apple scab. Other types of apoplastic pathogens grow between host mesophyll cells, such as *Leptosphaeria maculans*, oilseed rape phoma stem canker, or *Cladosporium fulvum*, tomato leaf mould (Stotz *et al.*, 2014).

With the pathogen occupying one of these spaces, the first line of host defence is pathogen recognition, a process governed by pattern recognition receptors (PRRs), proteins that can recognize conserved pathogen-associated molecular patterns (PAMPs). This recognition by PRRs activates the first line of active host defence known as PAMP-triggered immunity (PTI), which leads to intracellular signalling, activation of defence-related genes and generation of a set of defence responses that counter pathogen colonisation. Adapted pathogens may then release effector proteins to enhance virulence and suppress PTI. Effectors are encoded by pathogen *Avr* genes and released in a phase known as effector-triggered susceptibility (ETS) (Ngou *et al.*, 2021).

As a response, a resistant host activates resistance (*R*) genes, that encode for proteins that will recognise pathogenic effectors. This *R* gene-mediated host response will differ between cell-penetrating and apoplastic fungal pathogens. Against cell-penetrating pathogens, recognition of intracellular effectors is mediated by nucleotide-binding leucine-rich repeat (NLR) receptors. This recognition typically triggers a strong and rapid immune response known as effector-triggered immunity (ETI). This is often associated with a hypersensitive response, a mechanism used by the host that results in localised programmed host cell death at the infection site, that aims to eliminate the pathogen and prevent the spread of the disease (Jones & Dangl, 2006). Otherwise, if the pathogen effectors are released in the extracellular space by apoplastic fungal pathogens, the host uses cell surface receptor-like proteins (RLPs) and PRRs for recognition. This will initiate an effector-triggered defence (ETD), associated with host cell death after an elapsed period of endophytic pathogen growth (Stotz *et al.*, 2014).

Overall, the ETD response against apoplastic fungal pathogens is significantly slower than the ETI response against cell-penetrating pathogens since it does not resort to hypersensitive response to limit the spread of the disease. Both ETI and ETD are effector-triggered responses that rely on *R* genes, which encode for NLRs or RLP receptors able to recognise pathogen effectors, products of pathogen *Avr* genes, and aid in protecting the plant from infection. According to the 'gene for gene hypothesis' by Harold Flor (1971) each *R* gene has specificity for a *Avr* gene; therefore, a plant that has a specific *R* gene can resist the infection of pathogens with their corresponding *Avr* gene. This kind of host resistance, though effective, has the risk of being overcome by pathogen mutations: if *Avr* products change they will no longer be recognised by *R* gene-encoded receptors, leading to the breakdown of disease resistance (Watson, 1970). A more durable, broad-spectrum, form of host resistance is quantitative disease resistance (QDR).

QDR is a type of plant immunity that leads to a reduction of disease severity rather than completely preventing it. QDR is controlled by multiple quantitative trait loci (QTL), genomic regions that interact with each other and with the environment, and each provide a partial level of resistance to a pathogen. These interactions can be additive, pyramiding the effects of many QTLs can result in high levels of resistance (French *et al.*, 2016), but these loci can also display complex epistatic interactions. Epistasis in QDR refers to any non-additive interaction between multiple resistance genes, in which their combined effect is more complex than the sum of their

individual effects. Epistatic interactions can be “suppressing”, where the alleles of a QTL can mask the effects of the alleles at another locus, or “enhancing”, creating a synergistic effect, where the result from the interaction is more extreme than if their effects were simply added (Mackay & Anholt, 2024).

QDR has also proven to be more durable than *R* gene-mediated resistance (Parlevliet, 2002). *R* genes exert a strong selection pressure on the pathogen population, where a single mutation in a pathogen’s *Avr* gene could overcome the effect of the *R* gene (McDonald & Linde, 2002). Since QDR is controlled by multiple genes with only partial effects, the selection pressure on pathogen populations is significantly lower, and the pathogen variants that manage to overcome QTLs only gain a marginal advantage; thus, resistance breakdown is less common (Poland *et al.*, 2009). Furthermore, QDR is often race-nonspecific, the combined effects of QTLs confer a broad-spectrum of resistance to disease, contributing to its long-term stability (Parlevliet, 2002).

However, unlike the molecular mechanisms responsible for *R* gene-mediated resistance, QDR has not been as widely studied due to its complexity; QTLs are difficult to characterise on a molecular level due to their complex genetic architecture. In comparison to the amount of *R* genes published, there are only a few well characterised QTLs, for example the *STAYGREEN* (*SGR*) gene (Thomas & Ougham, 2014). This QTL is highly conserved in plants and has been characterised and found to be related to delayed senescence and extension of the photosynthetic period, leading to an increase in biomass and yield. It has been found in multiple plants species and crops, such as wheat, rice and maize. A specific loss-of-susceptibility mutation in the *SGR* gene in cucumbers (*CsSGR* gene) has proven to provide durable and broad-spectrum disease resistance to multiple pathogens, registered for over 50 years in the USA (Wang *et al.*, 2019).

### 1.1.3 Integrated Disease Management

It is crucial to develop effective pest and disease management strategies that ensure global food security. Conventional methods for crop protection are not sufficient to manage the spread of epidemics: traditional cultural practices, breeding programs for single disease-resistance genes or single-target fungicide spraying are not enough. For a better protection of crops from fungal disease, the best approach would need to combine different strategies and various technical innovations to manage the spread of disease. It would also require close collaboration between the agricultural industry, farmers, breeders, researchers and policymakers and governments (Stukenbrock & Gurr, 2023). For this reason, Integrated Crop Protection (ICP) or Integrated Pest Management (IPM) has become the most efficient way to limit disease impact and protect crop yields.

IPM is a holistic approach that combines different tactics and tools for prevention, monitoring, pest and disease management, and mitigation, always prioritising natural preventative measures to minimise disruption to the ecosystem and soil health (Figure 1.1). It is a tiered model that prioritises prevention by using conventional cultural practices such as crop rotation or late sowing to reduce the presence fungal spores around new seedlings and disrupt pest and disease cycles. Another recommended method of prevention is sowing crop varieties and cultivars that are highly resistant against pest and diseases (Xing, 2024). The next focus of IPM is forecasting

and monitoring disease levels, which requires a good understanding of the fungal diseases, their life cycles, the periods of crop susceptibility and the influence of abiotic factors on them. The rapid identification and early detection of fungal disease is a highly effective management practice that can aid in the selection of the of the most appropriate treatment (Jain *et al.*, 2019). Disease intervention involves the use of different methods to reduce reliance on any single method and strengthen crop protection, minimising both the environmental impact and the risk of the fungal pathogens developing resistance towards a particular control method.

For disease mitigation and treatment, IPM implements a combination of mechanical, biological and chemical controls to reduce the risk of infection in an environmentally conscious way. Mechanical and physical strategies include barriers such as nets or sticky traps to protect from insects, traps for bigger animals, or manual removal of weeds or infected leaves, amongst others. It also includes sanitation practices consisting of removing and destroying pest-infected plant material or crop debris from the fields in use and their surrounding areas (Zhou, W. *et al.*, 2024). Biological controls involve using predatory species to remove the pest such as bacteria or viruses pathogenic to specific pathogen species or using biopesticides with active ingredients originating from natural sources (Marrone, 2019). Chemical controls such as fungicides are a primary tool to manage fungal diseases but it is fundamental to only use them when strictly necessary, as a last resort. It is important to reduce the agricultural industries over reliance on chemicals to reduce their negative environmental impact and to minimise the chances of fungal strains developing resistance to the fungicides (Xing, 2024).

## **1.2 *Brassica napus***

Oilseed rape (*Brassica napus*), also known as rapeseed, is a member of the flowering Brassicaceae family, which shows a high genetic, morphologic and metabolic diversity. More specifically, it is part of the *Brassica* genus, which comprises around 40 species (Schmidt & Bancroft, 2011). Based on recent genome studies, *B. napus* is a relatively young crop species that originated around 7,500 years ago (Lu *et al.*, 2019), a recently formed polyploid containing two genomes as a result of an interspecific hybridisation between the diploids *Brassica rapa* and *Brassica oleracea*, resulting in an amphidiploid species (genome  $A_nA_nC_nC_n$ ,  $2n=38$ ), in agreement with the “Triangle of U” model (Figure 1.2; Xue *et al.*, 2020). The genome size of *B. napus* ranges between 850 Mb and 1130 Mb, with variations depending on the specific cultivar (cv.) and sequencing method used. Winter oilseed cv. Darmor-*bzh* was assembled with long-read [>700 base pairs (bp)] 454 GS-FLX+ Titanium and Sanger sequencing, with a total size of approximately 850 Mb (Chalhoub *et al.*, 2014).

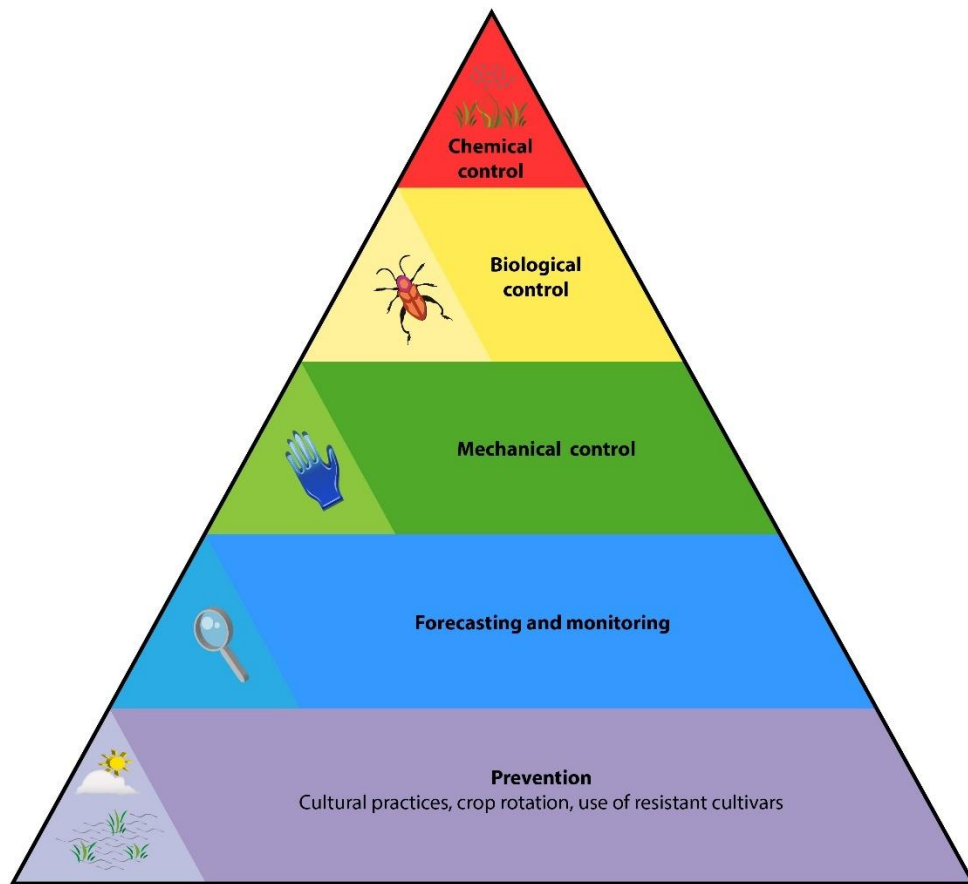


**Figure 1.1.** Key components of an Integrated Pest Management (IPM) program.

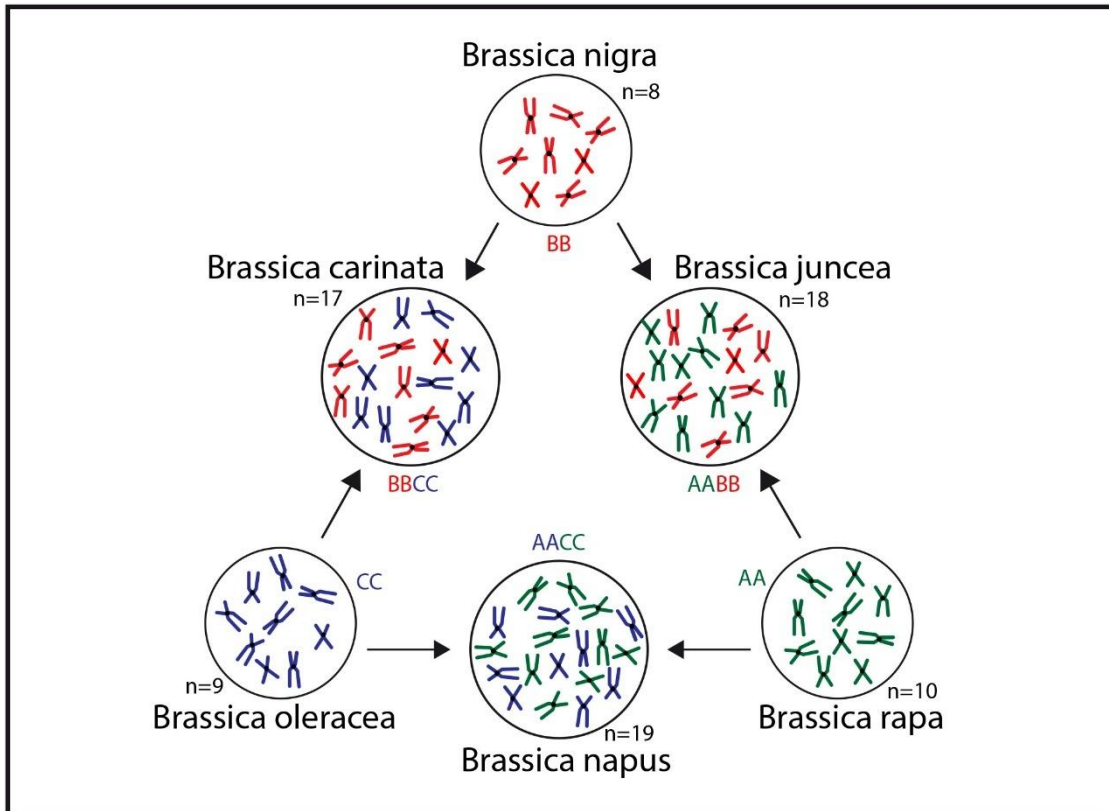


**Figure 1.2.** Brassica genome origins according to the 'Triangle of U' theory. The three ancestral diploid genomes from *Brassica rapa*, *Brassica nigra* and *Brassica oleracea* are representing A, B and C genomes. The derived tetraploid species *Brassica juncea*, *Brassica napus* and *Brassica carinata* are hybrids consisting of combined AB, BC and AC genomes.

This covers 79% of the 1130 Mb genome and includes 95.6% of *Brassica* expressed sequence tags. The genome consists of 19 chromosomes, 10 of which are from the A<sub>n</sub> subgenome (314.2 Mb), contributed by *B. rapa*, and 9 of which are from the C<sub>n</sub> subgenome (525.8 Mb), contributed by *B. oleracea*. Both subgenomes are similar in size to the progenitors' respective genomes (C<sub>n</sub>=540 Mb and A<sub>n</sub>=312 Mb). The genome contains 101,040 gene models, 91,167 of which matched with the progenitors, showing a large colinear correspondence between them.

Since there are no records of truly wild *B. napus* populations in nature, it is believed that the initial hybridization occurred in a cultivated context. Oilseed rape has been a relevant crop since its appearance as one of the earliest cultigens. Its genetic diversity was significantly reduced during the last 1000 years due to unconscious selection in artificial breeding. It is likely that its cultivation as an early form of winter rapeseed crop began in Europe around the 16<sup>th</sup> century and spread worldwide. However, it was not until the 1960s that oilseed rape became a major crop around the world, before that it was mainly used in rotation farming to boost the yield of cereal crops (Gomez-Campo & Prakash, 1999). Over the past 50 years, the total demand for oils has continuously grown worldwide at a 5% rate per year. Rapeseed production currently is, after soybeans, the second most produced oilseed in the world, providing about 12% of the total vegetable oils, the most abundant in temperate regions (USDA, 2021).

Occasional gene flow events between the two progenitors into *B. napus*, have contributed to the acquisition and improvement of complex traits in the species (Lu *et al.*, 2019). Additionally, artificial selection and intensive breeding have been used to improve pathogen resistance, optimise seed oil content and lipid biosynthesis, while promoting glucosinolate gene loss, a compound responsible for an unpleasant bitter taste in the oil (Chalhoub *et al.*, 2014). Another relevant compound artificially selected against is erucic acid, a long-chain, omega-9 monounsaturated fatty acid. It constitutes about 30-60% of the total fatty acids in Brassicaceae plants. Some studies published in the late 1970s have linked it to myocardial lipidosis, heart lesions and high blood cholesterol in laboratory rats (Heijlenskjöld & Ernster, 1975). This led to authorities like the U.S. Food and Drug Administration (FDA) and the European Food and Safety Authority (EFSA) to regulate food-grade rapeseed oil to less than 2% erucic acid by weight, establishing a tolerable daily intake of 7 mg/kg body weight (Vetter *et al.*, 2020). The development of lower erucic acid rapeseed (LEAR) cultivars opened new markets for rapeseed oil for human or animal consumption, as opposed to high erucic acid rapeseed (HEAR) cultivars, exclusively intended for industrial uses (Woodfield & Harwood, 2017). Currently, 60% of oilseed rape is used in the food industry and 3% for animal feed. The remaining 38% is used for industrial purposes, the production of biofuel being of increasing importance (Woodfield & Harwood, 2017).

Winter oilseed rape is predominantly cultivated in areas with cold climates since the flowering of the crop requires a prolonged exposure to the cold of winter (vernalisation). According to the Food and Agriculture Organisation of the United Nations (FAOSTAT) and the United States Department of Agriculture (USDA), approximately 86 million metric tonnes of oilseed rape were produced worldwide in 2024, the main producers were Canada and Europe, with a production of 19.24 and 16.84 million metric tons, respectively. The United Kingdom contributes 10% to the total European production (Figure 1.3).

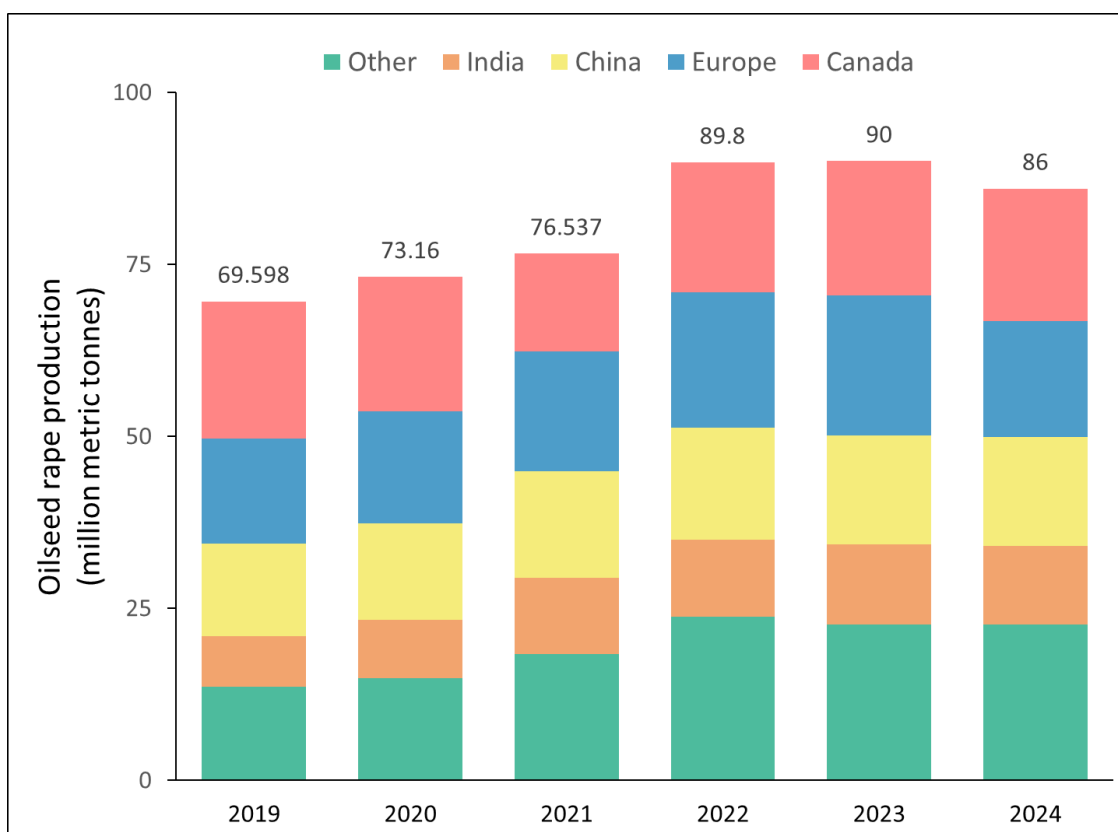


**Figure 1.3.** Oilseed rape production from 2019 to 2024, measured in million metric tonnes, according to FAOSTAT and USDA.

### **1.3 Light leaf spot disease and its economic impact**

Despite the consistent increase in oilseed rape global production throughout the decades, the growth rate in yield has stagnated in Europe in the last decade, a phenomenon associated with biotic stress and decreasing availability of active ingredients for chemical control (Noleppa, 2017). A survey by the Department for Environment, Food & Rural Affairs, released in August of 2025 showed a decline of 19% in area of rapeseed crops in Great Britain, resulting in the smallest cultivated area since 1983, continuing the downward trend seen in the past decade. This has been attributed to a combination of variable weather and the increasing cost pressures from pest control (DEFRA, 2025; AHDB, 2025).

Researchers have recorded 16 diseases and 37 insect pests affecting oilseed rape, as well as nematodes, slugs and snails. Of these pathogens, light leaf spot (LLS) was reported to be in the top ten most significant diseases affecting the winter oilseed rape across continental Europe and the most damaging disease in the UK (Zheng *et al.*, 2020). In the UK alone, more than 90% of the cultivated crops have been affected by this epidemic, causing annual yield losses of 30% worth £100-160M (CropScience Bayer: Farmpep.net), a dramatic increase from the £20M loss in 2005 (CropMonitor; AHDB). Furthermore, LLS can also severely affect the quality of the fresh Brassica vegetable, the blemishes caused by infection later in the season reduces its market value (Karandeni Dewage *et al.*, 2018). LLS has recently surpassed phoma stem canker (blackleg) as the major disease of rapeseed crops. The increasing incidence of LLS seemed to coincide with the use of cultivars with the major resistance gene *Rlm7* for effective control of phoma stem canker, which often were susceptible to LLS (Karandeni Dewage *et al.*, 2021).

### **1.4 *Pyrenopeziza brassicae* life cycle**

*Pyrenopeziza brassicae* is classified within the class Leotiomyces and phylum Ascomycota. It is an apoplastic subcuticular fungus responsible for the LLS disease, with an anamorphic form known as *Cylindrosporium concentricum* that was first documented in the 1820s (Greville, 1923). The teleomorph was reported more than a century and a half later, in 1978, four years after the first recorded major LLS epidemic in the UK (Rawlinson *et al.*, 1978).

It is an hemibiotrophic pathogen, with an initial biotroph-like phase, in which the pathogen takes up nutrients from the living host to survive, followed by a necrotrophic stage, where the pathogen kills the host cells and feeds on the dead tissues (Boys *et al.*, 2007). The necrotrophic phase coincides with the sexual stage of the fungus; the transition into consuming the dead biomass is unrelated to a higher nutritional requirement for sexual reproduction and the development of ascospores, since it has been proven that both sexual and asexual sporulation have similar energetic demands. It is rather due to a trade-off between pathogen growth and reproduction (Clément *et al.*, 2010). Furthermore, sexual reproduction can be very advantageous for pathogens since the allele recombination it generates can increase genotypic diversity. This can improve pathogenic fitness and facilitate the adaptation to its hosts (Zhan *et al.*, 2007), as well as to abiotic factors, since ascospores are more infective and durable than conidia, with a higher resistance to environmental elements such as heat or radiation, making more suitable to survive in adverse conditions and restart the life cycle (Gilles *et al.*, 2001).

*Pyrenopeziza brassicae* is a polycyclic pathogen, meaning it can produce several cycles of infection per season, increasing its impact on crop production (Boys *et al.*, 2007). As illustrated in Figure 1.4, the cycle starts in late summer-early autumn when airborne ascospores produced on debris from previous crops come into contact with winter oilseed rape crops as the primary inoculum. Ascospores germinate and hyphae penetrate the leaf cuticle with the aid of extracellular cutinases through enzymatic degradation (Li *et al.*, 2003). Somatic divisions of the hyphae generate a hypomycelium that occupies the subcuticular space, establishing itself between the cuticle and the epidermal cells of the leaf, and slowly colonising the host (Ashby, 1997; Davies *et al.*, 2000; Li *et al.*, 2003).

At early stages of the colonisation, the biotrophic phase of growth within the host is established: the pathogen feeds on the host nutrients whilst suppressing host defence responses; this is also referred as a symptomless phase (Lee & Rose, 2010). This stage continues for 1-4 months, after which sufficient accumulation of subcuticular fungal biomass leads to asexual sporulation, marking the end of the symptomless phase. The first visible symptoms are white salt-like structures, typically found in concentric rings, resulting from the development of acervuli, asexual sporulating structures containing conidia. They break through the leaf surface, often causing lesions on the leaf lamina (Fitt *et al.*, 1998b; Horbach *et al.*, 2011).

Later in early spring, the conidia contained in the acervuli are then dispersed by rain-splash and cause secondary infections in other organs. *Pyrenopeziza brassicae* can spread to all aerial parts of the plant, as well as to adjacent plants, and infection points can act as entry sites for other pathogens, increasing the damage to the plant (Gilles *et al.*, 2000). In addition to the presence of acervuli, other common foliar symptoms are the appearance of lesions which may enlarge and often develop a halo of chlorosis around them. Leaf deformations, such as leaf curling or leaf distortion, have also been reported associated with LLS and, in cases of severe infection, can lead to the death of the leaves (Karandeni Dewage *et al.*, 2021). The pathogen can also induce the formation of 'green islands' in the leaves to redirect host nutrients to the infected tissue (Ashby, 1997). LLS can spread to the stem and branches, producing brown necrotic lesions and cracks along them. It also spreads to pods, causing them to shatter prematurely and leading to significant seed losses (Karandeni Dewage *et al.*, 2018). Some cultivars show other symptoms, such as necrotic flecking on the leaf surface that has been correlated with resistance against the pathogen (Karandeni Dewage *et al.*, 2021). Production and dispersal of conidia is favoured by the characteristic wet weather of spring, which explains the increased severity of LLS epidemics in northern England and Scotland (Fitt *et al.* 1998b; Gilles *et al.*, 2000).

At this stage, usually at the beginning of summer, the pathogen undergoes a physiological transition to a necrotrophic phase in which it initiates the destruction of host cells by secreting cell-wall degrading enzymes. This coincides with the sexual phase, characterised by mating (sexual reproduction) between opposite mating types: *MAT-1* and *MAT-2*. This leads to the formation of apothecia, structures that produce ascospores, on the surface of senesced leaves (Singh & Ashby 1998). Ascospores are released throughout the summer and the first half of autumn, and the wind-dissemination of these ascospores begins the cycle again in new crops. Interestingly, *Leptospphaeria* spores are released from mid-autumn to the end of the year, so there is some overlap between both pathogens (West, 2021).

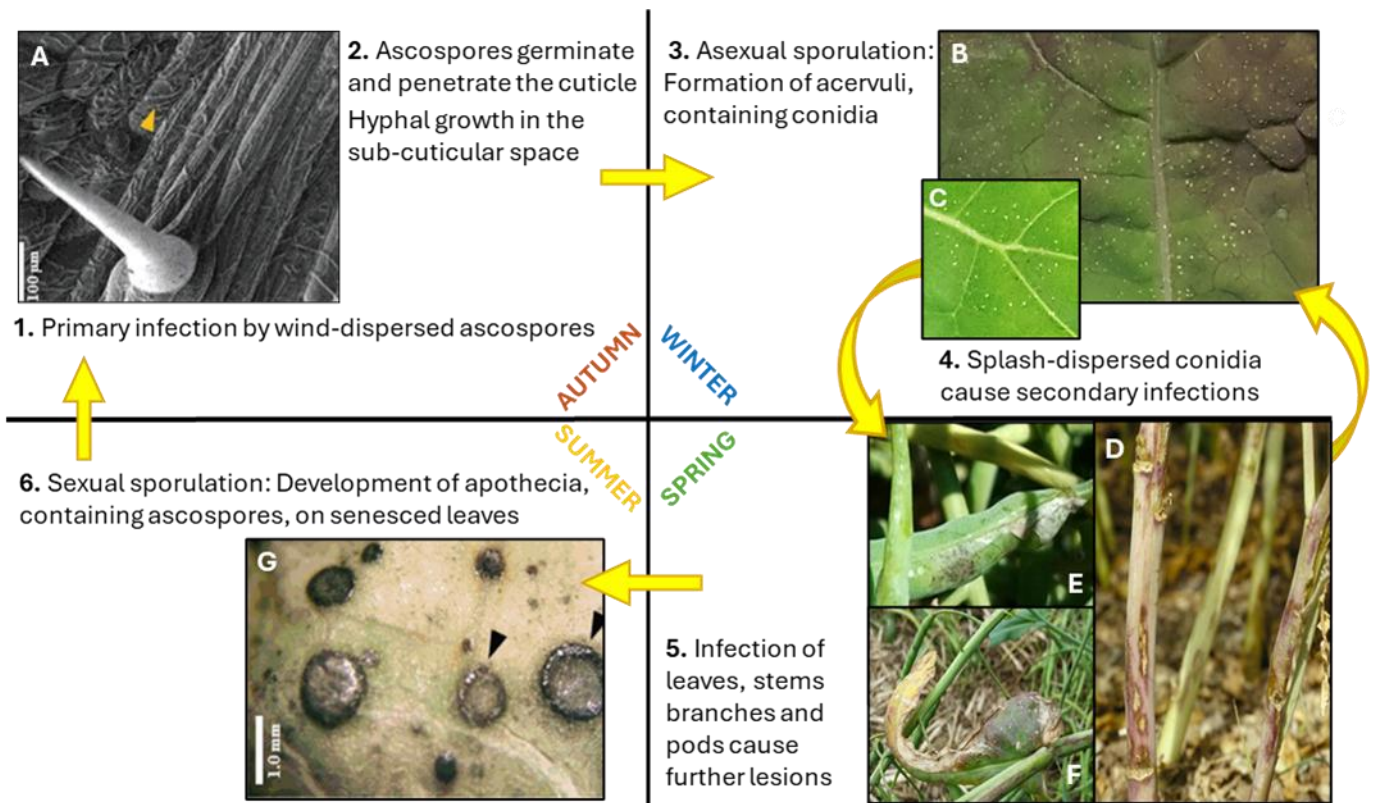


**Figure 1.4.** Life cycle of *Pyrenopeziza brassicae* in winter oilseed rape (*Brassica napus*). The cycle starts in early autumn when airborne ascospores from the previous season crop debris germinate on leaf surfaces and penetrate the leaf cuticle to grow in the sub-cuticular apoplastic space. The pathogen proliferates and colonises the host tissue in an asymptomatic phase for four months (A). During winter, lesions on the leaf surface start to appear as the pathogen forms acervuli containing conidia (B,C), which are released and splash-dispersed during spring, causing secondary infections of other leaves, stems, branches and pods, as well as adjacent plants. The increased damaged tissue leads to horizontal cracks along the stem (D), leaf distortion (F) and pods that shatter early, losing the seeds (E). Finally, during summer, *P. brassicae* initiates its sexual phase by developing apothecia that contain ascospores (G), which, when dispersed, will restart the cycle in autumn. Photograph credit: (A,G) Karandeni Dewage et al., 2018. (D,E,F) Bayer Crop Science.

*Pyrenopeziza brassicae* can also infect other vegetable crop species in the *Brassica* genus, suggesting that LLS can spread between them. The effects of the pathogen can be seen from the early stages of cropping season, by reducing plant vigour and even killing seedlings, producing significant yield losses. The impact on crops increases later in the season. The infection causes light brown patches and a large amount of visible acervuli on the leaves which reduces the market value of the produce. LLS has been recorded on kale, cabbage and Brussels sprouts (*B. oleracea* varieties *acephala*, *capitata* and *gemmifera*, respectively). It can also affect Chinese cabbage (*B. rapa* ssp. *Pekinensis*), turnip (*B. rapa* ssp. *rapa*), swede (*B. rapa* ssp. *rapifera*) and black mustard (*B. nigra*) (Karandeni Dewage *et al.*, 2018).

## **1.5 Disease management of LLS**

LLS has become the biggest disease threat for winter oilseed rape across the UK. It has proven challenging to control the disease due to several factors, such as a long symptomless phase that interferes with pathogen detection, growing insensitivity to fungicides (Carter *et al.* 2013, 2014), and the lack of knowledge of the pathosystem. To achieve an effective control of the disease epidemics, IPM has been established as the most efficient approach, combining different tactics and tools to manage the spread of disease, always prioritising natural preventative measures to minimise disruption to the ecosystem. Control of LLS affecting oilseed rape crops emphasises the importance of monitoring and disease forecasting as a fundamental pillar, with cultural practices, chemical control and cultivar resistance control strategies (Bayer Crop Science UK, 2012; Huang *et al.*, 2025).

### **1.5.1. Monitoring**

The first step for an efficient application of control measures is an early detection system for the pathogen. Morphological identification was used as an initial approach for *P. brassicae* identification by growing isolates obtained from diseased crops on artificial media and generating single-spore isolates (Karandeni Dewage *et al.*, 2021). These traditional methods are typically used in combination with molecular diagnostics because the morphological features of *P. brassicae* may be similar to those of other pathogens. Molecular identification methods provide higher sensitivity and specificity for a better identification.

Polymerase chain reaction (PCR) is widely used for identification of *P. brassicae* in air samples and in asymptomatic plant tissue samples, collected before the appearance of symptoms. Specific primers are used to amplify *P. brassicae* ITS (internal transcribed spacer) region, a highly variable, non-coding ribosomal DNA segment, widely used as a barcode to detect the presence of *P. brassicae* and distinguish from other fungi (Karolewski *et al.*, 2006). Furthermore, mating types of the *P. brassicae* isolates can be identified and differentiated using a three primer PCR (Foster *et al.*, 2002; Muthayil-Ali, 2023). Additionally, quantitative PCR (qPCR) or reverse transcriptase qPCR (RT-qPCR) can be used to also quantify *P. brassicae* DNA (Karolewski *et al.*, 2012). Another molecular diagnostics tool for early detection of *P. brassicae* is the loop-mediated isothermal amplification (LAMP) assay. This fast and cost-effective alternative target either ITS or  $\beta$ -tubulin DNA in *P. brassicae* for early detection (King *et al.*, 2017).

### 1.5.2. Cultural practices

Cultural control strategies are some of the oldest practices used by farmers to manage disease epidemics. They can reduce the concentrations of inoculum in the environment and reduce disease spread in *Brassica* crops, therefore they are typically integrated in IPM programmes against LLS disease in rapeseed crops as preventive methods rather than to suppress disease outbreaks.

One of the main cultivation techniques used by growers is burying deep into the soil the crop debris from the previous season before sowing the new crops. Airborne ascospores, responsible for initiating the epidemics, are still present in the stubble from harvested *B. napus* crops. This acts as the primary source of inoculum for new emerging crops, therefore ploughing or tilling are often used to prevent the release of spores into the air and reduce the risk of starting the infection cycle and contaminating the new seedlings (Gilles *et al.*, 2001).

Changing the sowing date is another common disease management practice. Oilseed rape is commonly sown between late August and early September, with variations depending on geographic location. Delaying the sowing by approximately two weeks can significantly reduce crop exposure to airborne *P. brassicae* ascospores during their periods of maximum release. Subsequently, the reduction of primary inoculum leads to decreasing the severity of LLS epidemics. This, however, runs the risk of poor establishment of the crop before the onset of the winter (Welham *et al.*, 2004). This method is not exclusive to the control of LLS disease, it is commonly used to stall the development of other autumn foliar diseases, such as phoma stem canker, since the ascospores of the responsible pathogens (*L. maculans* and *L. biglobosa*) are released at similar times (Huang *et al.*, 2011; West, 2021).

Additionally, crop rotation with non-host crops can help reduce the risk: by not growing the crop continuously, the amount of ascospores that serve as first inoculum is substantially reduced (Zheng *et al.*, 2020). It has been observed that the severity of LLS disease increased significantly when oilseed rape was grown in successive cropping seasons (Figuerola *et al.*, 1994). Traditionally, oilseed rape is grown in three-to-four-year rotations with cereals, however recent intensification of agriculture has changed farming practices, leading to shorter crop rotations (Karandeni Dewage *et al.*, 2018).

Despite these strategies being commonly used by farmers and growers, they can only partly contribute to reducing the severity of the disease, but on their own, they are not enough to control the epidemics unless they are complemented with other strategies such as chemical control.

### 1.5.3. Fungicide applications

The use of fungicides is an important strategy to manage the LLS disease in the UK and limit yield loss. However, fungicide applications have an economic impact on farmers, since more than £20 million are spent every year on fungicide control. Efficient fungicide usage is crucial to maximise the effect of the chemical control as well as minimising the expense; hence it is fundamental to optimise the timing of the spray applications. The first spray during autumn can largely decrease the incidence of the disease (Gilles, 2000); however, it is challenging to time application accurately during the symptomless phase of the disease (Fitt *et al.*, 1998a). For this reason, monitoring airborne ascospore release and reliable weather-based forecast models can help

determine the most optimal timing for chemical treatment. This is commonly followed by another fungicide spray in late winter to decrease the secondary infections, and the last one after the flowering phase to control pod infection and avoid premature shattering (Fitt et al., 1998a). To improve the effectiveness of fungicide treatment, it is recommended to use mixtures of fungicides with different modes of action, for example mixing azoles with strobilurins for broader disease protection (Ijaz & Honermeier, 2012). However, the most efficient way to develop anti-sensitivity is minimising the use of fungicides, only applying them when justified. Furthermore, the development of novel fungicides with new modes of action is expensive and time-consuming (Leadbeater, 2015); thus, it is highly important to preserve the few current available treatments.

However, fungicide applications have substantial setbacks, as the availability of fungicides has decreased over time. Chemical control has become less efficient because of an increase in fungicide insensitivity of *P. brassicae* in Europe; this includes MBC (methyl benzimidazole carbamate) and azole fungicides (Carter et al., 2013; 2014; King et al., 2021; Bucur et al., 2024).

It was reported that reduced sensitivity to azoles was mainly due to mutations in the *P. brassicae* *CYP51* gene, which encodes for the enzyme sterol 14 $\alpha$ -demethylase, which is a precursor in the biosynthetic pathway of ergosterol, a fundamental component of the fungal membrane structure. It is the primary target for common azoles, that inhibit ergosterol synthesis, damaging fungal membranes and leading to cell death. Changes to the *CYP51* gene and, subsequently, to the enzyme, results in the decrease of binding affinity for azoles. This reduction in its enzyme activity is associated with reduced azole sensitivity (Carter et al., 2014). Mutations have also been found in the promoter region of the *CYP51* gene in insensitive *P. brassicae* strains. Inserts of different sizes were associated with induced *CYP51* overexpression upon exposure to azole fungicides (King et al., 2021). As for the MBC fungicide, Carter et al. (2013) showed that the reduced sensitivity associated with this fungicide was caused by an amino acid substitution in  $\beta$ -tubulin gene, which is the MBC's target.

Mutations drive the adaptation of pathogens to pesticides and fungicides. In *P. brassicae*, selection for fungicide insensitivity is favoured by the rapid genetic diversification of the pathogen and its polycyclic life cycle. This increases the speed at which more fungicide-resistant strains appear (McDonald & Linde, 2002). The development of insensitivity to a fungicide can be a major issue, rendering the treatment as completely ineffective as the pathogen becomes desensitized to its specific mode of action, which can persist for many years. For this reason, alternative non-chemical control methods are prioritised, such as the use of resistant cultivars.

## **1.6 Resistant cultivars**

### **1.6.1. Molecular mechanisms of host resistance**

The control of LLS disease is very challenging due to its polycyclic nature of the disease with both sexual reproduction and frequent asexual sporulation cycles; this leads to rapidly evolving large and diverse populations. Cultural and chemical practices are not sufficient to manage the spread of the disease. For this reason and following the IPM model, that prioritises the use of preventive control strategies, studying host resistance and breeding for cultivar resistance could significantly reduce epidemics. Using more resistant cultivars could reduce costs, as well as the environmental impact, hence being the most effective and sustainable method for LLS control.

To properly understand how *B. napus* detects the presence of *P. brassicae* and responds to its infection, it is essential to study the molecular mechanisms of host resistance. The infection starts when the pathogen germinates and penetrates the leaf cuticle. The cuticle is a hydrophobic barrier that covers the epidermal cells of the foliar surface. The main components of the cuticle are cutin, an insoluble lipid polymer, and soluble waxes. The waxes can be either within the cutin matrix or accumulated on the external surface of the cuticle forming an epicuticular wax film layer. The epicuticular wax protects the plant against water loss and external environmental stresses (Yeats & Rose, 2013). It has been demonstrated how the hydrophobicity of the cuticular wax is able to repel the water drops containing fungal spores, preventing pathogen survival and infection (Koch, 2004). Furthermore, the structural variations and components of the cuticle have proved to enhance host resistance against *P. brassicae* (Muthayil-Ali, 2023).

The only known pathogenicity effectors released by *P. brassicae* during its infection of *B. napus* that have been studied in this pathosystem are extracellular cutinases, extracellular proteases and cytokinins. The cutinase, *Pbc1*, is crucial for infection, since its enzymatic activity aids in the degradation of the cuticle component cutin, allowing for cuticular penetration, breaking the major structural physical barrier of the host (Li *et al.*, 2003).

Proteases, such as *Psp1*, are released during the subcuticular fungal growth and are suspected to break down *B. napus* extracellular matrices, degrade plant cell walls and membranes to divert nutrients to the apoplast, promoting hyphal growth. Potentially, proteases also degrade host signalling proteins involved in resistance responses, inhibiting the plants immune system (Batish *et al.*, 2003). As for cytokinins, these phytohormones were reported to modify host metabolism and, as *Psp1*, divert nutrients to the site of the infection (Ashby, 1997).

*Pyrenopeziza brassicae* does not induce a hypersensitive response in *B. napus* upon infection, in a similar way to other apoplastic pathogens such as *R. commune* and *V. inaequalis* on their respective hosts (Stotz *et al.*, 2014); therefore, it is likely that, during posterior pathogen's colonisation of the apoplast, *R*-gene mediated resistance and QDR are involved in the inhibition of hyphal growth and the reduction of asexual sporulation. Major loci were found to play a role in the prevention of asexual reproduction, with cv. Imola showing a significant reduction in the conidia produced by acervuli and black flecking resistant phenotypes (Bradburne *et al.* 1999; Boys *et al.* 2012).

### **1.6.2. *R* gene-mediated host resistance and QDR in *Brassica napus***

*R* gene-mediated resistance has been favoured by breeders due to its ability to prevent the disease completely and its ease of selection, since they have very clear phenotypic effects that can be rapidly detected in glasshouse tests using seedlings. However, quantitative resistance has proven to be more durable than *R* gene-mediated resistance, therefore the use of cultivars with this kind of resistance would improve the efficiency of LLS management. Subsequently, the use of resistant cultivars would increase the yield whilst reducing the need for fungicides (Brun *et al.*, 2010; Boys *et al.* 2007), following the environmentally conscious IPM model that prioritises prevention of the epidemics over mitigation of the disease and chemical control.

In comparison to other diseases affecting oilseed rape, such as phoma stem canker caused by the fungal pathogen *L. maculans*, LLS has not been as widely studied and less is known about its

host resistance. In *B. napus*, both major gene-mediated resistance and QDR against *P. brassicae* have been identified (Table 1.1) and constitute useful resources for resistant cultivar breeding and selection. Pilet *et al.* (1998) reported six quantitative trait loci (QTL) in a doubled haploid (DH) population derived from crossing susceptible cv. Yudal with moderately resistant cv. Darmor-bzh. Bradburne *et al.* (1999) identified two major resistance loci, *PBR1* and *PBR2*, positioned on chromosomes A01 and C06, respectively. *PBR1* resulted in a phenotype with no specific symptoms, whilst *PBR2* resulted in black necrotic flecking and reduced asexual sporulation. Boys *et al.* (2012) studied resistance against *P. brassicae* in a DH population and discovered that the resistance mediated by the *PBR1* gene resulted in black flecking after inoculation and no asexual sporulation of the fungus, although it did not stop sexual reproduction after leaf senescence. This suggested that this locus could be a QDR locus instead of an *R* gene, since it was associated with a significant decrease in hyphal growth rather than pathogen death typically linked to *R* gene-mediated resistance. Karandeni Dewage *et al.* (2022) found four QTLs that were involved in QDR on linkage groups C01, C06 and C09. Two of these QTLs were identified on chromosome C06, and the QTL found in C01 significantly reduced the asexual sporulation.

Fell *et al.* (2023) screened 195 *B. napus* accessions against *P. brassicae*, and identified four QTL in linkage disequilibrium, found in chromosomes A02, A09, C02 and C08. Furthermore, eight gene expression markers (GEMs) associated with QDR against *P. brassicae*. They were located at chromosomes A03, A04, A05, A09, A10, C02, C05 and C07, and although QTLs had been identified in some of these chromosomes before (Pilet *et al.*, 1998), these markers were on different regions.

Seven of the identified GEMs were positively correlated with resistance, and included genes involved in vesicle trafficking ( *$\beta$ -adaptin*), lignification (*C4H*), universal stress protein (*USP*) and a pathogenesis-related gene (*PR1*). Muthayil-Ali (2023) showed that these QDR genes are expressed at different stages during the *P. brassicae* infection of *B. napus*. The expression of *USP* and  *$\beta$ -adaptin* were induced at the first day post-inoculation (dpi) and were associated with limited spore germination, whilst *PR1* was able to reduce the penetration of the pathogen at 2 dpi, and *C4H* reduced the pathogen's branching in the subcuticular space at 4 dpi. The expression of these four GEMs proved to be significantly higher in resistant *B. napus* cultivars than in susceptible ones, linked with a reduced colonisation of the pathogen.

The expression of one of the GEMs was correlated with susceptibility to the pathogen, suggesting that it might be a susceptibility gene. This gene belongs to the enzyme family BAHD-acyltransferase, CoA-dependent enzymes with the capability to acylate multiple primary and specialised secondary metabolites (Xu *et al.*, 2023). The acylated products are generally more stable and have higher lipid solubility and bioavailability. Members of this family commonly share two conserved amino acid motifs. The first motif is HXXXD, which is generally located in the central region of the protein and has been associated with catalytic activity; the histidine of this motif often acts as a catalytic residue and the aspartate residue, that points away from the active site, seems to play a structural role (Xu *et al.*, 2023). The DFGWG motif is found around the carboxyl terminus of the protein and has been associated with CoA binding (D'Auria, 2006).

**Table 1.1.** Known resistance in *Brassica napus* against *Pyrenopeziza brassicae*.

| Reference                             | Host resistance            | R gene/QTL                                                                                  | Chromosome                             |
|---------------------------------------|----------------------------|---------------------------------------------------------------------------------------------|----------------------------------------|
| Pilet <i>et al.</i> (1998)            | Quantitative resistance    | Identification of 6 QTLs at linkage groups                                                  | A02, A06, A07, A09, C04                |
| Bradburne <i>et al.</i> (1999)        | R gene-mediated resistance | Identification of major resistance locus ( <i>PBR1</i> )                                    | A01                                    |
|                                       |                            | Identification of major resistance locus ( <i>PBR2</i> )                                    | C06                                    |
| Boys <i>et al.</i> (2012)             | Quantitative resistance    | Characterisation of <i>PBR1</i>                                                             | A01                                    |
| Karandeni Dewage <i>et al.</i> (2022) | Quantitative resistance    | Identification of 4 QTLs                                                                    | C01, C06, C09                          |
| Fell <i>et al.</i> (2023)             | Quantitative resistance    | Identification of 4 QTLs                                                                    | A02, A09, C02, C08                     |
|                                       |                            | Identification of 8 gene expression markers associated with QDR against <i>P. brassicae</i> | A03, A04, A05, A09, A10, C02, C05, C07 |

## 1.7 Gene mapping

Genetic studies reveal the backbone of host resistance by identifying *R* genes and QTLs, which is crucial for the selection of resistant cultivars and the development of breeding programs. The study of genetic variation and trait inheritance has drastically evolved from 19<sup>th</sup>-century simple Mendelian principles to the 21<sup>st</sup>-century data-driven genomic revolution. The focus has shifted from the interpretation of discrete and qualitative traits controlled by few genes with clear dominance and recessive inheritance to more complex, polygenic traits, as well as the study of quantitative traits and population genetics (Mersha, 2023). Development of complex technological advances in the past few decades have revolutionised the study of genetics, now driven by molecular, statistical, mathematical and computational methods. Advancements such as Next-Generation Sequencing (NGS) and Genome-Wide Association Studies (GWAS) have improved the resolution and power of QTL analysis and gene mapping, refining gene-trait association studies (Mahmood *et al.*, 2022).

There are multiple strategies that aim to find the DNA regions or loci that influence complex traits. Despite the multiple advantages of GWAS as a mapping tool, it solely focuses on genetic variations such as Single Nucleotide Polymorphisms (SNPs). It does not consider the essential influence of gene regulation in how genetic polymorphism contributes to the complex genetic architecture behind phenotypic traits. For this reason, associative transcriptomics (AT) was developed as an advanced integrative genomics approach that combines GWAS with transcriptome profiling, enhancing the resolution and sensitivity of trait discovery (Jayabharathi *et al.*, 2025). AT uses RNA-Seq to examine variation in both gene sequence and gene expression by studying both SNPs and GEMs, respectively, and providing with a more precise method of establishing direct links between genetic variation and observable characteristics (Stower, 2012).

Another mapping approach used for the identification of QTLs associated with a complex trait is the bulked segregant analysis (BSA), first developed by Michelmore *et al* (1991). This technique compares the pooled DNA of two bulks of individuals from a segregating population, each bulk with extreme opposite ends of a trait's phenotypic distribution, such as resistance vs susceptibility to a particular disease. The contrast between DNA pools can result in the identification of genetic markers or genomic regions with divergent allele frequencies, which can lead to the discovery of genes associated with specific traits. Modern BSA integrated NGS, commonly renamed as BSA-seq, significantly improves the efficiency of allele frequency comparison, increasing mapping resolution and speed.

For a more complete and in-depth analysis of disease resistance, epigenetic studies can expand on how transcriptional regulation can contribute to resistance mechanisms (Fujimoto *et al.*, 2012). The most common methods for activating or silencing genes are DNA methylation and demethylation, histone modification and chromatin remodelling (Kamiya *et al.*, 2023). This insight into epigenome mechanisms for disease resistance can improve molecular breeding programs. Epigenomic analysis performed in multiple *Brassica* vegetables have shown that DNA methylation and histone modifications are involved in the regulation of disease resistance mechanisms. This is achieved through the differential expression of genes involved in the ethylene/salicylic acid/jasmonic acid signalling pathways (Akter *et al.*, 2025). The identification of epigenetic markers associated to *R* genes and QTLs in *Brassica* vegetables would significantly improve the development of disease resistance breeding programs.

## **1.8 Aims and objectives**

Light leaf spot disease is considered the most economically damaging disease affecting winter oilseed rape in the UK. Current cultural and chemical control practices have proven insufficient to manage the spread of epidemics. The IPM model emphasises the importance of prevention and monitoring over disease mitigation, often linked with disruption of the environment; hence, it is fundamental to prioritise the use of resistant cultivars and to improve the current methods for disease detection. To contribute to LLS control, this project aims to expand on the existing knowledge of the *B. napus*-*P. brassicae* pathosystem.

For an improved resistant cultivar selection, the study of the genetic basis of host resistance is crucial, focusing on QDR, which offers a more durable defence against pathogens and has not yet been widely researched. The identification and characterisation of new quantitative sources of resistance or susceptibility to *P. brassicae* will aid in the selection of more resistant *B. napus* cultivars that could withstand the LLS epidemics. Furthermore, it would contribute to the improvement of breeding programs to develop biotic stress resilient *B. napus* cultivars. Additionally, this project aims to improve monitoring strategies by exploring different phenotyping quantitative approaches for pathogen identification, early detection *in planta* and disease severity determination. The development of more reliable techniques would aid in the selection of the most adequate control methods to use, allowing for targeted and localised treatment, instead of blanket spraying.

Both the selection of more resistant cultivars and the improvement of monitoring strategies would significantly reduce the yield loss and the use of disruptive control techniques, which would significantly benefit breeders and farmers. Moreover, a lower reliance on fungicide treatments would be favourable for the ecosystem, since it would minimise the environmental risk to beneficial insects, water and soil health, as well as reducing the development of fungicide resistance.

The main objectives are:

1. To assess the reliability of different quantitative phenotyping methods and compare them with the qualitative method, visual assessment, in identifying and detecting the presence of *P. brassicae* in *B. napus*. It is hypothesized that quantitative strategies will provide more accurate and reliable data than qualitative methods.
2. To characterise the function of a BAHD acyltransferase gene, potentially involved in susceptibility to *P. brassicae*, through the analysis of *B. napus* TILLING mutants. It is hypothesized that this gene will be partially responsible for the susceptibility to the pathogen.
3. To improve the understanding of the genetics of QDR by identifying QTLs involved in resistance or susceptibility to LLS through a bulked segregant analysis. It is hypothesized that QTLs will be identified and associated with QDR in *B. napus*.

## Chapter 2: General materials and methods

### **2.1. Media preparation for *Pyrenopeziza brassicae* germination and growth**

To prepare 500 mL of malt extract agar (MEA), 15 g of malt extract (Oxoid LP0039, UK) and 7.5 g of bacteriological agar powder (Oxoid LP0011, UK) were weighed and suspended into 500 mL of sterile distilled water (SDW) in a glass Duran bottle with a screw cap. The mix was autoclaved at 121°C for 15 minutes, and once it had cooled down to 50°C, 250 µl of antibiotics streptomycin and penicillin (50 µg/mL) were added to avoid bacterial growth on plates. The media was then poured into 9 cm diameter Petri dishes (approximately 20 ml per Petri dish) and left to solidify without the lid inside the laminar flow cabinet for 20 minutes. The conditions for *P. brassicae* growth in MEA plates were described in Chapter 3.2.1 (*Pyrenopeziza brassicae* populations and isolates).

### **2.2. Growing *Brassica napus* under controlled environment conditions**

#### **2.2.1. *Brassica napus* seed germination**

With aseptic tweezers, *Brassica napus* seeds were placed in Petri dishes containing damp Whatman filter paper, soaked with deionized water. The seeds were incubated in this humid environment at 20°C in the dark for 48 hours or until germinated (Figure 2.1).

#### **2.2.2. Planting pre-germinated seeds into soil**

To sow the pre-germinated *B. napus* seeds, 40-well seed trays were filled with a 50:50 mixture of Miracle-Gro multipurpose compost and John Innes No.3 compost. A small hole was made on the surface of the soil in each well and a germinated seed placed inside, then covered again with the compost mix and a layer of vermiculite to increase soil aeration and porosity. Seed trays were placed in outer trays over capillary sheets soaked with water to maintain water content. Plants were incubated either in a glasshouse or in controlled-environment (CE) cabinets (FITOCLIMA D1200, ARALAB, Rio de Mouro, Portugal or Conviron A1000, Canada), at 20°C in light (250 µmol·m<sup>-2</sup>·s<sup>-1</sup>) for 16 hours and 18°C in dark for 8 hours, with a 60% relative humidity. In glasshouse conditions, though, relative humidity cannot always be as accurately controlled as in CE cabinets.

Usually after two weeks, seedlings reached the growth stage 1.0-1.1, corresponding to unfolded cotyledons and first true leaf emerged (Sylvester-Bradley & Makepeace, 1984). Seedlings were then repotted into 9x9 cm pots with the same compost mixture and incubated under the same light and temperature conditions until they reached growth stage 1.4-1.5, when the fourth to fifth true leaves were present, which commonly took another two weeks before they were ready for pathogen inoculation (Figure 2.2). Key growth stages in oilseed rape were shown in Appendix 1.

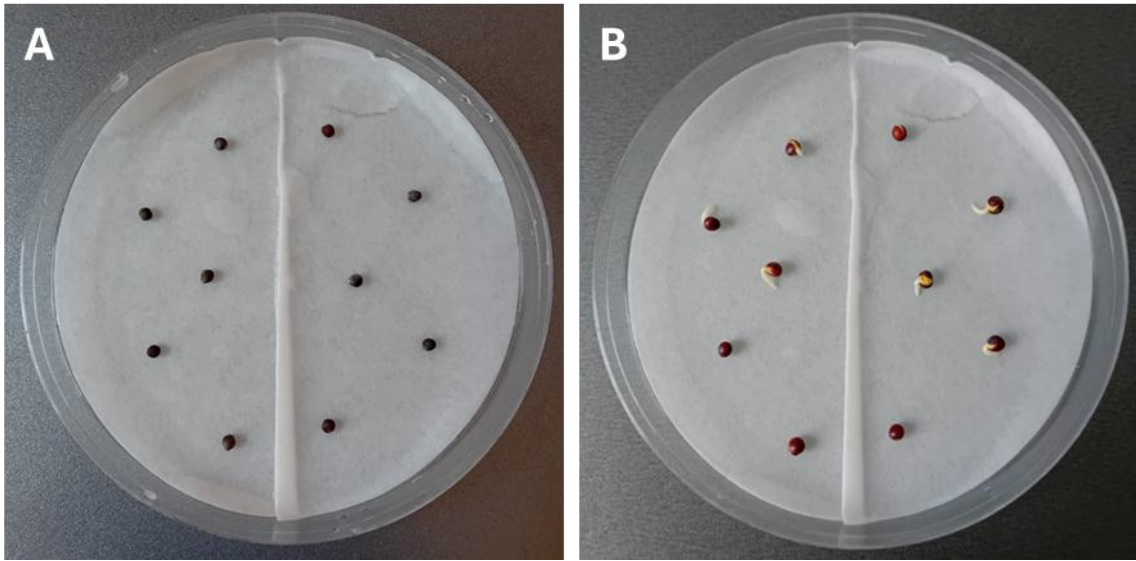


**Figure 2.1.** *Brassica napus* seeds placed on damp filter paper in a Petri dish **(A)** before and **(B)** after germination after a 48-hour incubation in the dark.



**Figure 2.2.** Growth stages of *Brassica napus*. **A)** Seedlings at day 8 after sowing; at stage 1.0 (i.e. unfolded cotyledons). **B)** Seedlings at day 14 after sowing; at stage 1.1-1.2 (i.e. cotyledons unfolded, and first true leaves emerging). **C)** Plants at day 19 after sowing; seedlings were transferred to bigger pots at stage 1.3. **D)** Plants at day 28 after sowing; at stage 1.4-1.5 (fourth to fifth true leaves are present), ready for pathogen inoculation.

### **2.3. *Pyrenopeziza brassicae* spray-inoculation method**

A *P. brassicae* conidial suspension was adjusted to a concentration of  $10^5$  spores/mL for use as an inoculum for spray-inoculations. Silicone surfactant Silwet was added to the suspension at a 0.005% concentration to act as a wetting agent, and the mix vortexed to avoid spores clumping together; the suspension was transferred to a spray bottle. Each plant was sprayed with 1-2 mL of spore inoculum, until the leaves were evenly and fully covered with fine droplets of the spore suspension (Figure 2.3). The sprayed plants were covered either with a plastic high dome propagator (Figure 2.3C) or with transparent polyethylene sheets and incubated for 48 hours under the following conditions: 16°C for 12 hours in daylight and 14°C for 12 hours in the dark. These conditions enhance high humidity, facilitating the *P. brassicae* conidial germination and host penetration (Muthayil-Ali, 2023). After removing the transparent plastic coverings, the plants were incubated in the same light and temperature conditions for another 21 days.

### **2.4. Diseased leaf washing and *P. brassicae* conidial suspension preparation**

To harvest *P. brassicae* spores, diseased leaves with light leaf spot symptoms were harvested at 21 days post-inoculation (dpi) and covered in tissue that was subsequently sprayed with SDW, sealed in a polyethylene bag and incubated at 4°C for 5 to 10 days. The high humidity environment and low temperature induced sporulation and produces visible conidia-containing acervuli (Figure 2.4) that can be washed with SDW into a beaker. Spore suspensions were filtered through autoclaved Miracloth (Merck Millipore, Watford, UK) and the spore concentration measured with a Bright-Line haemocytometer (Sigma-Aldrich, UK), under a GXM-L2800 microscope (GT vision, UK). Inoculum aliquots were stored at -20°C until required, and a few aliquots were stored at -80°C in 20% glycerol.

### **2.5. DNA extraction**

Leaf material was placed in Falcon tubes and either freeze-dried for 48 hours or frozen in liquid nitrogen, then ground to a fine powder using a sterile mortar and pestle. A DNA extraction kit (DNAMITE plant kit, Microzone Ltd., UK) was used to extract genomic DNA (gDNA). Approximately 50-75 mg of powdered leaf material were transferred to a clean 2 ml screw-cap tube (enough to fill the conical base), and an extra step was added to the manufacturer's protocol by adding three stainless steel beads (3 mm diameter each) along with the 1 mL of Solution LA (cell lysing solution). The tubes were vortexed and agitated in a FastPrep machine (MP Biomedicals, UK) at 4 m/s for 40 seconds to homogenise the leaf material. The rest of the protocol was followed as indicated. Finally, the DNA pellet was suspended in 30 µl of sterile nuclease-free water (Promega), left overnight (16 hours) to rehydrate in the fridge at 4°C and stored at -20°C for further analysis. DNA concentration, measured in ng/µl, was quantified both with an Invitrogen™ Qubit™ 4 Fluorometer (Thermo Fisher Scientific, UK) using a broad range dsDNA kit (Thermo Fisher Scientific, UK), and with a Nanodrop ND-1000 spectrophotometer (Labtech International, UK), which also allowed to measure ratios of absorbance A260/280 and A260/230 to assess DNA purity.



**Figure 2.3.** Details of *Pyrenopeziza brassicae* inoculation. Comparison between non-inoculated *Brassica napus* leaf **(A)**, and a *B. napus* leaf after spray-inoculation with  $10^5$  spores/mL *Pyrenopeziza brassicae* conidial inoculum **(B)**. The picture was taken at day 28 after sowing (stage 1.4-1.5). **(C)** Plastic high dome propagator to cover *B. napus* plants just inoculated with *P. brassicae*, to maintain a high humidity environment and promote host penetration.

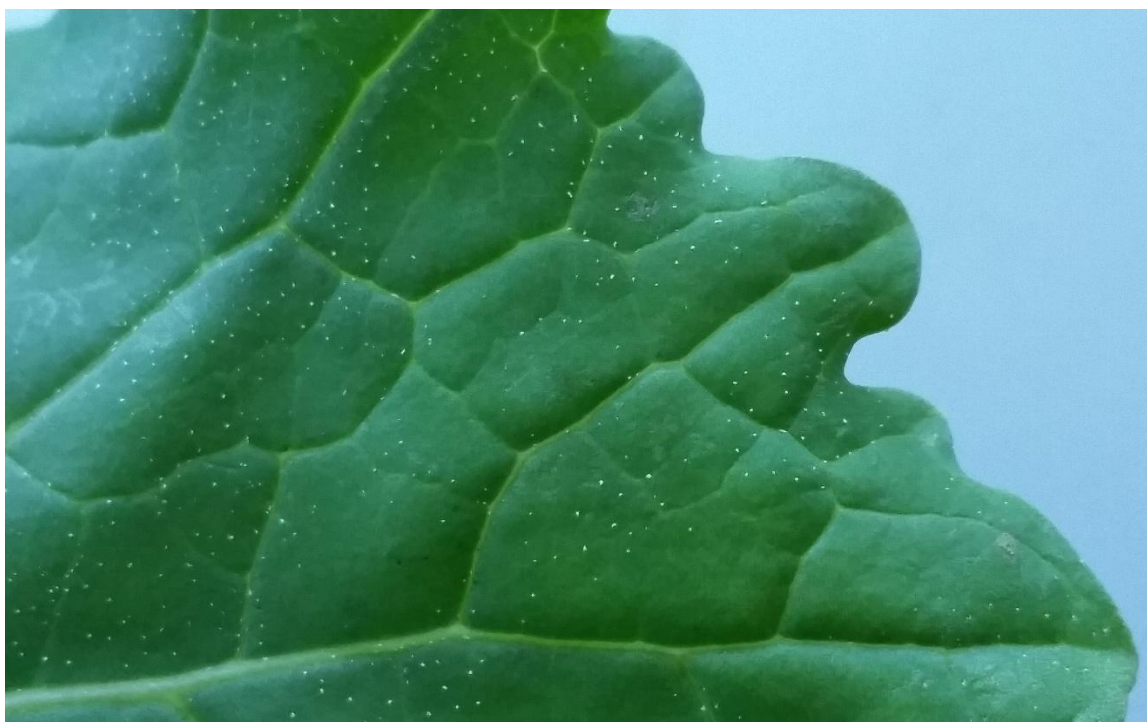


**Figure 2.4.** Visible *Pyrenopeziza brassicae* acervuli on the surface of *Brassica napus* leaves after 5-10 days at 4°C incubation under a high humidity environment.

## **2.6. Preparation of buffer for mycelium DNA extraction**

The DNA extraction buffer was prepared to aid in the extraction of mycelium DNA. This buffer solution is the result of mixing three different buffers. For buffer A, 12.75 g of sorbitol (0.35 M), 2.42 g of Tris-HCl (0.1 M, pH 8.0) and 0.37 g of EDTA (5 mM) were diluted in 200 mL of SDW. For buffer B, the DNA extraction base mix, 4 g of CTAB (2% w/v) and 2 g of PVP 40 (1% w/v) were diluted in 200 mL of a 2XTEN solution, which was prepared by diluting 12.11 g of Tris base, 9.31 g of EDTA and 58.44 g of NaCl in 500 mL of SDW and mixing until homogeneous. Finally, buffer C was prepared by diluting 5 g of sarkosyl into 100 mL of SDW.

Once these were ready, the DNA extraction buffer was prepared by warming 2.5 volumes of buffer B on a water bath at 65°C for five minutes. Then, under the fume hood, 1%  $\beta$ -mercaptoethanol were added to buffer B. This was later mixed with 2.5 volumes of buffer A, 1 volume of buffer C and around 0.048 volumes of proteinase K. It was gently mixed and stored in the fridge at 4°C until required.

## **2.7. Agarose gel electrophoresis**

The concentration of an agarose gel is measured in weight of agarose over volume of the buffer used (w/v). The amount of agarose powder dissolved in buffer depends on the purpose of the gel. Agarose gels were prepared by adding agarose powder (Sigma-Aldrich, UK) into an Erlenmeyer flask containing 1xTBE buffer. The mix was brought to the boil in a microwave and agitated manually until the agarose was completely dissolved. After the solution cooled down to approximately 50°C, 5  $\mu$ L of 1xGelRed™ were incorporated into the mix, which was then slowly poured in the gel tray with a well comb in place and allowed to solidify at room temperature for 20-30 minutes. The gel was placed in the gel box and filled with 1xTBE buffer, ensuring that all the wells are filled with it.

Loading buffer was added to each of the samples at a dilution of 1:6, and 5  $\mu$ L of each sample were loaded into separate wells, alongside 10  $\mu$ L of DNA ladder (Sigma Aldrich, UK). Gels were run for 70 minutes at 80V, and the result visualised under UV light (Syngene Ingenius 3 Imager, UK).

## **Chapter 3: Assessment of different methods to quantify *Pyrenopeziza brassicae* causing light leaf spot in *Brassica napus***

### **3.1 Introduction**

#### **3.1.1 Visual assessment as the current phenotyping strategy**

Crop protection and safeguarding sustainable agriculture requires reliable and accurate phenotyping methods that determine the severity of the disease affecting the crops. With oilseed rape (*Brassica napus*) it is crucial to study the effect and severity of the light leaf spot disease, caused by the pathogen *Pyrenopeziza brassicae*. As part of its life cycle, the fungus develops asexual fruiting bodies, known as acervuli, which appear as white pustules that break through the surface of the leaves. These sporulating structures contain conidia, that will be released to produce secondary infections in the plants as part of this step in this pathogen's life cycle. Current strategies for assessment of susceptibility to the light leaf spot (LLS) pathogen are based on the visual assessment of *P. brassicae* sporulation on leaf surfaces, a method that relies on a subjective estimation of the percentage of leaf area covered by acervuli.

For field trials, the Agriculture and Horticulture Development Board (AHDB) uses a 9-point scale to assess the severity of the LLS disease on oilseed rape plots (Appendix 2), where a high number (like 6 or above) indicates good resistance. A rating of 1 indicates that all plants in the plot were stunted or dead due to their high susceptibility to *P. brassicae*. The score gradually increases as the percentage of sporulation on plants diminishes, up to a rating of 9, that indicates there were no traces of disease observable, associated with the highest level of resistance (Boys *et al.*, 2012). For smaller-scale experiments and trials done in glasshouses or controlled environment (CE) cabinets, a 1-6 severity scale is commonly adopted for phenotype screening (Karandeni Dewage *et al.*, 2021). The range for this method stretches from 1, when no sporulation is observed, gradually increasing in incidence of acervuli on the leaves, up to score 6, with 75-100% of the leaf surface covered with sporulation. These ways of rating the severity of disease can help breeders and growers to choose the *B. napus* cultivars that are less susceptible to the LLS disease, contributing to an integrated disease management strategy alongside other practices.

#### **3.1.2 Alternative quantitative phenotyping strategies**

Quantitative disease resistance (QDR) is a highly desirable trait but is difficult to precisely phenotype. The common visual assessment currently being used to estimate disease severity can serve as a fast and easy phenotyping method however, it has been demonstrated to be linked to assessor bias. The subjective nature of these assessments can lead to error and the categorical data they produce could be inaccurate, they are challenging to reproduce and prone to human error, with research showing a high variability in assessments made by the same and different scorers (Ayliffe *et al.*, 2010).

To properly examine the interaction between the pathogen and its host, and study the genetic basis of resistance, it is fundamental to establish phenotyping methods that can accurately quantify disease severity. To minimise the error of subjective assessment, different quantitative

phenotyping approaches were studied. These methods measure the presence of the pathogen on or in the host tissue. The selected strategies were acervuli quantification, spore counting, wheat-germ agglutinin chitin (WAC) assay and quantitative PCR (qPCR) of pathogen DNA.

#### 3.1.2.1 Acervuli quantification

As established by the traditional visual assessment phenotyping method, there is an observable and clear contrast between *B. napus* leaves that have different levels of disease susceptibility to the pathogen *P. brassicae*. However, instead of visually estimating the percentage of leaf surface affected by sporulation and assigning a rating in the form of categorical data, it was assumed that the number of acervuli could be related to the severity of the LLS disease. The acervuli are visible to the naked eye on the surface of the leaf, therefore they could be quantified macroscopically, potentially offering a better method for phenotyping than visual assessment.

#### 3.1.2.2 Spore counting

After infection of *B. napus* plants, *P. brassicae* germinates, penetrates and colonises the host (Muthayil-Ali, 2023). Laboratory experiments have shown that *P. brassicae* conidia that develop on the surface of the host's leaves can be dispersed in water droplets as small as 30 µm formed during the splash of a raindrop as a dispersion mechanism (Patemi & Fitt, 1983), therefore conidia can be collected by washing leaves with sterile distilled water (SDW). The method of spore counting can be used to determine whether there is a correlation between disease severity and the spore concentration from individual plants (Guimarães *et al.*, 2004).

#### 3.1.2.3 WAC assay

Wheat-germ agglutinin (WGA) is a lectin, a carbohydrate-binding protein that is highly specific to specific sugar residues of macromolecules and abundantly found in wheat (*Triticum*) kernels. Structurally, WGA is a homodimer and each of its monomers consists of four hevein-like domains (i.e. chitin-binding domains) noncovalently associated to form four independent ligand-binding sites (Van Damme *et al.*, 2014), thus containing a total of eight binding domains per WGA molecule, with a high binding specificity for the monosaccharide *N*-acetylglucosamine (GlcNAc), the monomeric subunit of the chitin polymer (Wright *et al.*, 1991; Mishra *et al.*, 2019). In the natural environment, chitin is one of the main polysaccharide constituents of the cell walls of fungi and of the exoskeleton of insects and other arthropods like crabs, shrimps, etc. Some of these are potential threats to wheat crops, and lectin WGA has been shown to have anti-fungal and anti-insect properties, playing a significant role in the innate immunity response of the plants (Peumans & Van Damme, 1995). WGA can recognise a variety of pathogens by specifically interacting with the residues of GlcNAc in their chitin structures, forming reversible linkages that will trigger a host immune response, enhancing the response to external stimuli and even contributing to the inhibition of fungal growth by synergistically acting with chitinases, disrupting the cell's structural integrity (Ciopraga *et al.*, 1999; De Hoff *et al.*, 2009; Carcea *et al.*, 2024).

The natural binding selectivity of WGA for GlcNAc makes it an ideal protein to detect the presence of a fungal pathogen containing chitin as a prominent component in their cell. WGA can therefore

be conjugated with the fluorophore fluorescein isothiocyanate (FITC), and its fluorescence generates a quantitative signal that can be related to the amount of pathogen present in the host tissues. This information can be used for correlations to the severity of the disease (Ayliffe *et al.*, 2013). The use of WGA-FITC to quantify fungal biomass using a fluorescent assay, known as the WAC assay, was reported and Schnippenkoetter *et al.* (2021) tested it with *Leptosphaeria maculans* in *B. napus*.

#### 3.1.2.4 qPCR

The presence of *P. brassicae* DNA can be detected through DNA-based PCR techniques (Calderon *et al.* 2002). qPCR was used to quantify the amount of *P. brassicae* DNA inside the infected host to study plant-fungus interactions (Klosterman, 2011) and the severity of the disease, which can thus be used as a phenotyping technique (Qi and Yang, 2007; Brunner *et al.*, 2009).

To quantify the presence of *P. brassicae* in the host, the internal transcribed spacer (ITS) region was targeted for amplification. The ITS region is present in fungal ribosomal DNA (rDNA), located between the small subunit and large subunit rRNA genes. It is used as a universal fungal DNA barcode for identifying and distinguishing fungal species through PCR analysis (Martin & Rygiewicz, 2005), as well as for fungal quantification (Karolewski *et al.*, 2006).

### **3.1.3 Aims and objectives**

In the study of the effect of light leaf spot disease on oilseed rape, it is crucial to establish a reliable method to determine the severity of the disease. The aim of this chapter was to compare different quantitative methods to detect the presence of the *P. brassicae* pathogen in *B. napus*, and determine their precision, accuracy, reliability and utility, in contrast to subjective visual assessment methods currently being used as the predominant phenotyping strategy for this pathosystem.

It was hypothesized that these quantitative strategies will show better accuracy and generate more reliable data than the visual assessment scoring method, thus potentially replacing it as the main phenotyping strategy to study the *P. brassicae*-*B. napus* interaction.

The separate objectives contained in this chapter were:

1. To obtain *P. brassicae* populations and isolates to work with.
2. To use acervuli quantification, spore counting, WAC assay, and qPCR as quantitative phenotyping methods for the *P. brassicae*-*B. napus* pathosystem.
3. To contrast the quantitative methods to the visual assessment qualitative method, and compare accuracy, reliability and utility.

## **3.2 Materials and methods**

### **3.2.1 *Pyrenopeziza brassicae* populations and isolates**

Different populations and isolates of *P. brassicae* inoculum were used to inoculate *B. napus* plants with the pathogen. In addition, the mycelia of certain isolates was harvested and freeze-dried for other experiments.

*Pyrenopeziza brassicae* isolates that differ in pathogenicity were obtained and *P. brassicae* populations collected. This was done through a spore multiplication process (Karandeni Dewage *et al.* 2021), resulting in concentrated first-generation stocks that could be used for different experiments, as illustrated in Figure 3.1.

*Pyrenopeziza brassicae* populations were collected from oilseed rape field trials in Thriplow, Cambridgeshire (KWS) and from glasshouse experiments at Rothamsted Research (Muthayil-Ali, 2023). Spore inoculum generated from these trials was used for bulking, in a spore multiplication experiment performed *in planta* by spray-inoculating *B. napus* cvs Cabriolet and Ambassador. Plants were grown following the procedure described in chapter 2.2 (Growing *Brassica napus* in a controlled environment), inoculated as described in chapter 2.3 (*Pyrenopeziza brassicae* spray-inoculation method) and the inoculum obtained as described in chapter 2.4 (Diseased leaf washing and *P. brassicae* conidial suspension preparation).

The *P. brassicae* isolates selected for further work were 15WOSR81-SS1, a less aggressive strain, and 17WOSR-I4, a more aggressive strain, which are both mating type 2; and 15WOSR64-SS1, mating type 1 (Karandeni Dewage *et al.* 2021). Isolates were grown on MEA plates, which were prepared as described in chapter 2.1 (Media preparation for *Pyrenopeziza brassicae* germination and growth) for a month in the dark at 15°C. Once the mycelia had grown all over the surface of the medium (Figure 3.2), 10 ml of SDW were added and the *P. brassicae* cultures scraped with a sterile L-shaped spreader to release the spores. Cultures were left to incubate in the water for 5 minutes. The suspension was collected and filtered through autoclaved Miracloth (Merck Millipore, Watford, UK). The inoculum was then quantified using a Bright-Line haemocytometer (Sigma-Aldrich, UK), concentrated to 10<sup>5</sup> spores/ml and stored at -20°C. This inoculum, however, cannot directly be used to inoculate plants for experiments because *P. brassicae* can lose pathogenicity when continuously grown on plates. Therefore, the spore inoculum obtained from plates was combined with 0.005% of Silwet and used to spray-inoculate *B. napus* plants. Spores were collected after infection and incubation, forming spore inoculum that can then be reliably used in plant experiments.

Additionally, mycelia were collected from the cultured *P. brassicae* in MEA plates. The mycelium was scraped off the surface without scratching the media and collected in tubes that were later freeze-dried for 48 hours and stored for future experiments.

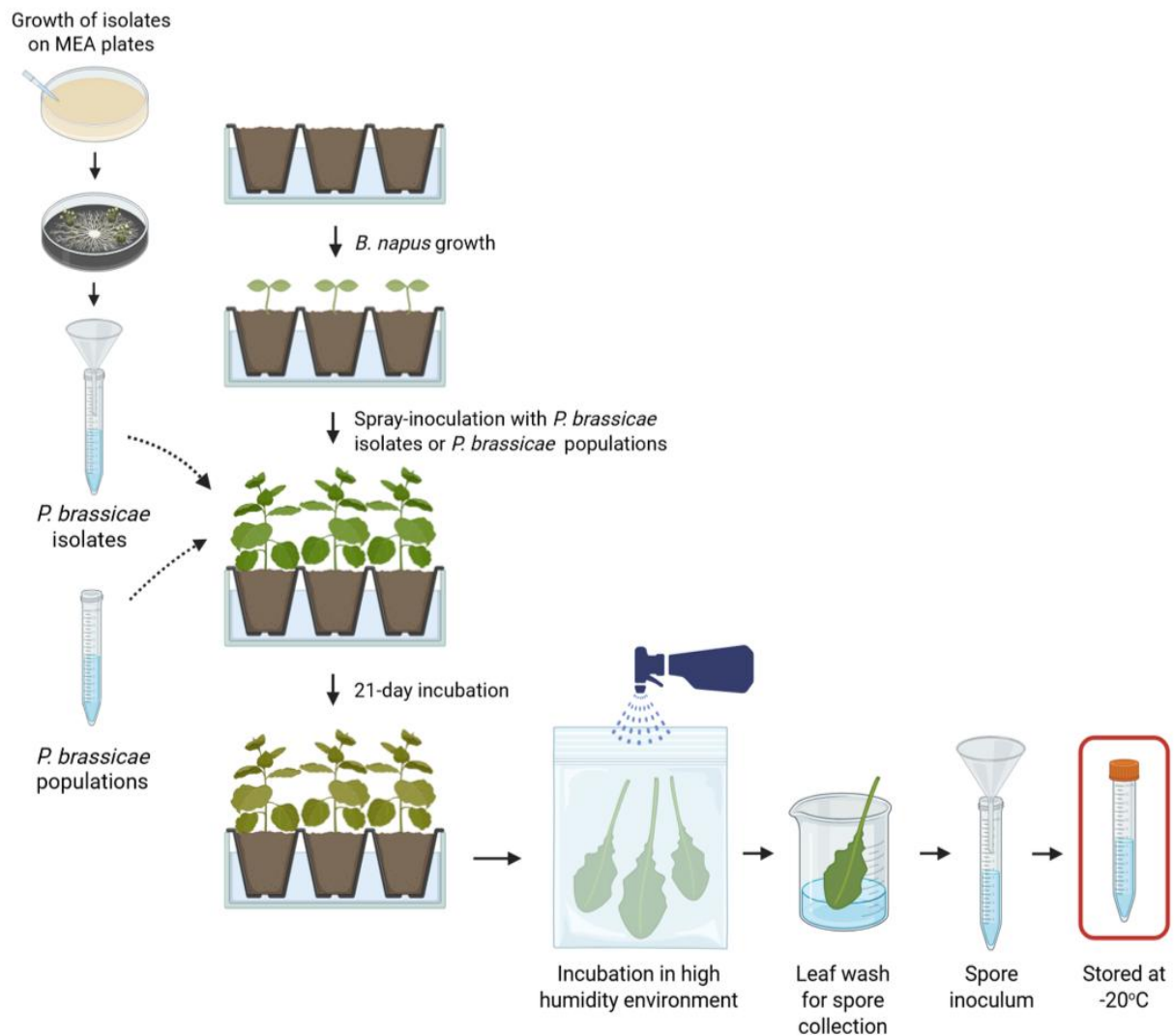


**Figure 3.1.** Scheme of the spore multiplication process for *Pyrenopeziza brassicae* isolates and populations. Spore inoculum from populations was directly used to spray-inoculate the *Brassica napus* plants, whereas *P. brassicae* isolates were cultivated on malt-agar extract (MEA) plates from which spores were collected and used to spray-inoculate the plants. After inoculation and a 21-day incubation, leaves were harvested, and spores were washed from the leaves to form the spore inoculum that could reliably be used in experiments; it was stored at -20°C until needed. (Created with BioRender.com).

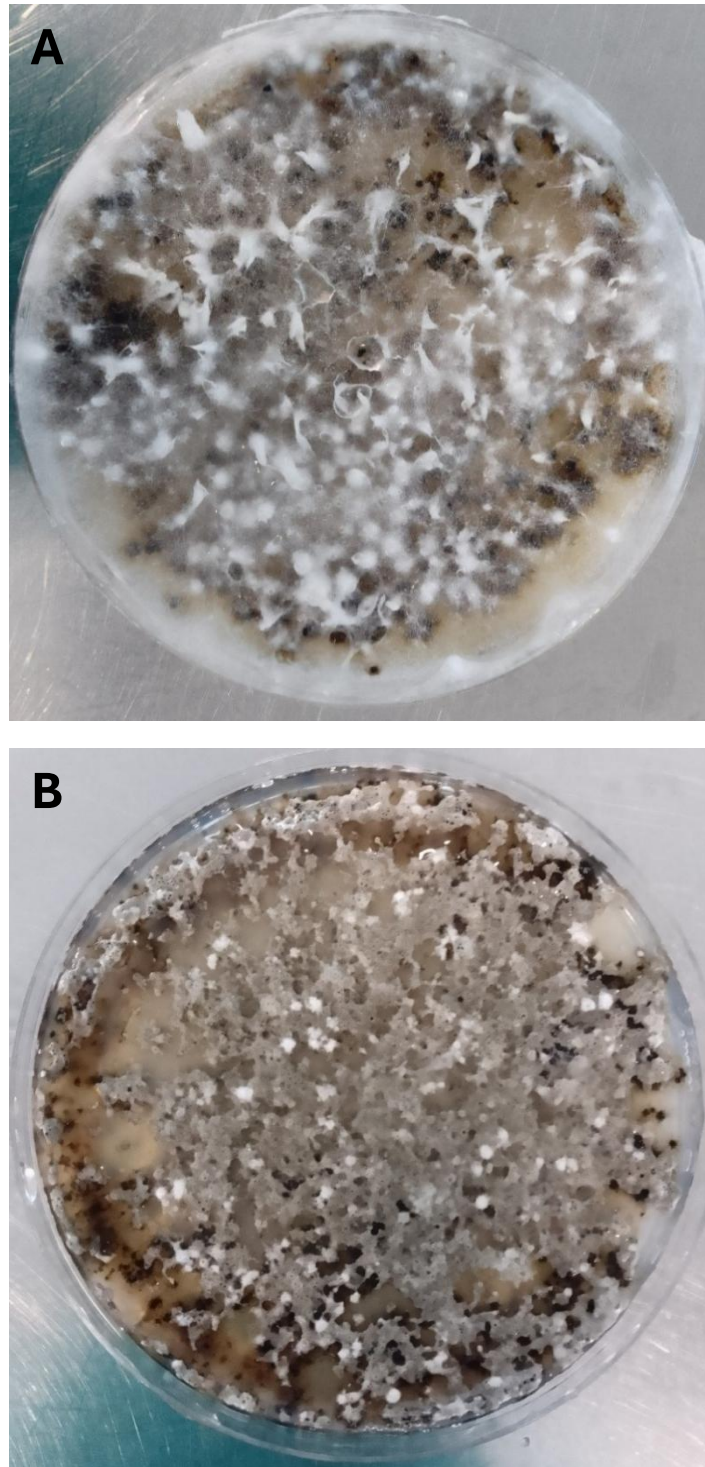


**Figure 3.2.** *Pyrenopeziza brassicae* cultures on malt-agar extract (MEA) plate, after 1 month of growth in the dark at 15°C. **A)** *P. brassicae* isolate 15WOSR81-SS1. **B)** *P. brassicae* isolate 17WOSR-I4.

### 3.2.2 Selection of *Brassica napus* lines

Prior to the start of this project, a funded collaboration between the University of Hertfordshire and the John Innes Centre established disease scores for 195 *Brassica* accessions in glasshouse experiments (Fell *et al.*, 2023), 21 of which (Table 3.1) were further studied in a field trial at Thriplow, Cambridgeshire (managed by KWS), as part of a BBSRC Follow on Fund project. The cultivars (cvs), which varied from susceptible to resistant, were sown in the field in September 2021, distributed in duplicate blocks as shown in Table 3.2, for statistical analysis. Except for cvs Moana and Cabriolet, that did not germinate, all were well established, as seen in Figure 3.3. They were inoculated with the *P. brassicae* population that originated from Thriplow, from the same fields the trial took place, that had been prepared in a previous season. Samples were collected in March, May and June of 2022, by harvesting 10 leaves per block picked at random and later assessed using different phenotyping strategies.

The severity of the disease was initially established using visual assessment for all 19 *B. napus* cultivars, followed by spore counting. To study the other quantitative phenotyping methods (acervuli quantification, WAC assay and qPCR), samples from resistant *B. napus* cvs Lipid and Nikita, and susceptible cv. Ambassador and line RV31 were selected, as they were in opposite extremes of the disease scale. These lines were inoculated with *P. brassicae* and analysed to determine the effect and severity of LLS disease on them. These four lines were examined along with a negative control, an uninoculated cv. Cabriolet grown in the glasshouse and inoculated with the *P. brassicae* population that originated from the Thriplow fields.

### 3.2.3 Visual assessment

To visually assess *B. napus* plants infected with *P. brassicae*, leaves were harvested and incubated at 4°C for 7-10 days wrapped in damp paper towels, placed inside polyethylene bags to create a high humidity environment that induced sporulation. After this, the acervuli could be visualised macroscopically on the leaf surfaces, presenting as small white spots. Depending on the percentage of sporulation, the leaves were categorised individually using the scale of 1-6 (Karandeni Dewage *et al.*, 2021) and given a score according to it (Figure 3.4). If the leaves showed no signs of sporulation, score 1 was used. If sporulation affected around 10% of the leaf surface, score 2 was assigned. Scores 3, 4 and 5 were given to leaves that showed sporulation percentages of 10-25%, 25-50% or 50-75%, respectively. If more than 75% of the leaf surface was covered with acervuli, the highest score 6 was used. The scores of all the leaves from an individual plant were averaged to obtain a disease severity rating.

**Table 3.1.** Light leaf spot disease scores obtained through visual assessment using the 1-6 scale of disease severity in a glasshouse experiment led by Heather Fell and Ajisa Muthayil-Ali (Fell *et al.*, 2023). Colour formatted with green, yellow and red ranges, from lower to higher susceptibility to *P. brassicae*.

| <i>Brassica napus</i><br>cultivar/line | Brassica sub-type | Disease score<br>(glasshouse) |
|----------------------------------------|-------------------|-------------------------------|
| Imola                                  | Spring OSR        | 1.00                          |
| Cubs Root                              | Spring OSR        | 1.19                          |
| POSH                                   | Winter OSR        | 1.53                          |
| Dwarf Essex                            | Forage rape       | 1.58                          |
| SWU Chinese 1                          | Semiwinter OSR    | 1.84                          |
| Zenith                                 | Winter OSR        | 2.90                          |
| Yudal                                  | Spring OSR        | 2.71                          |
| Lipid                                  | Modern Winter OSR | 2.77                          |
| Nikita                                 | Winter OSR        | 3.00                          |
| Aurelia                                | Winter OSR        | 3.40                          |
| Temple                                 | Winter OSR        | 3.90                          |
| Barbados                               | Winter OSR        | 3.70                          |
| Tapidor                                | Winter OSR        | 3.87                          |
| Leopard                                | Winter OSR        | 4.77                          |
| Campus                                 | Winter OSR        | 5.20                          |
| Laser                                  | Modern Winter OSR | 5.37                          |
| Moana                                  | Fodder rape       | 5.58                          |
| Ambassador                             | Winter OSR        | 5.60                          |
| Sansibar                               | Modern Winter OSR | 5.68                          |
| Cabriolet                              | Winter OSR        | 5.69                          |
| RV31                                   | Winter OSR        | 5.90                          |

**Table 3.2.** Distribution of the 21 *Brassica napus* accessions in duplicate plots (green and blue), sown in the field in Thriplow, Cambridgeshire, in September 2021.

| Row 1   | Row 2       | Row 3         | Row 4         | Row 5   |
|---------|-------------|---------------|---------------|---------|
| Discard | POSH        | RV31          | Zenith        | Discard |
| Discard | Nikita      | Sansibar      | Yudal         | Discard |
| Discard | Moana       | SWU Chinese 1 | Temple        | Discard |
| Discard | Lipid       | Tapidor       | Tapidor       | Discard |
| Discard | Leopard     | Temple        | SWU Chinese 1 | Discard |
| Discard | Laser       | Yudal         | Sansibar      | Discard |
| Discard | Imola       | Zenith        | RV31          | Discard |
| Discard | Dwarf Essex | Ambassador    | POSH          | Discard |
| Discard | Cubs Root   | Aurelia       | Nikita        | Discard |
| Discard | Campus      | Barbados      | Moana         | Discard |
| Discard | Cabriolet   | Cabriolet     | Lipid         | Discard |
| Discard | Barbados    | Campus        | Leopard       | Discard |
| Discard | Aurelia     | Cubs Root     | Laser         | Discard |
| Discard | Ambassador  | Dwarf Essex   | Imola         | Discard |



**Figure 3.3.** Different accessions of *Brassica napus* growing in a field in Thriplow, Cambridgeshire, managed by KWS, in **A)** February 2022 and **B)** May 2022.

| Score | Description                        | Supporting images                                                                    |
|-------|------------------------------------|--------------------------------------------------------------------------------------|
| 1     | No sporulation                     |    |
| 2     | <10% leaf area with sporulation    |    |
| 3     | 10-25% leaf area with sporulation  |   |
| 4     | 25-50% leaf area with sporulation  |  |
| 5     | 50-75% leaf area with sporulation  |  |
| 6     | 75-100% leaf area with sporulation |  |

**Figure 3.4.** Phenotypic visual assessment on a scale of 1-6 to determine the severity of *Pyrenopeziza brassicae* symptoms on *Brassica napus* leaves (Karandeni Dewage et al., 2021). Acervuli can be seen macroscopically, allowing for categorising the leaves into the specified scores according to the percentage of leaf surface covered.

### 3.2.4 Acervuli quantification

After a 21-day incubation post-inoculation with *P. brassicae*, glasshouse grown *B. napus* plants were harvested and incubated at 4°C to induce sporulation, as specified in chapter 2.4 (Diseased leaf washing and *P. brassicae* conidial suspension preparation). After 5-10 days, the leaves were prepared to photograph them in the laboratory by carefully placing them on a flat and clean surface, under controlled, even, high intensity but indirect lighting to avoid harsh shadows and bright spots. The leaves were secured with tape to minimise the effect of leaf distortion. A tripod was placed on top to ensure the camera was stable, steady and positioned so the camera lens was parallel to the samples. The photographs were taken with an Olympus E-510 digital camera (Olympus Corporation, China).

The photographs were then segmented into twelve pictures (Figure 3.5) and processed by increasing the contrast to highlight the white spots and facilitate the acervuli quantification. The number of acervuli in each picture were counted twice.

### 3.2.5 Spore counting

After 21 days post-inoculation, leaves were harvested and incubated at 4°C to induce sporulation as specified in Chapter 2.4 (Diseased leaf washing and *P. brassicae* conidial suspension preparation). After 5-10 days, infected leaves of an individual plant were washed in 15 ml of SDW to recover the conidia, which were collected in a liquid suspension that was filtered through autoclaved Miracloth to remove dust and larger particles. To determine its concentration in spores/ml, 10 µl of the spore suspension were placed in a Bright-Line haemocytometer (Sigma-Aldrich, UK) and the spores were counted using a GXM-L2800 microscope (GT vision, UK).

To normalise the data, the area of the plant surface also had to be considered, quantifying the data as spores/cm<sup>2</sup>, for which the leaf area was calculated. Leaves were placed flat on a standard GraphPad that included a 26 cm reference ruler. Pictures were taken with the reference code for each plant individual, and the photographs were analysed with ImageJ software. The reference ruler in the GraphPad was used to set the scale in cm (Figure 3.6A), after which the leaf area was accurately traced and measured in cm<sup>2</sup> (Figure 3.6B). To confirm the calculation of the leaf area was correct, the method was calibrated by measuring the area of a square of known size (1cm x 1cm) from the GraphPad using ImageJ. If it yielded 1cm<sup>2</sup>, the results were considered reliable. This was done at least once for every batch of pictures.

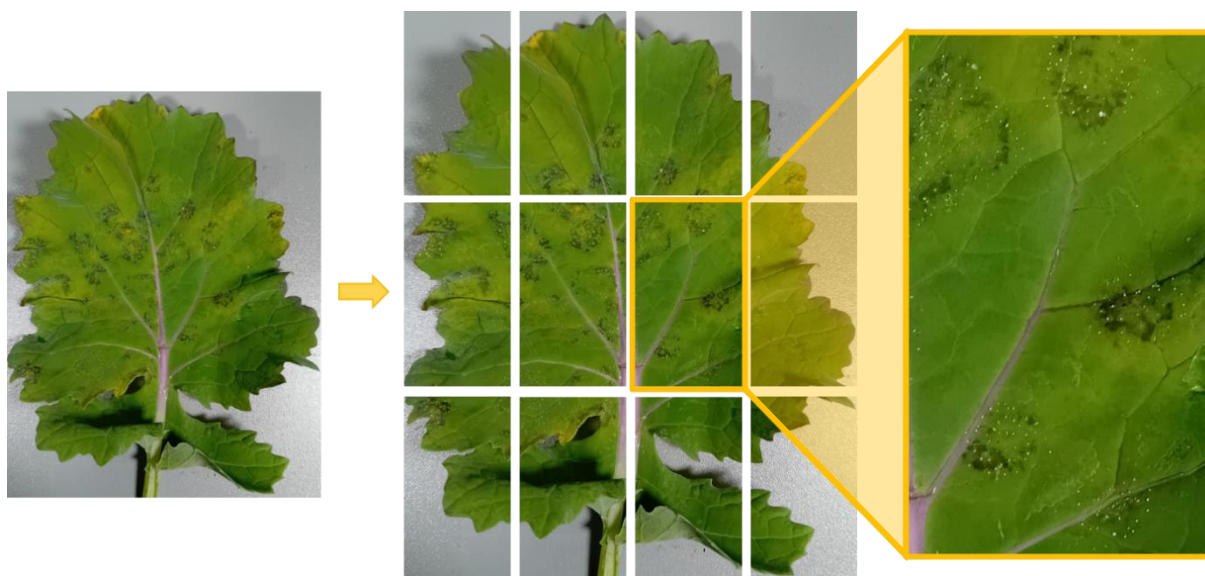


**Figure 3.5.** Segmented photograph of a leaf from *Brassica napus* susceptible line RV31, covered with visible *Pyrenopeziza brassicae* acervuli.

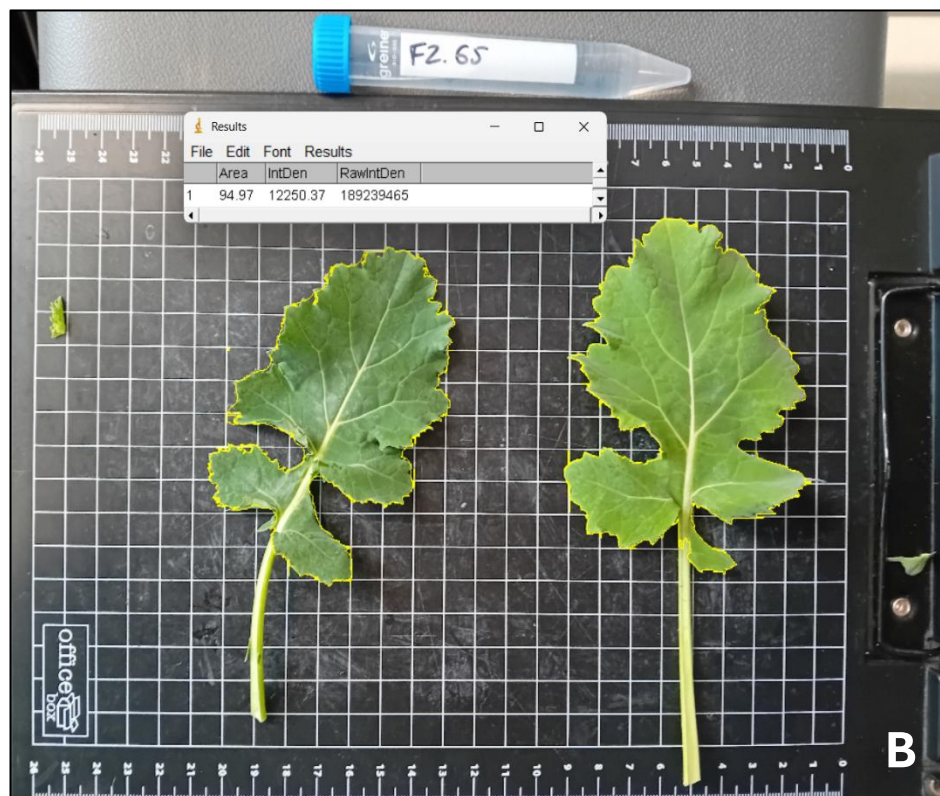
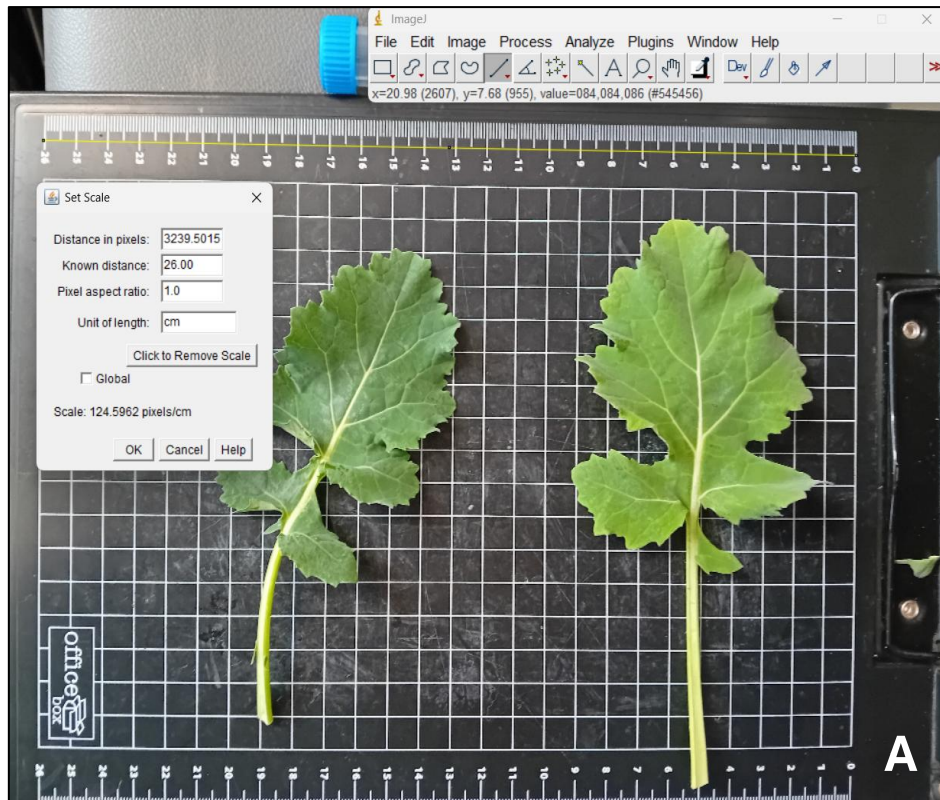


**Figure 3.6.** Use of ImageJ software to quantify the area in  $\text{cm}^2$  of the *Brassica napus* leaves. **A)** Scale was set to 26 cm. **B)** Leaf area was selected and quantified in  $\text{cm}^2$ .

### 3.2.6 WAC assay

The methods described by Ayliffe *et al.* (2013), Schnippenkoetter *et al.* (2021) and Kovács-Simon & Fones (2024) for quantifying fungal biomass in infected plant tissue through a WAC assay were adapted to the *B. napus* - *P. brassicae* pathosystem. For each experiment, mean values for each cultivar and their respective standard errors were then calculated from relative fluorescence units (RFU) values.

Firstly, an experiment was conducted with WGA-FITC as a positive control to test if the reagent reacted as expected, to confirm its functionality and ensure accurate and reliable results when using it. For it, the fluorescence of unbound WGA-FITC at different concentrations was measured. A serial dilution was prepared from a 1 mg/ml WGA-FITC conjugate stain (Sigma-Aldrich, UK) as shown in Figure 3.7. From each dilution, in triplicate, 5 µl were mixed in 150 µl of nuclease-free water by pipetting. The mix was added to a well in a black non-transparent 96-well plate, along with 3 wells containing nuclease-free water as blanks. The fluorescence of the samples was measured using a CLARIOstar®*Plus* Microplate Reader (BMG Labtech, Germany) at 485 nm excitation and 520 nm emissions.

Following this, WGA-FITC was tested with chitin from shrimp shells in powder form (Sigma-Aldrich, UK) diluted at different concentrations in triplicate, for which 5 mg of chitin were diluted in 5 ml of solvent Tris-HCl pH 7.5, vortexed and placed in a rocker for a day to completely dilute the powdered chitin. From this 1:1 dilution, dilutions at concentrations 1:5, 1:15, 1:25 and 1:50 were prepared and vortexed. From these, 6 technical replicates of 200 µl were pipetted into clean Eppendorf tubes, three of which were used as control with no WGA-FITC addition, and the remaining three were mixed with 5 µl of 1 mg/ml WGA-FITC through vortex. All the tubes were incubated at room temperature for an hour with constant movement in a rocker and later centrifuged at 3000×g for 3 minutes. The pellet containing the chitin bound to WGA-FITC was resuspended in 150 µl of Tris-HCl and transferred to the wells of a black non-transparent 96-well plate. Blank controls were included by adding triplicates of 200 µl of nuclease-free water and Tris-HCl. An additional WGA-FITC positive control was included by transferring a dilution of 200 µl of solvent Tris-HCl and 5 µl WGA-FITC (1 mg/ml) in triplicate into the wells. The fluorescence of the samples was measured using a CLARIOstar®*Plus* Microplate Reader (BMG Labtech).

To test the reaction of WGA-FITC directly to the fungal tissue, *P. brassicae* isolate 17WOSR-14, grown in MEA plates and collected as described in chapter 3.2.1, was freeze-dried for 48 hours to remove all the water inside the tissue. The dried fungal material was grounded into powder and diluted in 1M KOH into serial dilutions 1:1, 1:5, 1:15, 1:25 and 1:50. The dilutions were incubated at 60°C for 2 days, subjected to mixing at least twice daily. Suspensions were centrifuged at 2000×g for 3 minutes at room temperature, and the supernatant with the remaining KOH discarded. The pellets were washed by resuspending in 400 µl of Tris-HCl pH 7.5 and repeating the centrifuge and resuspension steps 3 additional times to neutralise the solution and completely remove the KOH. The pellets are resuspended in 600 µl of Tris-HCl, and three 200 µl technical replicates prepared from each tube, pipetted into new 0.5 ml tubes along with 5 µl of WGA-FITC (1mg/ml). The mix was vortexed and incubated for an hour at room temperature, then centrifuged at 2000×g for 3 minutes, and the supernatant containing the unbound WGA-FITC discarded. Pellets were washed with Tris-HCl by resuspending in 400 µl of the buffer and centrifuged (2000×g, 3 minutes) 4 times. The pellets were finally resuspended in 150 µl of Tris-

HCl and transferred to the wells of a black non-transparent 96-well plate. Blank controls were included by adding triplicates of 200 µl of nuclease-free water and Tris-HCl. An additional WGA-FITC positive control was included by transferring a dilution of 200 µl of solvent Tris-HCl and 5 µl WGA-FITC (1 mg/ml) in triplicate into the wells. The fluorescence of the samples was measured using a CLARIOstar®*Plus* Microplate Reader (BMG Labtech).

Finally, the test was performed with *B. napus* leaf tissue infected with *P. brassicae*. The *B. napus* cultivars selected for the analysis were resistant cvs Lipid and Nikita, and susceptible cv. Ambassador and line RV31. As a negative control, a non-inoculated *B. napus* cv. Cabriolet was used along the resistant and susceptible samples. For this experiment, modifications were introduced following the method for chitin quantification described by Kovács-Simon & Fones (2024). Infected host tissue was freeze-dried and ground to a powder, initially using mortar and pestle, and later using a TissueLyser® at 0.5 Hz for 3 minutes. Tubes were prepared with 0.05 g of ground leaf tissue per sample, and incubated at 100°C for 30 minutes, suspended in 600 µl of 1M KOH, to remove the chlorophyll and clear the leaf tissue, as well as aiding on fluorescence staining, improving the resolution. Suspensions were centrifuged at 2000×g for 4 minutes, supernatants containing KOH were discarded. Pellets were washed by resuspending in 1000 µL 50 mM Tris-HCl pH 7.5 buffer and centrifuging for 30 minutes at 15000×g. The supernatant was discarded, and the pellet resuspended in 800 µl of Tris-HCl, vortexed and allowed to equilibrate overnight at 4°C. Prior to sample pipetting, the ends of pipette tips were removed, and homogenates were regularly agitated to ensure that a uniform sample was collected. The suspensions were divided in two equal volumes and 20 µL WGA-FITC 1 mg/ml were added and incubated at room temperature for 15 minutes. Following this incubation, 1000 µL Tris-HCl were added and then centrifuged for 15 minutes at 15000×g. Supernatants were discarded to remove the excess of WGA-FITC. Pellets were washed 3 times by, subsequently, resuspending them in 500 µL Tris-HCl, centrifuging at 15000×g for 3 minutes and discarding the supernatant. The final pellet was resuspended in 600 µL of Tris-HCl, and 200 µL of each were transferred to a black non-transparent 96-well plate. Blank controls were included by adding triplicates of 200 µl of nuclease-free water and Tris-HCl. An additional WGA-FITC positive control was included by transferring a dilution of 200 µl of solvent Tris-HCl and 5 µl WGA-FITC (1 mg/ml) in triplicate into the wells. Relative fungal biomass was measured using a CLARIOstar®*Plus* Microplate Reader (BMG Labtech) at 485 nm excitation and 520 nm emissions wavelengths.

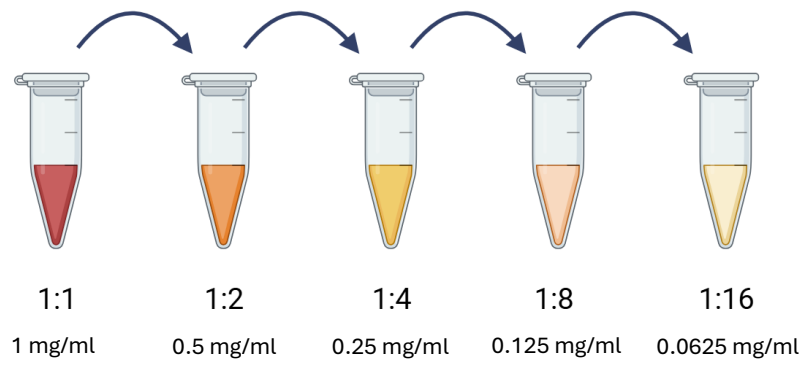


**Figure 3.7.** Serial dilution of WGA-FITC. (Created with BioRender.com)

### 3.2.7 qPCR

To quantify *P. brassicae* DNA found in infected *B. napus* plants, it was necessary to plot a standard curve, for which a *P. brassicae* DNA serial dilution was prepared to be included as standards. The extraction of *P. brassicae* DNA (isolate 15WOSR64-SS1) used mycelium grown in MEA plates (chapter 3.2.1), which was freeze-dried for 48 hours, after which 20 mg were taken and put inside a screw-cap tube along with 3 stainless steel beads. The mycelium tubes were placed in the FastPrep machine (MP Biomedicals, UK) for 20 seconds at 4.0 m/s to grind the tissue into powder. The powder was mixed with 800  $\mu$ L of DNA extraction buffer, which was prepared as detailed in chapter 2.6 (Preparation of buffer for mycelium DNA extraction), and vortexed briefly. Then, the samples were incubated in a water bath at 65°C for 30 minutes, inverting the tubes every 10 minutes, and then left to cool at room temperature. A volume of 800  $\mu$ L of the bottom phase of phenol:chloroform:isoamyl alcohol were added into the tube and vortexed briefly. The samples were placed into a microcentrifuge at 13000 rpm for 10 minutes at 4°C. The top aqueous phase, which is normally around 600  $\mu$ L, was carefully transferred into a clean 1.5 mL Eppendorf tube using filter tips. Next, 10  $\mu$ L of RNase A were added to each tube and incubated at 37°C in a hot plate for 30 minutes, after which 0.1 volumes (60  $\mu$ L for 600  $\mu$ L) of NaAc at room temperature and 0.66 volumes (396  $\mu$ L for 600  $\mu$ L) of cold isopropanol were added, mixed by inverting the tubes 6 times and incubated at room temperature for 5 minutes. The tubes were stored in a -20°C freezer overnight. The next day, the samples were placed into a microcentrifuge at 13000 rpm for 30 minutes at 4°C, to pellet the DNA. Subsequently, the supernatant was discarded, and the pellet was washed in 400  $\mu$ L of 70% ethanol. The samples were centrifuged again in the same conditions for 10 minutes, the ethanol carefully removed, and the pellet left to dry in the fume hood for 30 minutes. Finally, the DNA pellet was resuspended in 40  $\mu$ L of nuclease-free water and stored in a -20°C freezer until needed.

To plot a qPCR standard curve, a serial dilution of *P. brassicae* DNA was prepared at 5 different concentrations (1 pg, 10 pg, 100 pg, 1000 pg and 10000 pg), diluting the DNA in PCR grade water.

Following the preparation of the *P. Brassicae* serial dilution for the standard curve, DNA was extracted from 50 mg of freeze-dried leaf tissue of resistant *B. napus* cvs Lipid and Nikita, susceptible cv. Ambassador and line RV31, and a non-inoculated *B. napus* sample. The DNA extraction was done following the protocol described in chapter 2.5 (DNA extraction). The qPCR protocol required a minimum yield of 50 ng of DNA per sample.

The targeted sequence in the *P. brassicae* DNA was the ribosomal ITS4 region, and the primers used for the qPCR amplification were PbITS-Forward (5'-TTGAACCTCTCGAAGAAGTTCAGTCT-3') and PbITS-Reverse (5'-AGATTGGGGGTTGTTGGCTAA-3') (Karolewski *et al.*, 2006), with an expected amplicon size of 461 bp.

The Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix was used for the qPCR. A reaction mixture was prepared following the volumes in Table 3.3, for a final reaction mixture volume of 20  $\mu$ L, by adding 17.5  $\mu$ L of the master mix to each well of the 96 well-plate, then adding 2.5  $\mu$ L of the corresponding DNA template. Additionally, SDW was used as negative control substituting the DNA template, and the 1 ng *P. brassicae* DNA dilution was used as the positive control. Each sample was prepared in duplicate. The plate was covered with cap strips and centrifuged for 1 minute before running in a CFX Opus Real-Time PCR System qPCR instrument (Bio-Rad, UK).

Reaction conditions included an initial denaturation at 95°C for 2 minutes; 50 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 45 seconds, extension at 72°C for 45 seconds and a data collection step at 84°C of 15 seconds; followed by a final extension step at 95°C for 1 minute, 58°C for 30 seconds and 95°C for 30 seconds.

### **3.2.8 Statistical analysis**

The analysis of each data set generated from each phenotyping method was conducted using RStudio (R Core Team, 2023). Normal distribution was tested using Shapiro's test to decide whether to apply a parametrical or a non-parametrical statistical analysis. For normal distributed data, analysis of variance (ANOVA) was used, followed by Tukey's Honestly Significant Difference (HSD) to check the significant variances within or between groups. For non-normally distributed data, Kruskal-Wallis was used, followed by Dunn test. Furthermore, to check correlation between two groups or variables, Pearson's correlation test was used for parametrical datasets, whilst Spearman's correlation test was used for non-parametrical datasets.

To compare the different phenotyping methods, a multiple regression analysis was conducted, running an analysis of variance for each method to determine how many distinct groups can be significantly separated. The higher the number of distinct groups, the phenotyping strategy would be considered more effective or reliable in identifying difference in response to LLS between cultivars.

**Table 3.3.** *Pyrenopeziza brassicae* DNA qPCR, amounts of qPCR reagents added in each 96 well plate to quantify *P. brassicae* DNA from plant genomic DNA (final reaction mixture volume of 20  $\mu$ L).

| Reagents/Samples                             | Amount in $\mu$ L             |
|----------------------------------------------|-------------------------------|
| Brilliant III ultra-fast SYBR Green qPCR mix | 10 $\mu$ L                    |
| Sterile distilled water                      | 6.3 $\mu$ L                   |
| Primers (PbITSF and PbITSR)                  | 0.6 $\mu$ L each (10 $\mu$ M) |
| Template DNA                                 | 2.5 $\mu$ L (20 ng/ $\mu$ L)  |

### **3.3 Results**

#### **3.3.1 Visual assessment**

The visual assessment method was used as a baseline to compare with the alternative phenotyping strategies; hence it was the first step in categorising the 19 *B. napus* cultivars from the Thriplow field trial. Leaf samples collected in March and in May (2022) were scored. The comparison of the disease scores at different harvest time points showed there was a significant positive correlation between them (Figure 3.8). Similarly, the experimental design of the field trial included two replicated blocks per cultivar (Table 3.2), and a comparison of the disease scores of the duplicate blocks showed a significant positive correlation between them (Figure 3.9). ANOVA tests were used to confirm there was no significant effect of the harvest time or replicated blocks. These consistencies validated disease score results, reducing chance and error.

Given that the resistance to *P. brassicae* was scored on a 1-6 scale, the results indicated that most cultivars were more resistant than what was previously recorded, with lower disease scores than expected (Figure 3.10). Average disease scores ranged between 1 for Imola as the most resistant, and 3.5 for Leopard as the most susceptible, with most of the lines distributed just below score 2, which would be considered resistant. These genotypes have previously been scored higher than what the field trial data suggested (AHDB Recommendation List; Fell *et al.*, 2023). It is worth mentioning that cv. Ambassador appeared to be significantly more susceptible in the field trial, despite having high resistance scores in the AHDB Recommendation List, which is consistent with previous data from a glasshouse experiment (Muthayil-Ali, 2023). Nikita, Barbados and Aurelia are also in the AHDB recommended list, classified as resistant under glasshouse conditions. This fits with the low disease scores for Nikita and Barbados, but Aurelia appeared more susceptible than expected. Additionally, this was the first time *B. napus* line RV31 was examined in a field trial.

The 19 *B. napus* cultivars screened in the field trial had previously been assessed in a glasshouse experiment (Fell *et al.*, 2023; Muthayil-Ali, 2023), hence the disease scores for each cultivar could be compared across different environmental conditions to test the reliability and reproducibility of the method (Figure 3.11). There was a positive correlation between the data obtained in glasshouse experiments and the field trial. Notably, the x axis representing the disease scores obtained from the glasshouse experiment showed a wider range, from resistant score 1 to susceptible score 6, with a significant number of intermediate phenotypes around scores 3-4. Potentially, the controlled environment that a glasshouse experiment offers provides uniform conditions that are more suitable for the pathogen than those at a field setting, being able to adjust lighting, humidity, watering, etc. to the preferred settings. The less predictable natural environment of a field trial can interfere with disease establishment. LLS resistance could be efficiently studied in a glasshouse because the inoculation with *P. brassicae* was controlled to avoid contamination with any other pathogens, and the plants were under constant surveillance to check for any other infections affecting them during growth and incubation periods. This is not feasible in a field trial, where the crops are exposed to the natural environment without further protection. In the Thriplow field trial, it was especially challenging, controlling interactions with other organisms, such as pigeons at the beginning of the trial (October 2021) and aphids by the end of the trial (June 2022). These differences in the environmental conditions could be responsible for the divergence between the field trial and glasshouse disease scores.

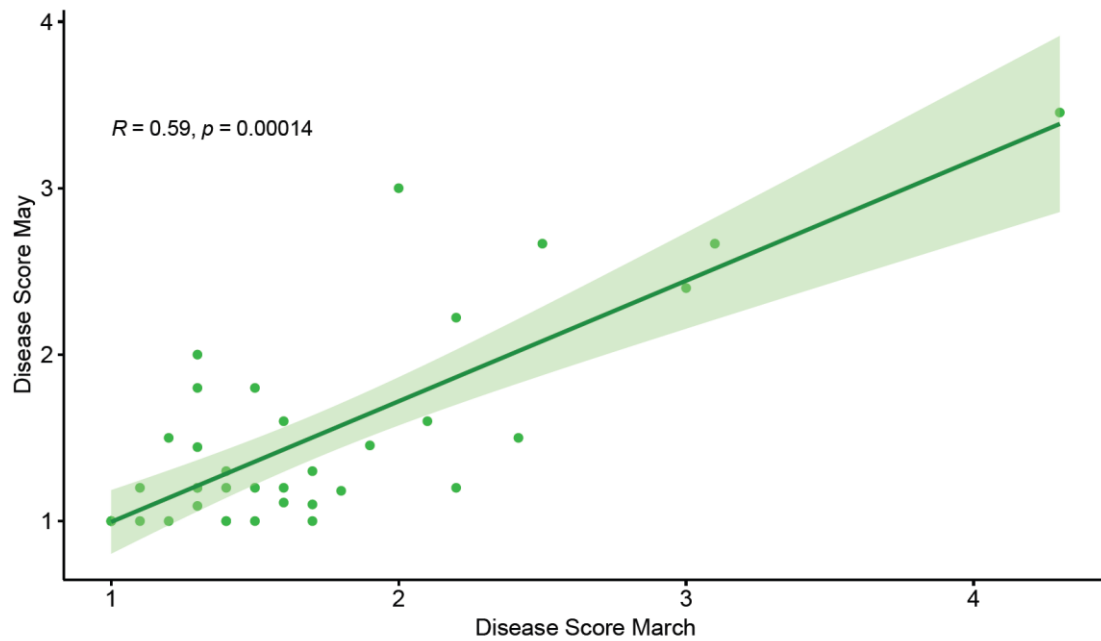


**Figure 3.8.** Correlation between the disease score of the *Brassica napus* samples harvested in March and May (2022), using Spearman correlation test, with a 95% confidence interval (green area).

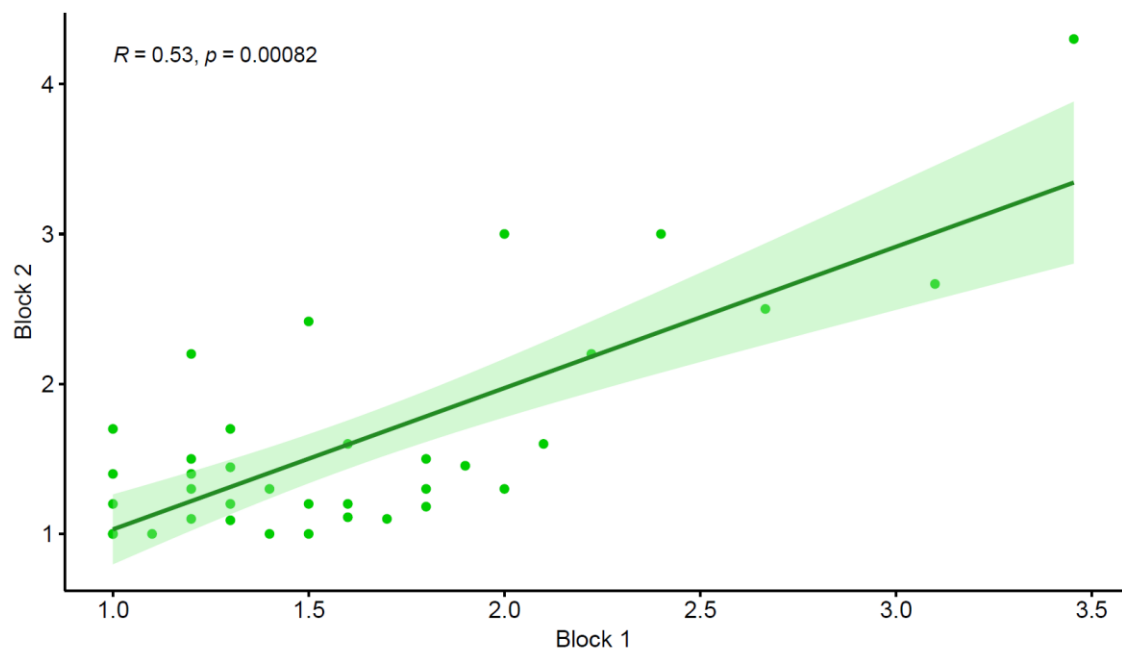


**Figure 3.9.** Correlation between the disease score of the *Brassica napus* samples harvested from duplicate plots, using Spearman correlation test, with a 95% confidence interval (green area).

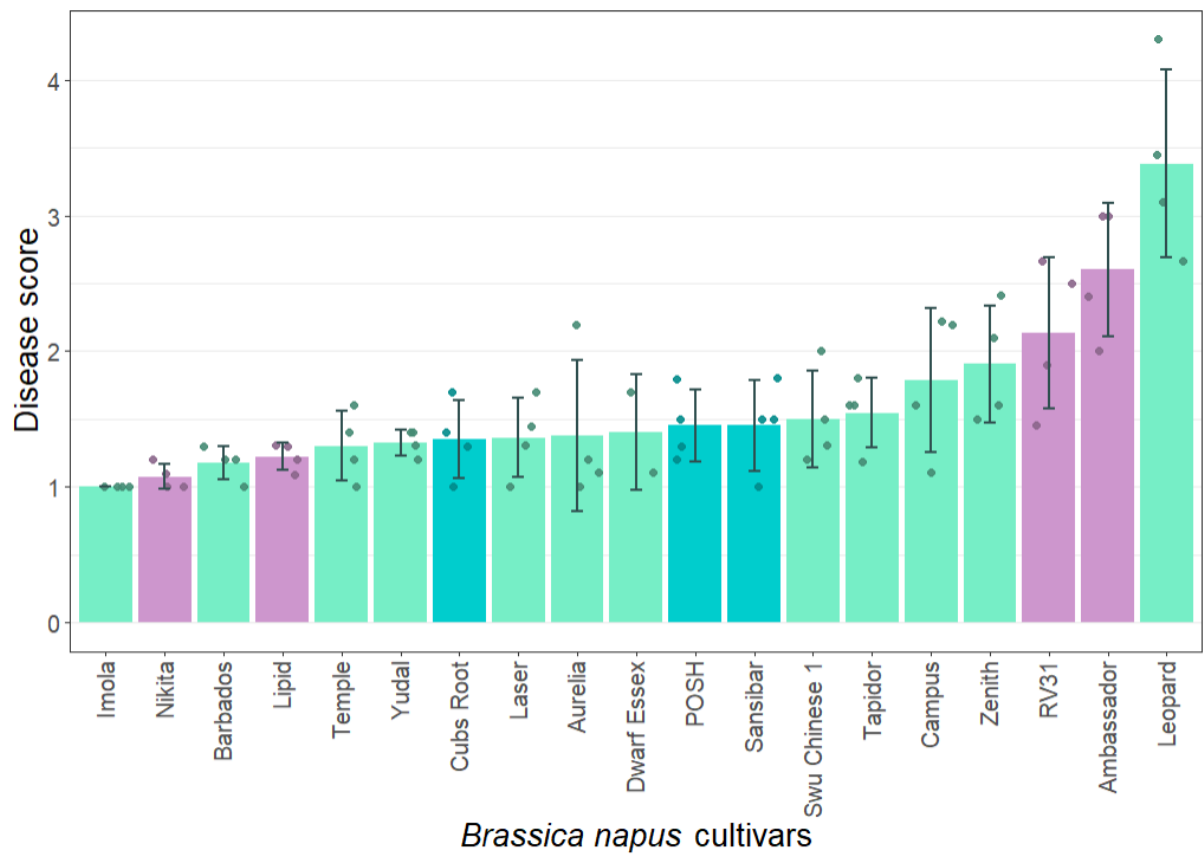


**Figure 3.10.** Disease score of the 19 *Brassica napus* cultivars grown in the field trial (Thriplow, Cambridgeshire, UK), ranked by the average score. It was screened using the visual assessment method (Karandeni Dewage *et al.*, 2021) to determine the severity of the light leaf spot disease, after inoculation with a *Pyrenopeziza brassicae* population from the Thriplow fields. Means and standard errors (error bars) are shown for four biological replicates. *B. napus* lines Nikita, Lipid, RV31 and Ambassador (in purple) were further examined in this chapter with different phenotyping methods, as they were in opposite extremes of the disease scale. *B. napus* lines Cubs Root, POSH and Sansibar (in blue) were included in an RNAseq analysis in chapter 4.

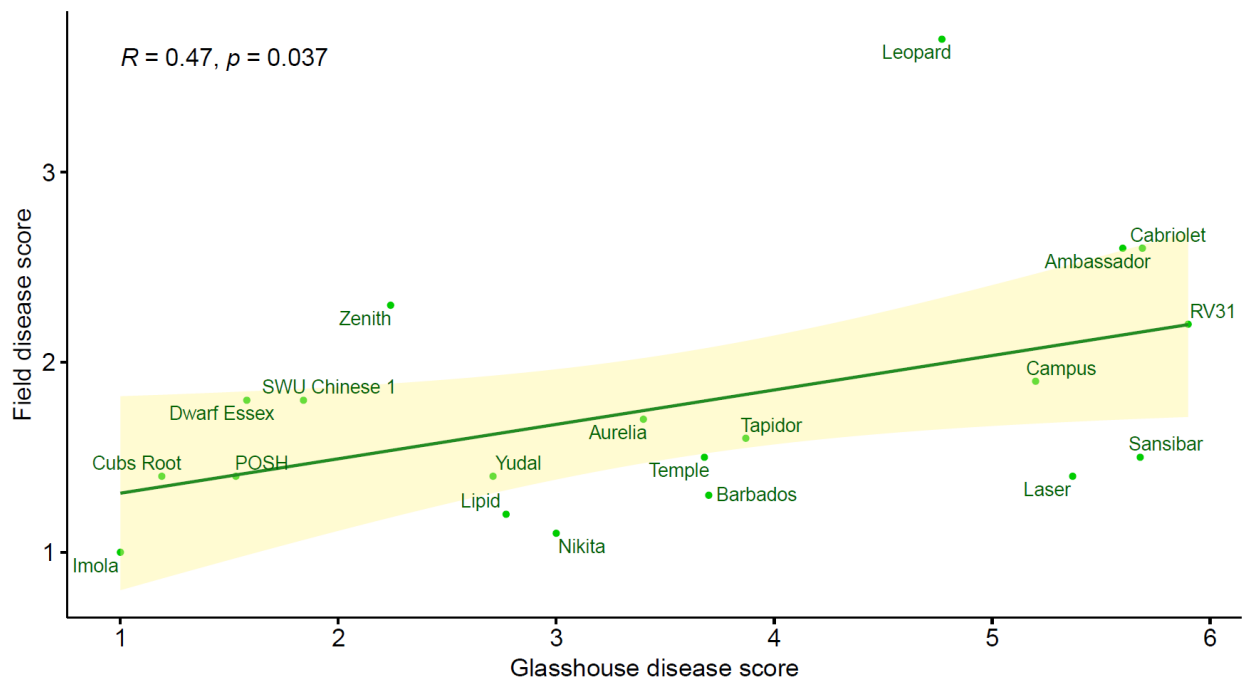


**Figure 3.11.** Regression analysis of the different *Brassica napus* cultivars inoculated with *Pyrenopeziza brassicae* and phenotyped after growth under different environmental conditions. The severity of the disease was assessed using the traditional method visual assessment. The line represents regression with associated Pearson's correlation. The yellow area represents the 95% confidence interval.

Amongst the discrepancies between field trial and glasshouse experiment, it should be noted that cvs Laser, Nikita and Barbados seemed to do better in the field, with lower disease scores. On the contrary, cvs Leopard and Zenith show a higher susceptibility to LLS in the field, growing significantly better in the glasshouse. Additionally, cvs POSH and Sansibar had more extreme disease scores in the glasshouse than in the field. POSH was significantly more susceptible to LLS in the glasshouse whilst Sansibar showed a higher resistance. These two cultivars were further studied in chapter 4, along with cvs Cubs Root and Cabriolet.

### 3.3.2 Acervuli quantification

As established by the traditional visual assessment phenotyping method, there is an observable and clear contrast between *B. napus* leaves that have different levels of disease resistance to pathogen *P. brassicae* (Figure 3.4). However, instead of visually and categorically estimating the percentage of leaf surface covered by sporulating structures (acervuli), they were individually counted.

For each of the cultivars assessed with this method, the logarithm of the number of quantified acervuli were represented in Figure 3.12. As expected, the non-inoculated control cv. Cabriolet showed a minimal number of acervuli on the leaf surface since it was not inoculated with the pathogen. Resistant *B. napus* cvs Lipid and Nikita had fewer acervuli than susceptible cv. Ambassador and line RV31, which harboured more acervuli, and significantly so in RV31. These results were completely compatible with the associated phenotype of each cultivar.

A Kruskal-Wallis non-parametric statistical analysis and a *post-hoc* Dunn test showed no statistical differences between cultivars within the same phenotypical group (i.e. resistant Lipid and Nikita were statistically similar; susceptible RV31 and Ambassador were statistically similar). The tests also showed a significant difference between the control (cv. Cabriolet) and susceptible cultivars ( $P < 0.05$ ), but the control did not show any difference with the resistant lines ( $P > 0.05$ ). The resistant cv. Nikita was statistically different from RV31, but surprisingly not from Ambassador. When processed as phenotypical groups (susceptible and resistant) instead of individual cultivars or genotypes the tests did show a significant difference between susceptible and resistant traits.

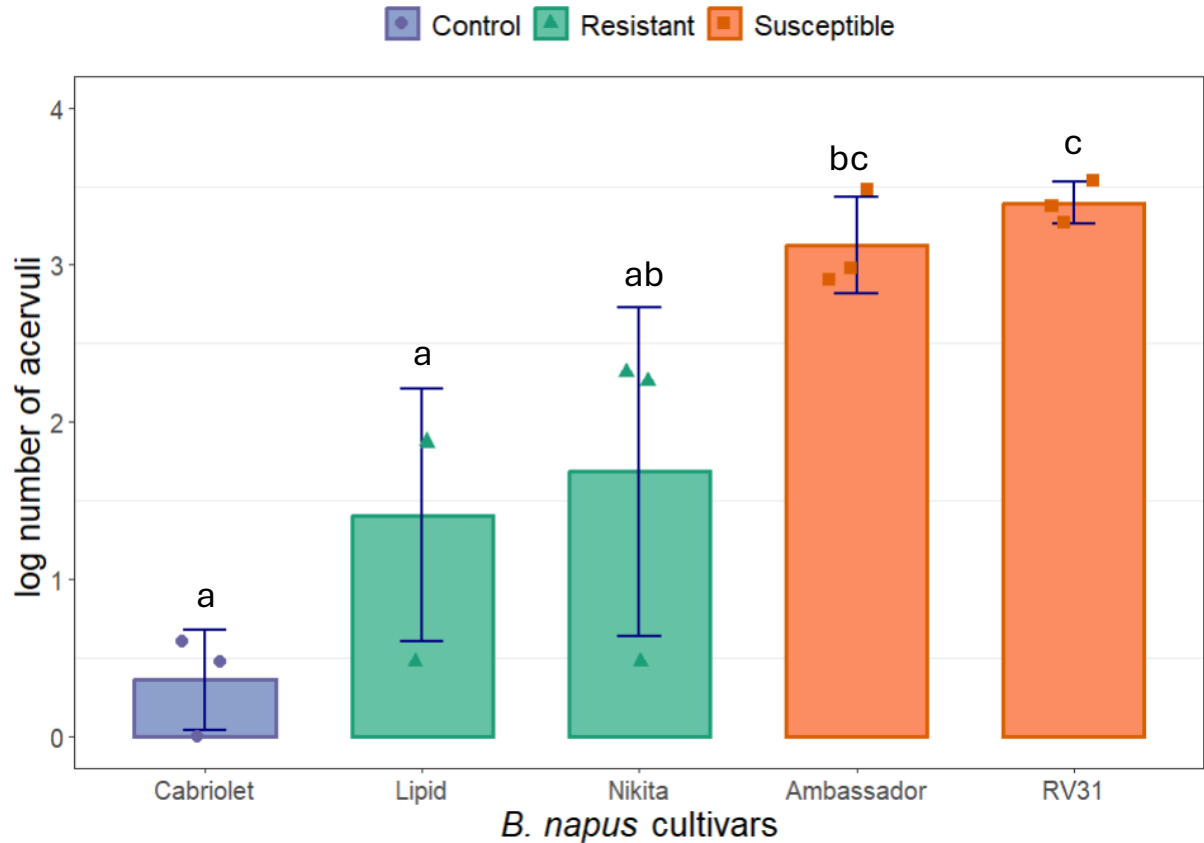


**Figure 3.12.** Comparison of the logarithm of the number of acervuli quantified on the leaf surface of different cultivars of *Brassica napus* after inoculation with a *Pyrenopeziza brassicae* population from the Thriplow fields. A non-inoculated *B. napus* control (cv. Cabriolet) was compared to resistant cvs (Lipid and Nikita) and to susceptible cvs (Ambassador and RV31). Values with the same letters are not significantly different (Kruskal-Wallis and Dunn test). Means and standard deviations (error bars) for three biological replicates per sample.

### 3.3.3 Spore counting

Spore inocula were placed in a haemocytometer to determine spore concentration under the microscope. *P. brassicae* spores were cylindrical, with an average size of 12  $\mu\text{m}$  x 3  $\mu\text{m}$ . They are clearly visible in Figure 3.13, that showed an observable difference in the number of spores harvested from cultivars with different LLS susceptibility. *B. napus* cv. Aurelia, a relatively resistant line, yielded a lower spore concentration ( $10^4$  spores/ml) than *B. napus* cv. Leopard, which is highly susceptible to LLS ( $10^6$  spores/ml). This supports the hypothesis that there is a correlation between the number of spores harvested and the severity of the disease.

The spore concentration results obtained from the Thriplow field trial in March and May (2022) were compared to the visual assessment data, showing a better positive correlation between both timepoints (Figure 3.14). The duplicate blocks per cultivar also correlated better than the visual assessment data (Figure 3.15). These improved consistencies amongst the replicates and the harvest timepoints, confirmed with ANOVA tests, validated the results obtained through spore counting and indicated this phenotyping method provided with a higher accuracy than visual assessment.

The spore counts (log spores/cm<sup>2</sup>) for each cultivar were plotted in Figure 3.16. Like the results obtained from the visual assessment phenotyping method, cvs Imola, Nikita and Aurelia appear to have lower spore concentrations which corresponds to the expected values for resistant lines. Likewise, cv. Leopard and line RV31 have the highest spore concentrations that could be associated with susceptible lines. Cultivar Ambassador, which is traditionally classified as a resistant cultivar (AHDB Recommendation List), appeared to be highly susceptible. This, however, does align both with previous data from a glasshouse experiment (Muthayil-Ali, 2023; Fell *et al.*, 2023) and with the disease score from this same field trial. This consistency further suggests that the previous classification by AHDB should be updated. Also on the AHDB Recommended List are cvs Nikita, Barbados and Aurelia, the three of them classified as resistant under glasshouse conditions, which fitted with the low spore counts from the field trial. Nonetheless, the lower spore counts corresponding to cv. Aurelia were in contrast with the higher disease scores it presented when phenotyping the cultivar with visual assessment. The rest of the cultivars assessed through spore counting show intermediate spore concentration values comparable to the disease scores, with the notable exception of SWU Chinese 1. This cultivar presented as highly susceptible when phenotyped with spore counting, as opposed to when scored with the visual assessment strategy.

As done for the visual assessment method, the spore concentrations obtained from the field trial were plotted against spore concentration values from Muthayil-Ali's (2023) parallel glasshouse experiment. It should be noted that the glasshouse experiment did not include cvs Imola, Temple or Tapidor, therefore these were excluded from the comparison. Figure 3.17 shows a positive correlation between the different environmental conditions. Notably, the x axis representing the spore concentration obtained from the glasshouse experiment showed a higher range, starting at value 6.0 (log spores/cm<sup>2</sup>) for cultivars associated with resistance against LLS, and reaching values proximate to 8.0 (log spores/cm<sup>2</sup>) for cultivars associated with susceptibility to LLS. These lower spore concentration values from the field trial, in comparison to the glasshouse experiment, could be associated to the challenges linked with the lack of control over the environment in the field.

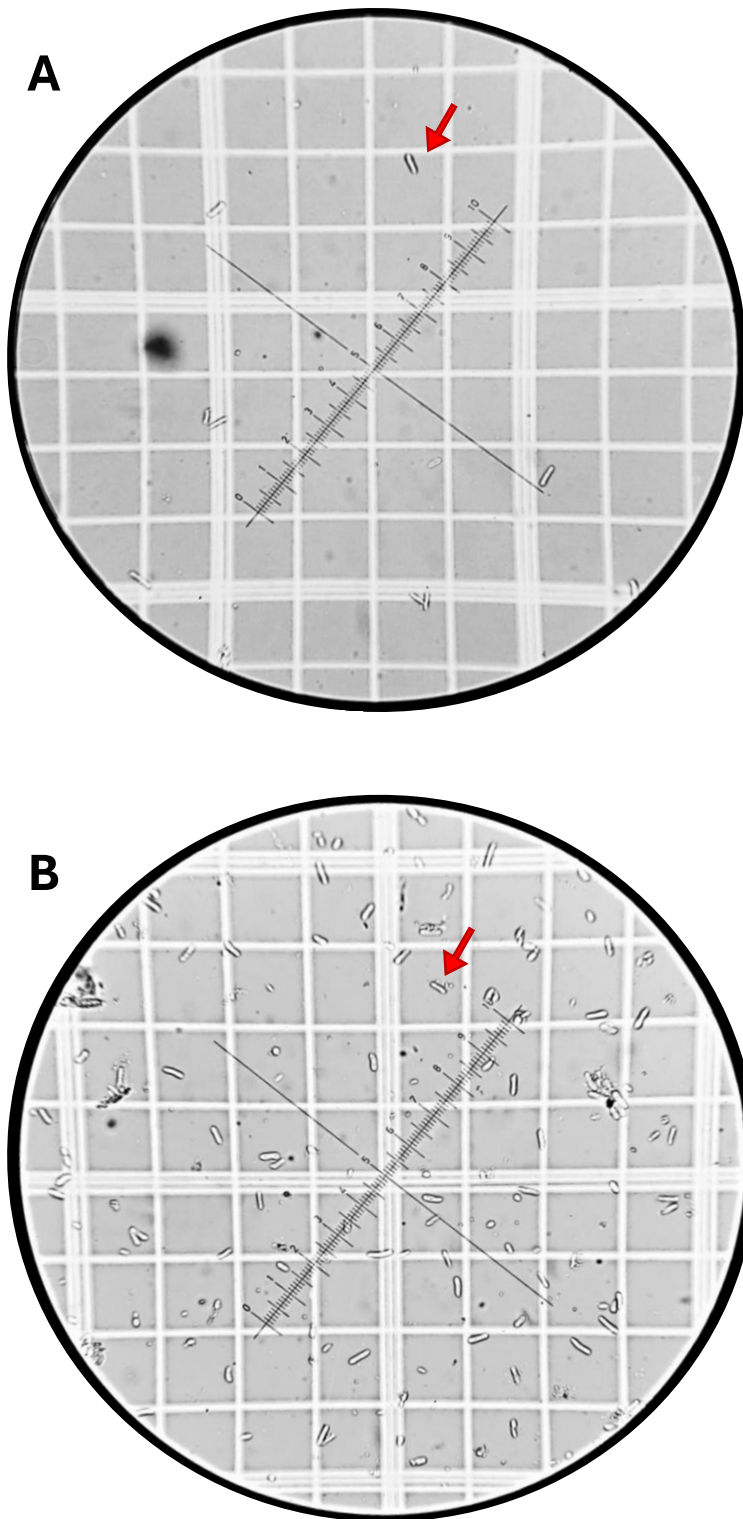


**Figure 3.13.** *Pyrenopeziza brassicae* spore suspensions harvested from different *Brassica napus* cultivars, seen in the haemocytometer under the microscope. Spores are indicated by red arrows. The scale bar indicates 0.25 mm. **A)** Spore suspension collected from *B. napus* cv. Aurelia, with a concentration of  $10^4$  spores/ml. **B)** Spore suspension collected from *B. napus* cv. Leopard, with a concentration of  $10^6$  spores/ml.

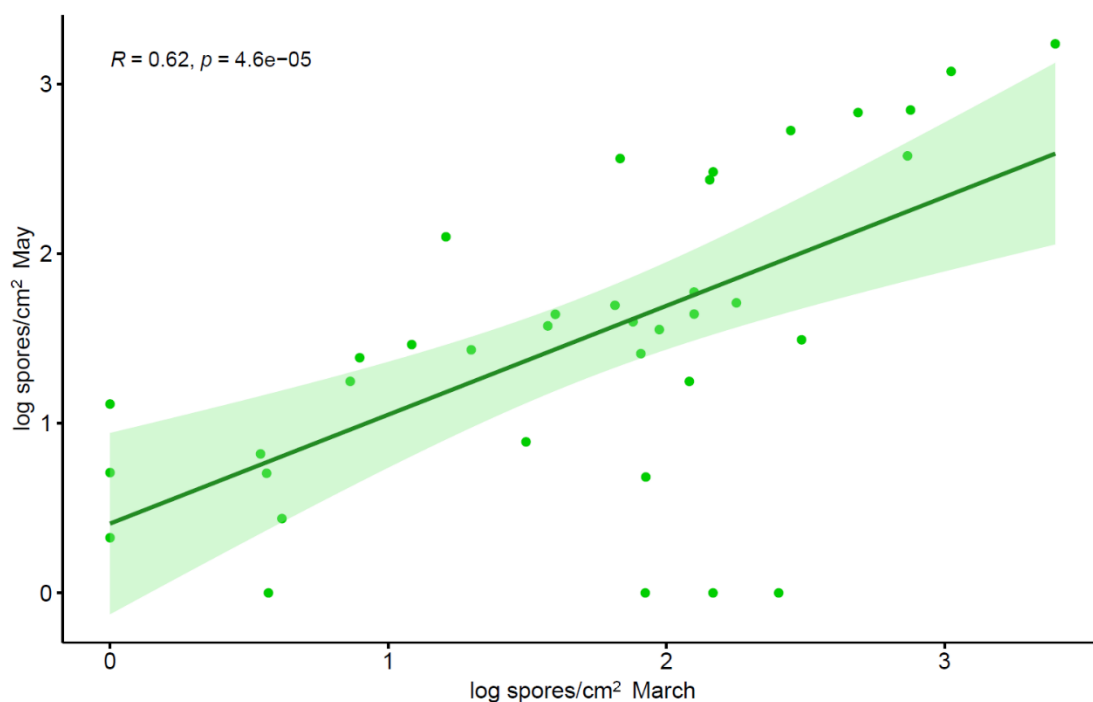


**Figure 3.14.** Correlation between the spore concentration ( $\log \text{ spores/cm}^2$ ) harvested from *Brassica napus* cultivars in March and May (2022), using Pearson correlation test, with a 95% confidence interval (green area).

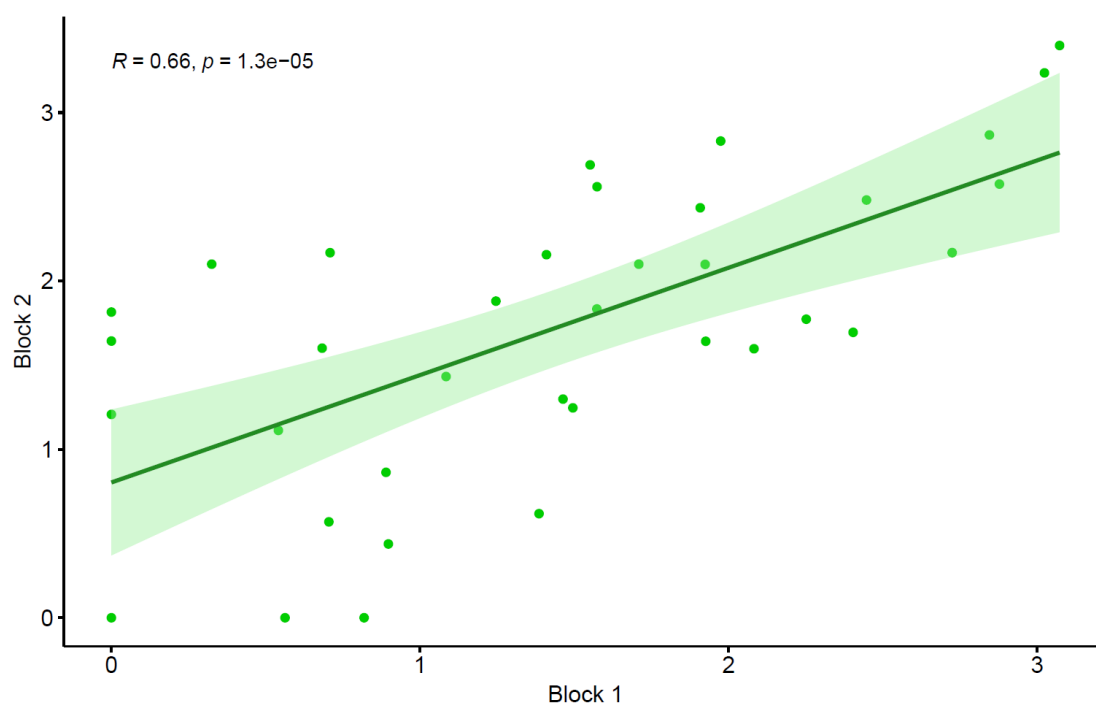


**Figure 3.15.** Correlation between the spore concentration ( $\log \text{ spores/cm}^2$ ) of the *Brassica napus* samples harvested from duplicate plot blocks, using Pearson correlation test, with a 95% confidence interval (green area).

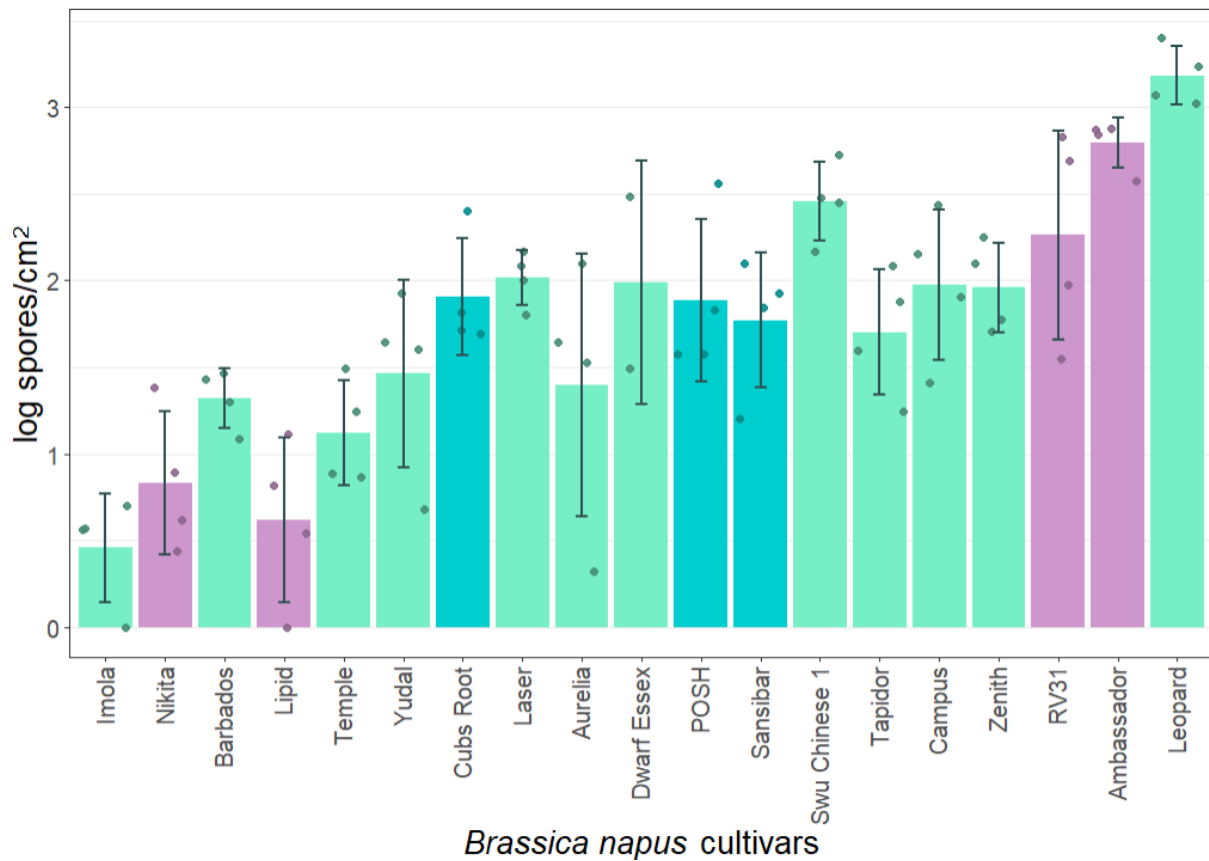


**Figure 3.16.** Spore concentration (log spores/cm<sup>2</sup>) harvested from 19 *Brassica napus* cultivars grown in the field trial (Thriplow, Cambridgeshire, UK). Lines were screened using the spore counting method to determine the severity of the light leaf spot disease, after inoculation with a *Pyrenopeziza brassicae* population from the Thriplow fields. Means and standard errors (error bars) are shown for four biological replicates. *B. napus* lines Nikita, Lipid, RV31 and Ambassador (in purple) were further examined in this chapter with different phenotyping methods, as they were in opposite extremes of the disease scale. *B. napus* lines Cubs Root, POSH and Sansibar (in blue) were included in an RNAseq analysis in chapter 4.

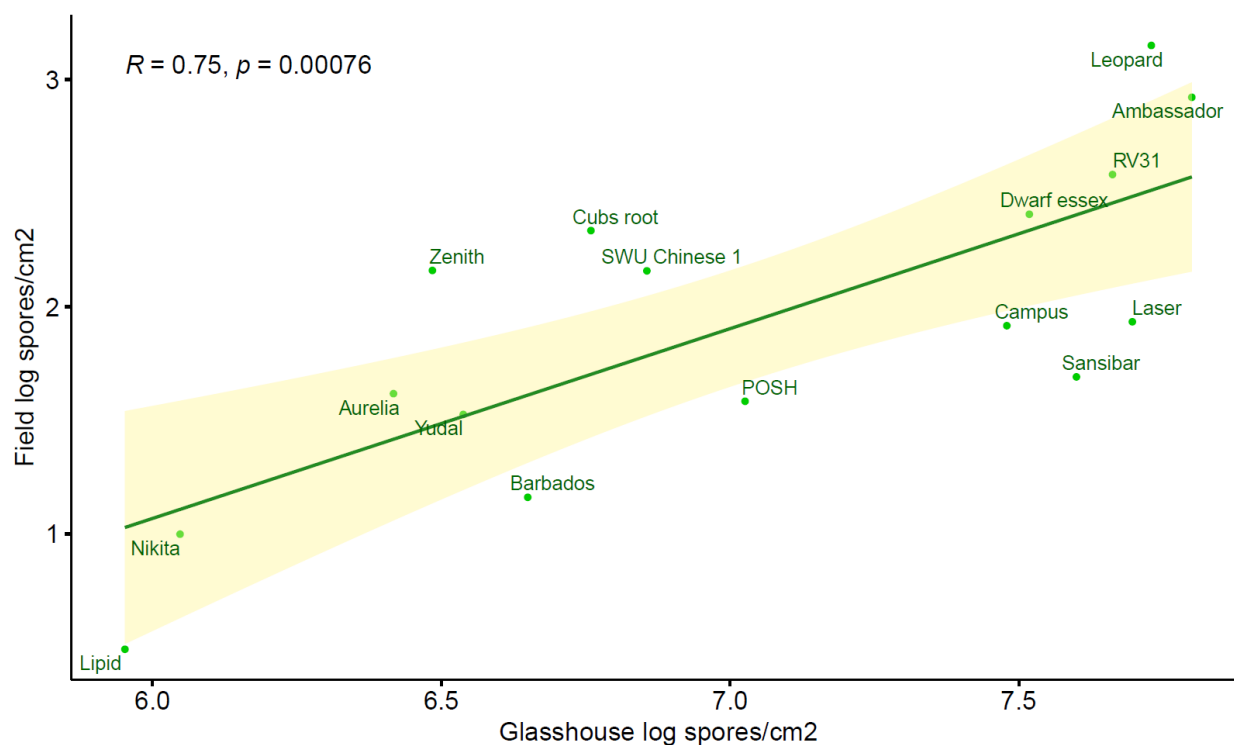


**Figure 3.17.** Regression analysis of different *Brassica napus* lines inoculated with *Pyrenopeziza brassicae* and phenotyped after growth under different environmental conditions. The severity of the disease was assessed using spore counting. The line represents regression with associated Pearson's correlation. The yellow area represents the 95% confidence interval.

### 3.3.4 Wheat-germ Agglutinin Chitin (WAC) assay

WGA-FITC was tested in a control experiment to confirm the reagent reacted as expected regarding its fluorescence. The relative fluorescence units (RFU) for the concentration of the WGA-FITC serial dilution (mg/ml), read at 485 nm excitation and 520 nm emissions, were used to plot a standard curve (Figure 3.18). Following testing of linear, quadratic and exponential regression models, the Bayesian Information Criterion (BIC) and the  $R^2$  confirmed the quadratic equation as the line of best fit (Table 3.4), with the lowest BIC and the highest  $R^2$ . The resulting standard curve showed an increment in the fluorescence values as the WGA-FITC concentration increased, as predicted, confirming the fluorescent properties of the reagent and ensuring the validity and accuracy of the following experiments that use this reagent.

To determine the binding of WGA-FITC to shrimp shell chitin, two chitin serial dilutions were prepared, one with WGA-FITC to bind to the chitin and a control serial dilution without any WGA-FITC. In addition, there were two controls: a negative control consisting of Tris-HCl buffer and a positive control consisting exclusively of WGA-FITC. The resulting fluorescence values (RFU) were plotted in Figure 3.19. This graph showed low values for the chitin serial dilution lacking WGA-FITC, whereas the values for the serial dilution containing WGA-FITC showed RFU values that gradually decreased as the chitin concentration was reduced, establishing a clear difference between chitin concentrations. After proper binding of the WGA-FITC reagent to chitin and wash of the unbound reagent, the concentration of these two compounds would be proportional. Thus, the fluorescence associated with WGA-FITC would directly represent the amount of chitin in the dilution. At its highest chitin concentration (1 mg/ml), 80% of the initial WGA-FITC had bound to the chitin molecules in the dilution. The controls were excluded from the graph due to the magnitude of their RFU values in comparison to the serially diluted chitin samples. The maximum RFU values yielded by the negative control did not exceed 800 RFU, whereas the positive control consisting of WGA-FITC doubled the values from the highest chitin dilution, with values around 230000 RFU.

The fluorescence values of the chitin serial dilution bound to WGA-FITC were also plotted as a dilution curve for chitin concentration (Figure 3.20). Following testing of linear, quadratic, logarithmic and exponential regression models, the Bayesian Information Criterion (BIC) and the  $R^2$  confirmed the logarithmic equation ( $y = 11000 + 29000x$ ) as the line of best fit (Table 3.5), with the lowest BIC and the highest  $R^2$ . This curve resembles a saturation curve approximating a plateau of saturation. Considering that homodimer WGA-FITC has eight binding sites with high specificity to the monomer unit of chitin (GlcNAc), a gradual increase in chitin concentration would translate to a proportional increase of the number of molecules interacting with the active sites. Consequently, this would result in a decline of free chitin-binding domains able to bind to the substrate and would eventually lead to WGA-FITC's maximum saturation.

The fluorescence of a *P. brassicae* fungal tissue serial dilution was measured to test its binding to WGA-FITC (Figure 3.21), along with a negative control consisting of Tris-HCl. A positive control consisting exclusively of WGA-FITC was also included in the test but excluded from the graph due to the magnitude of the fluorescence values that it yielded (approximately 220000 RFU) in comparison to the samples. The resulting graph shows a gradual increase in fluorescence as the concentration of *P. brassicae* tissue also increases, which aligns with the expected results. A non-parametric Kruskal-Wallis test indicated a significant difference between the *P. brassicae*

dilutions ( $P < 0.0001$ ). Furthermore, a Wilcoxon signed-rank test was done between groups, following the Benjamini-Hochberg (BH) method for  $p$ -value adjustment. The results suggested that all the *P. brassicae* dilutions were statistically different from each other, except for the two highest concentrated dilutions, containing 0.2 and 1 mg of dry weight *P. brassicae* diluted. They appeared to be statistically similar, despite being different from the rest, which could be explained by the saturation of WGA-FITC. Regression analysis of the data (Figure 3.22) revealed a curve that resembled the chitin saturation curve from Figure 3.20. The graph showed that the last two dilutions were approximating the saturation plateau. This could explain their statistical similarity despite their difference in *P. brassicae* dry weight and, thus, their different chitin concentrations.

In addition, the chitin calibration curve (Figure 3.20) was used to determine that the dried *P. brassicae* fungal tissue samples had approximately 3.5% of chitin content. This matches previous research that found that chitin content for fungi is between 3.8 and 13.6% (Izadi *et al.* 2025), and similar to the 2% chitin content of yeast (Chuene *et al.* 2024).

Plant material was used to compare the fluorescence of different *B. napus* cultivars that were inoculated with *P. brassicae* (Figure 3.23), along with a non-inoculated *B. napus* cv. Cabriolet sample and two controls: a positive control consisting exclusively of WGA-FITC and a negative control consisting of Tris-HCl. The positive WGA-FITC control was excluded from the graph due to the magnitude of the fluorescence values that it yielded (approximately 220000 RFU) in comparison to the samples. The negative control showed minimal fluorescence and Cabriolet showed the lowest fluorescence in the absence of pathogen, as expected. The fluorescence of reagent WGA-FITC, bound to the pathogens' chitin, indicated that resistant cvs Lipid and Nikita had lower concentration of chitin than susceptible cv. Ambassador and line RV31. An ANOVA statistical analysis showed a significant difference between the cultivars ( $P < 0.001$ ), which was further confirmed with *post-hoc* Tukey's HSD for a multiple pairwise-comparison. This test showed a significant difference between the non-inoculated control and the rest of cultivars, as well as a discernible difference between resistant and susceptible cultivars ( $P \leq 0.001$ ). However, the statistical test was unable to identify a significant difference between susceptible cv. Ambassador and line RV31, and between resistant cvs Nikita and Lipid ( $P > 0.05$ ).

Although cv. Cabriolet was never inoculated with *P. brassicae* and was grown in a glasshouse to minimise its contact with external potential pathogens, it is worth noting that it did show fluorescence. Despite showing lower fluorescent signal, it gives insight into the existence of GlcNAc-containing structures in plant cell walls, naturally occurring in plant glycoproteins (Olszewski *et al.*, 2009; Prabhu *et al.*, 2024). For this reason, there is a degree of background fluorescence in *B. napus* that should be taken into consideration when studying the fluorescence associated with pathogens in plants since it can reduce the sensitivity of measurements.

The percentage of fungal biomass present in each cultivar was calculated. Firstly, the fluorescence signal for each sample was adjusted by subtracting the RFU value of the control cv. Cabriolet, to take into consideration the background fluorescent signal previously mentioned. It was possible to then interpolate this adjusted fluorescent signal in the calibration curve for chitin concentration (Figure 3.20) to obtain the amount of chitin present in each cultivar, considering the measured aliquots were 200  $\mu$ L. Knowing that *P. brassicae* has a 3.5% of chitin content, the dry weight of fungal biomass for each sample was calculated. Seeing as these values correspond

to aliquots of 1/6 of the initial 50 mg of dry weight of leaf material, the percentage of *P. brassicae* present in *B. napus* samples was calculated (Table 3.6). Control cv. Cabriolet presented the lowest value as expected from a non-inoculated control. The rest of the *B. napus* lines show percentages between 1.66% and 1.83%. Cultivars Lipid and Nikita showed lower values than susceptible lines RV31 and Ambassador. A Kruskal-Wallis and a *post-hoc* Wilcoxon signed-rank demonstrated there was a significant difference between the resistant and susceptible lines, and between these and the control.

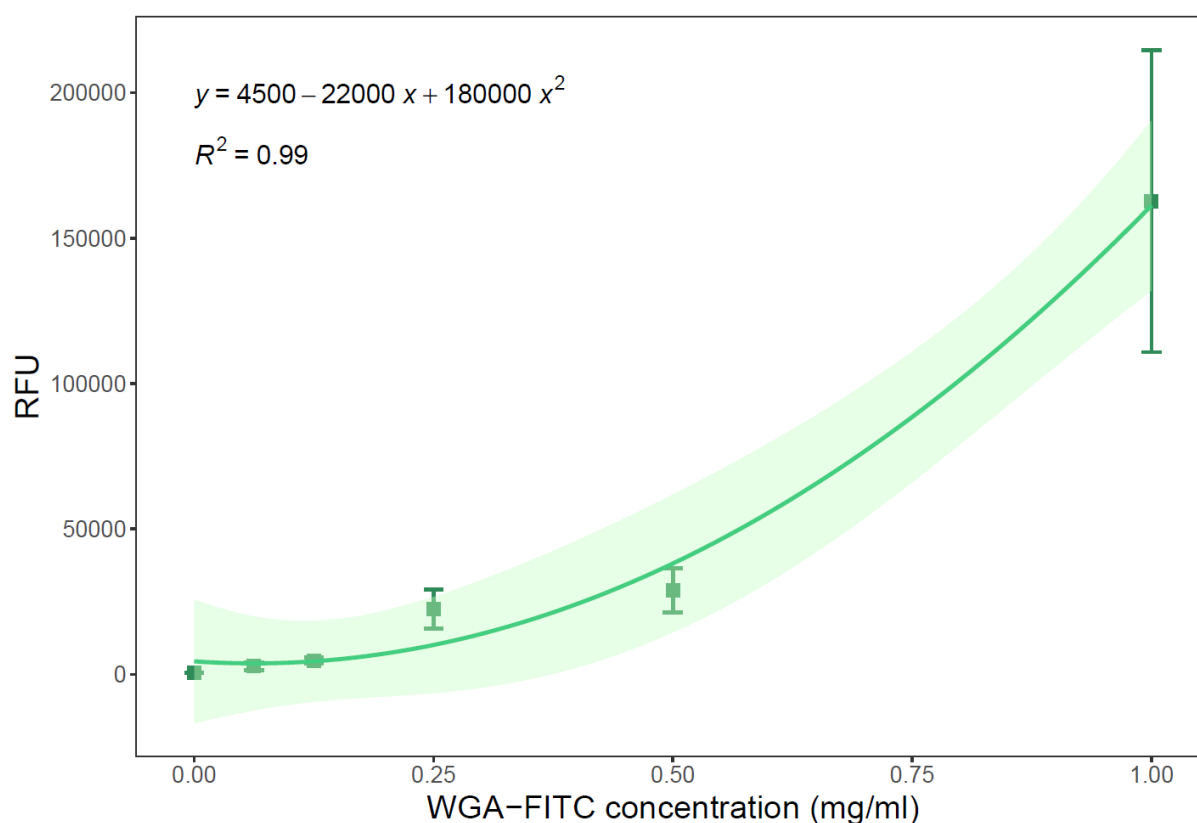


**Figure 3.18.** Standard curve of the concentration of WGA-FITC (mg/ml) vs relative fluorescence units (RFU) at 485 nm excitation and 520 nm emissions. The line of best fit follows the quadratic equation. Means and standard deviations (error bars) of technical triplicates are shown.

**Table 3.4.** Values for the Bayesian Information Criterion (BIC) and  $R^2$  for three potential regression models for the WGA-FITC standard curve.

| Regression model     | BIC      | $R^2$  |
|----------------------|----------|--------|
| Linear equation      | 139.9024 | 0.9023 |
| Quadratic equation   | 129.6253 | 0.9869 |
| Exponential equation | 134.4267 | 0.9608 |

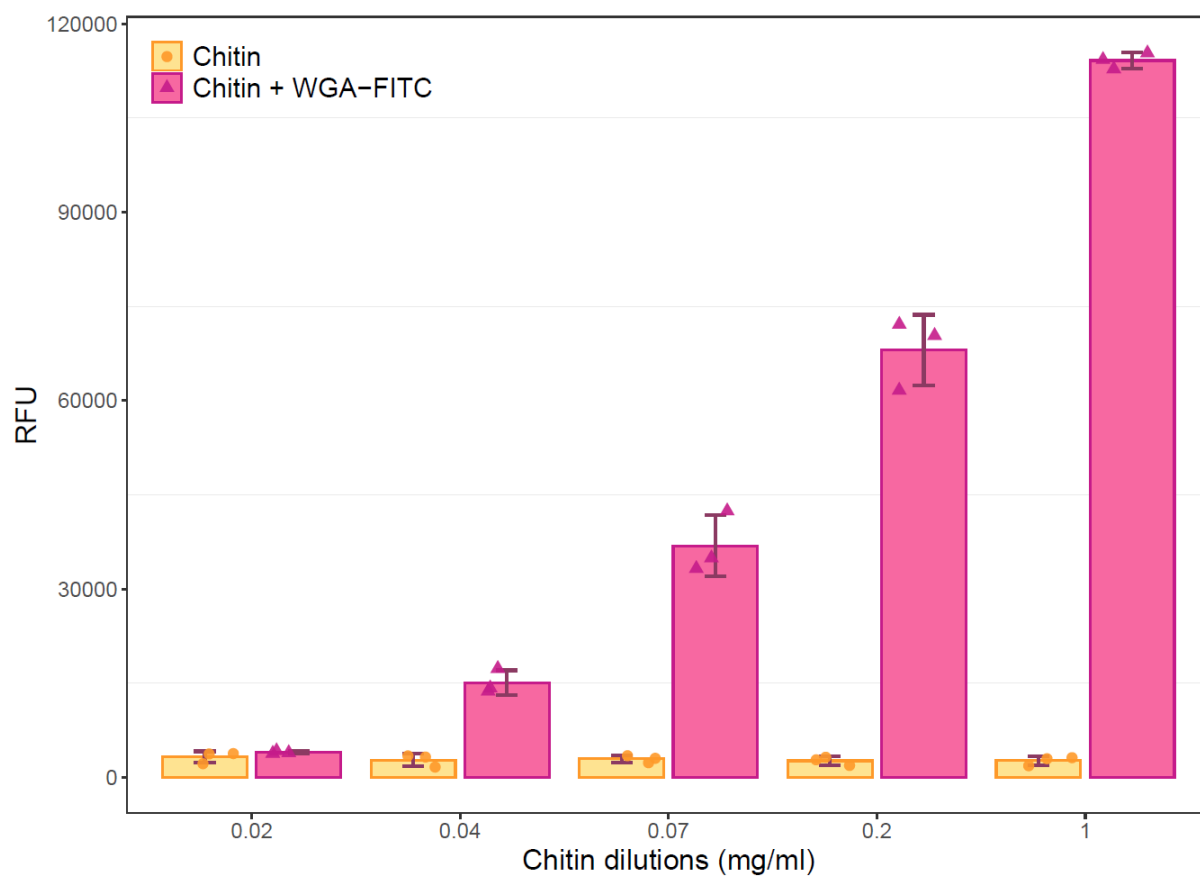


**Figure 3.19.** Comparison of relative fluorescence units (RFU) of serially diluted chitin incubated with WGA-FITC (1 mg/ml) and without it (pink and orange bars, respectively). Fluorescence was measured at 485 nm excitation and 520 nm emissions. Means and standard deviations (error bars) of technical triplicates are shown.

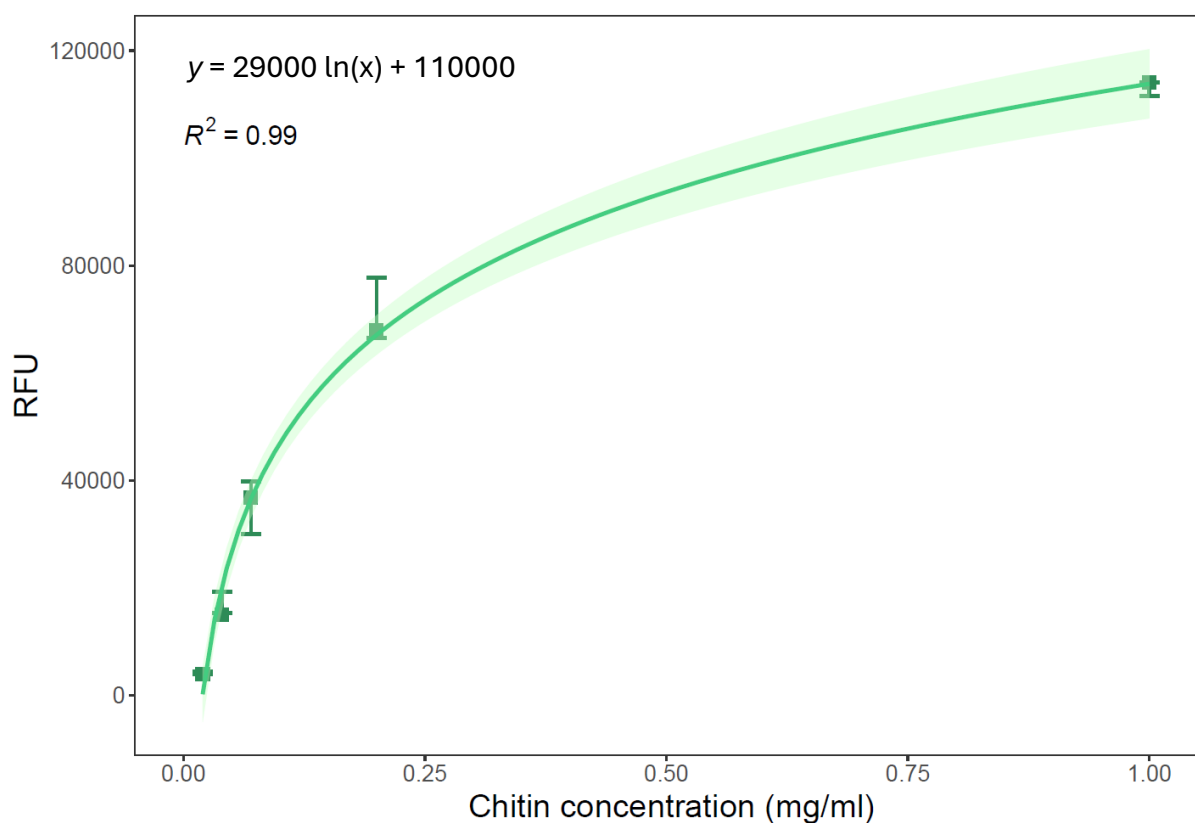


**Figure 3.20.** Standard curve of relative fluorescence units (RFU) of WGA-FITC bound to different concentrations of chitin. Excitation at 485 nm and emission at 520 nm were used. The line of best fit follows a logarithmic equation. Means and standard deviations (error bars) of technical triplicates are shown.

**Table 3.5.** Values for the Bayesian Information Criterion (BIC) and  $R^2$  for four potential regression models for the chitin standard curve.

| Regression model     | BIC      | $R^2$  |
|----------------------|----------|--------|
| Linear equation      | 341.9405 | 0.8300 |
| Quadratic equation   | 311.9755 | 0.9807 |
| Exponential equation | 345.5238 | 0.7841 |
| Logarithmic equation | 300.1475 | 0.9895 |

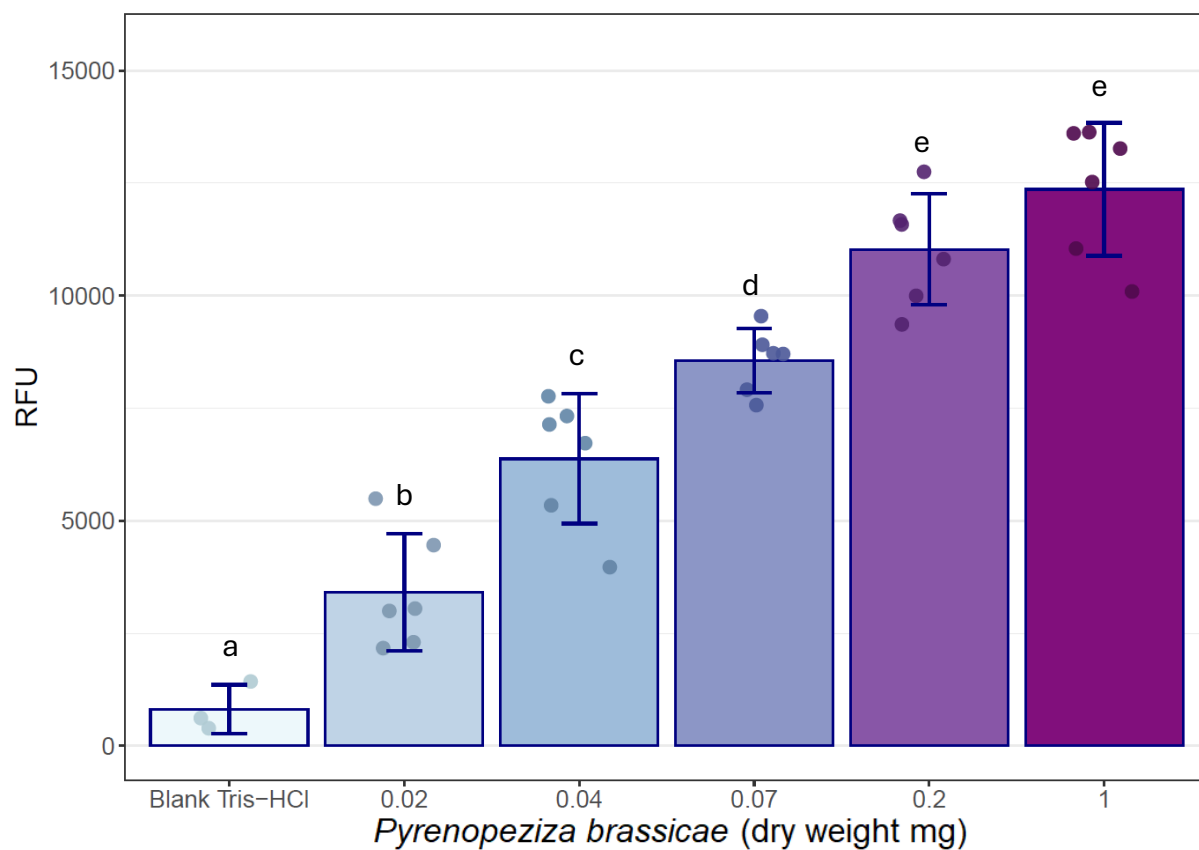


**Figure 3.21.** Comparison of the relative fluorescence units (RFU) of a *Pyrenopeziza brassicae* serial dilution bound to WGA-FITC (1 mg/ml), along a blank Tris-HCl control. Excitation at 485 nm and emission at 520 nm were used. Same alphabetic letter bars do not differ significantly (Kruskal-Wallis and Wilcoxon test). Means and standard deviations (error bars) of two biological replicates, each of them had three technical replicates.

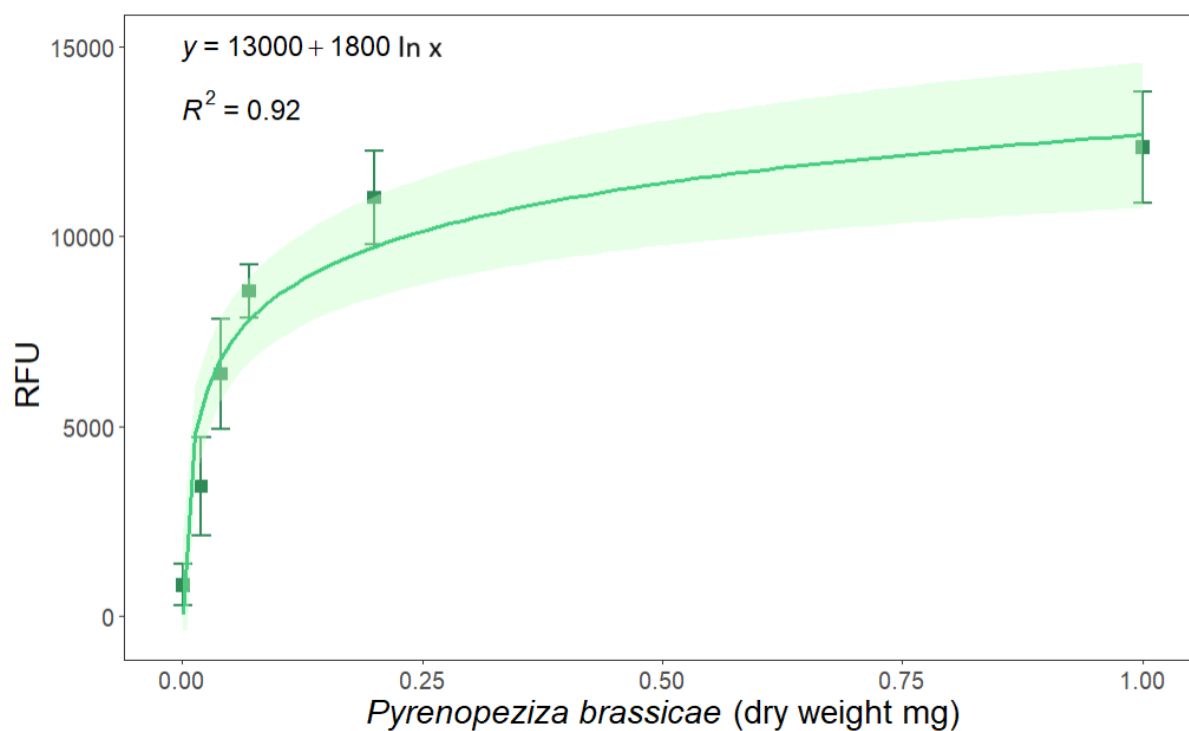


**Figure 3.22.** Standard curve of relative fluorescence units (RFU) of a *Pyrenopeziza brassicae* serial dilution bound to WGA-FITC (1 mg/ml), along a blank Tris-HCl control. Excitation at 485 nm and emission at 520 nm were used. The line of best fit follows a logarithmic equation associated with a saturation curve. Means and standard deviations (error bars) of two biological replicates, each of them had three technical replicates.

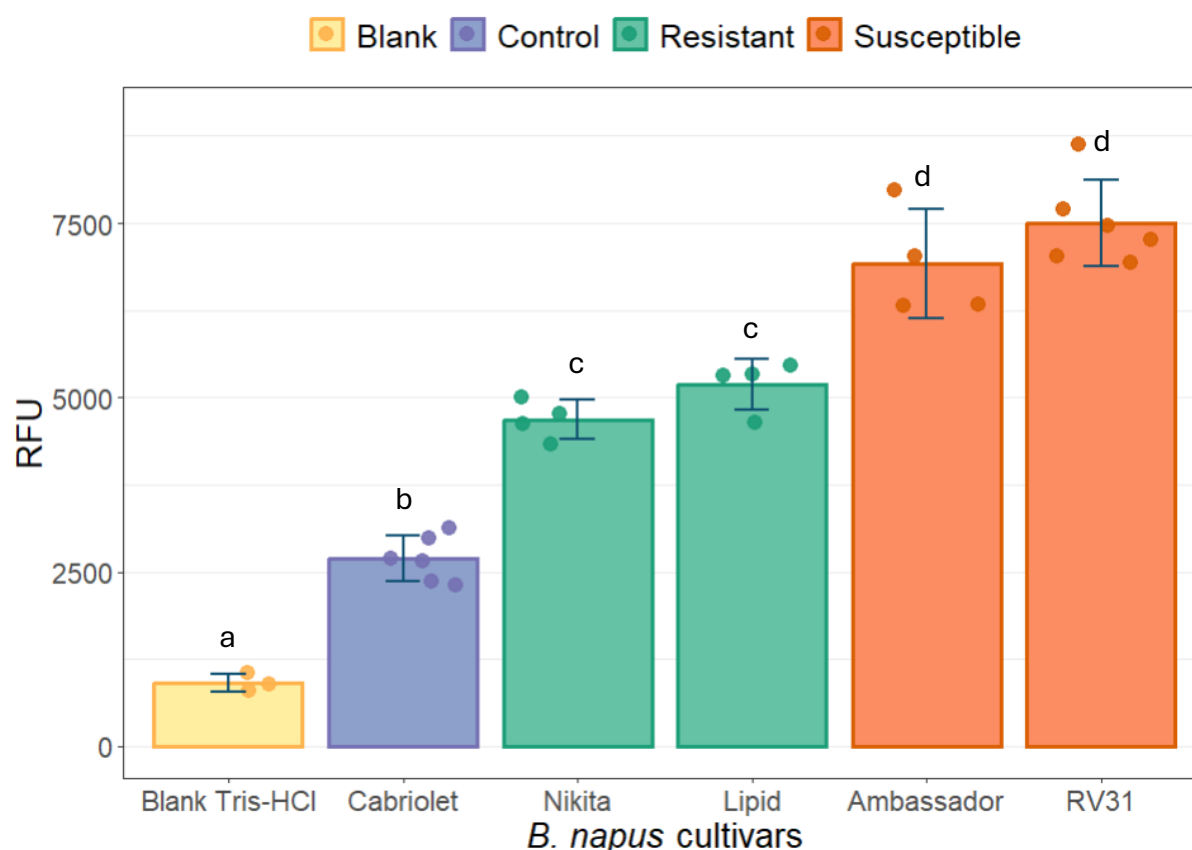


**Figure 3.23.** Comparison of the fluorescence (RFU) of *Brassica napus* cultivars after inoculation with a *Pyrenopeziza brassicae* population from the Thriplow fields, incubated with WGA-FITC (1mg/ml), measured at 485 nm excitation and 520 nm emissions. A non-inoculated *Brassica napus* control (cv. Cabriolet) was compared to susceptible cvs (Ambassador and RV31) and to resistant cvs (Lipid and Nikita), along with a blank Tris-HCl control. Same alphabetic letter bars do not differ significantly (ANOVA and Tukey's HSD tests). Means and standard deviations (error bars) of two biological replicates, each of them had three technical replicates.

**Table 3.6.** Percentage of *Pyrenopeziza brassicae* present in each *Brassica napus* sample.

| <i>B. napus</i><br>line/cultivar | RFU     | Adjusted<br>RFU | chitin<br>(mg/ml) | chitin<br>(mg) | <i>P. brassicae</i><br>dry weight (mg) | % Fungal<br>biomass |
|----------------------------------|---------|-----------------|-------------------|----------------|----------------------------------------|---------------------|
| Lipid                            | 5190.25 | 2501.75         | 0.025             | 0.0049         | 0.140                                  | 1.69 %              |
| Nikita                           | 4683.50 | 1995.00         | 0.024             | 0.0048         | 0.138                                  | 1.66 %              |
| Ambassador                       | 6917.50 | 4229.00         | 0.026             | 0.0052         | 0.149                                  | 1.80 %              |
| RV31                             | 7502.33 | 4813.83         | 0.027             | 0.0053         | 0.152                                  | 1.83 %              |
| Cabriolet                        | 2688.50 | 0.00            | 0.002             | 0.0004         | 0.012                                  | 0.02 %              |

### 3.3.5 Quantitative PCR of pathogen DNA

The Ct values (quantification cycles) for the standard dilutions corresponding to the *P. brassicae* DNA serial dilution were used to plot a standard curve of dilutions, as seen in Figure 3.24. The coefficient of determination has a value of  $R^2=0.99$ , indicating there is a strong linear relationship between the Ct and the logarithm of the concentration of *P. brassicae* DNA, which is essential for a reliable qPCR assay. The linear equation that defines the line of best fit ( $y=34-3.2x$ ) was used to interpolate the Ct values of the different *B. napus* cultivars assessed to obtain the quantified concentration of *P. brassicae* DNA that each sample contained. This data, presented in Figure 3.25, showed that *B. napus* cv. Cabriolet had the lowest presence of pathogen, as expected of the non-inoculated control. An ANOVA statistical analysis showed a significant difference in pathogen presence between the *B. napus* cultivars ( $P<0.001$ ). This was further confirmed with *post-hoc* Tukey's HSD for a multiple pairwise-comparison, that showed a discernible difference between the control (cv. Cabriolet) and the other cultivars ( $P<0.05$  for Nikita;  $P<0.001$  for Lipid, RV31 and Ambassador). This analysis also shows a significant difference between the resistant and susceptible cultivars as phenotypical groups ( $P<0.001$ ), since the pathogen's presence was significantly lower in resistant cvs Lipid and Nikita, in comparison to the susceptible cv. Ambassador and line RV31, that contained a higher concentration of *P. brassicae* DNA. Furthermore, this method is the only one of the quantitative phenotyping strategies considered that was able to statistically differentiate between cultivars within the same phenotypical trait, showing significant differences between the resistant cvs (Lipid and Nikita) and susceptible lines (Ambassador and RV31).

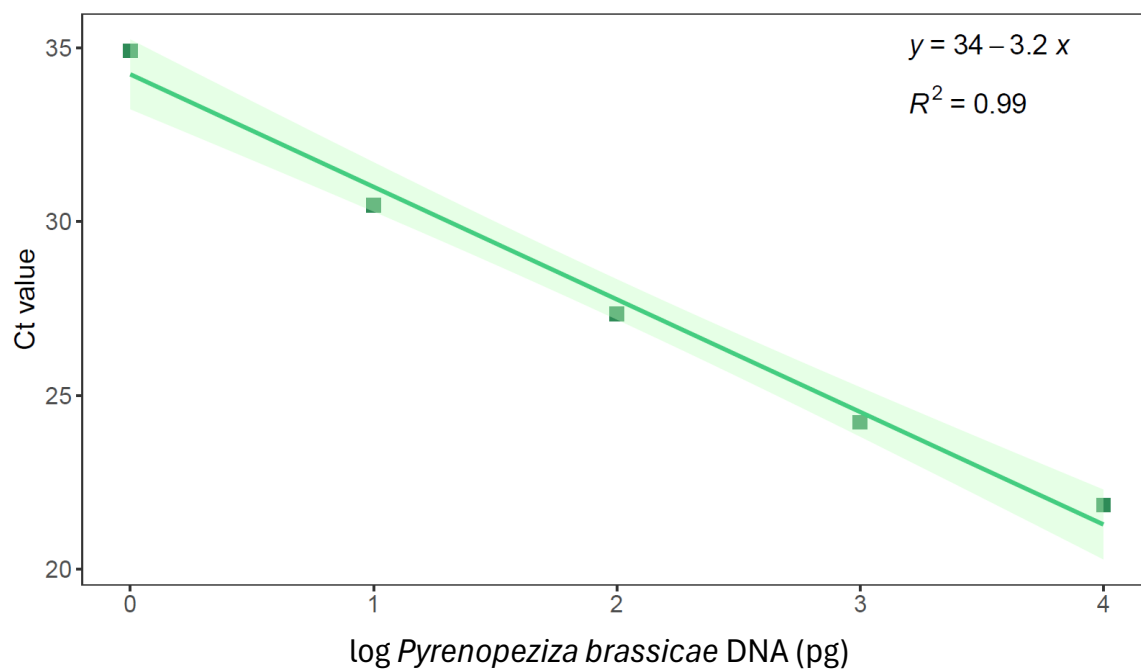


**Figure 3.24.** Standard curve establishing the relationship between the logarithm of the *Pyrenopeziza brassicae* DNA (pg) and the Ct value through qPCR. The line of best fit follows the linear equation. Means of technical triplicates are shown.

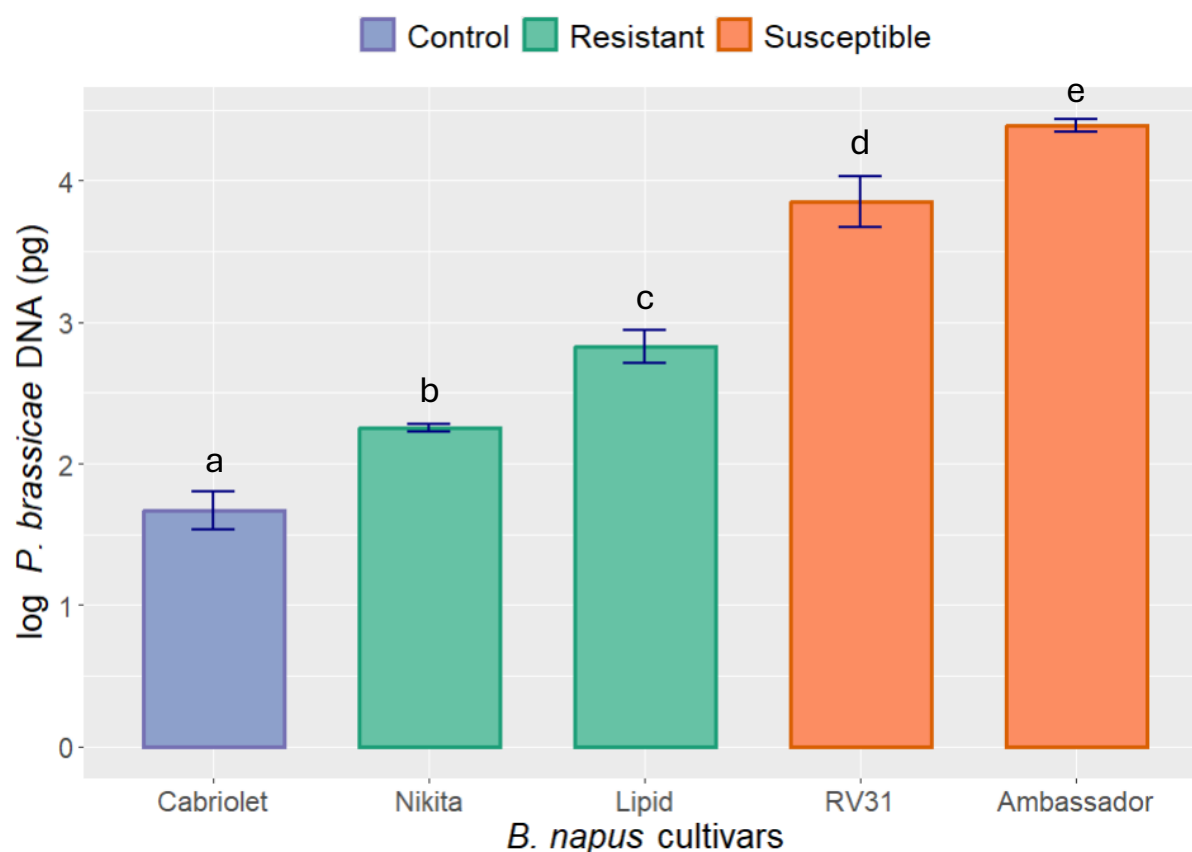


**Figure 3.25.** Comparison through qPCR of the concentration of DNA from a *Pyrenopeziza brassicae* population from Thriplow (Cambridgeshire, UK) used to inoculate the different *Brassica napus* cultivars. A non-inoculated *Brassica napus* control (cv. Cabriolet) was compared to susceptible lines (Ambassador and RV31) and to resistant cvs (Lipid and Nikita). Values with the same letters are not significantly different (ANOVA and Tukey's HSD). Means and standard deviations (error bars) of 2 biological replicates and technical triplicates.

### 3.3.6. Statistical comparisons of the different phenotyping strategies

A multiple regression analysis of variance was conducted to compare the different phenotyping methods (Table 3.7). Visual assessment only showed one statistically distinct group: the non-inoculated control cv. Cabriolet with highly susceptible cv. Ambassador. The alternative phenotyping strategies showed a higher number of distinct groups, suggesting that they are more appropriate to determine the severity of the LLS disease on oilseed rape.

Acervuli quantification showed three distinct groups amongst the selected *B. napus* cultivars (Table 3.7). It could statistically differentiate between the non-inoculated control (cv. Cabriolet) and the susceptible line RV31, and between the latter cultivar and resistant cvs Lipid and Nikita. This method did not show any significant difference between the control and the resistant lines, or between those resistant lines with one of the susceptible ones. These results show that this method is more discriminatory and a slight improvement over the traditional visual assessment; it is a slightly better indicator for quantifying disease severity.

Spore counting showed six distinct groups amongst the selected *B. napus* cultivars (Table 3.7). This method allowed for the differentiation of susceptible and resistant groups, although it cannot differentiate between the non-inoculated control and the resistant lines, and was unable to find any statistical distinction within the groups. A comparison between visual assessment and spore counting on the samples collected from the field trial (Figure 3.26) showed a significant positive correlation and a Pearson's correlation coefficient of  $R=0.82$ .

WAC assay showed eight distinct groups amongst the selected *B. napus* cultivars (Table 3.7). This phenotyping strategy could establish a clear and significant differentiation between cultivars, allowing for an accurate distinction between resistant and susceptible lines. It was also able to statistically distinguish the non-inoculated control from each of the phenotypical groups: resistant and susceptible cultivars. This was especially important when establishing a difference between the resistant cultivars and the control, much more similar in values than the susceptible cultivars. Nevertheless, it could not find a difference within the phenotypical groups, considering resistant cvs Lipid and Nikita as statistically equivalent. Likewise, there did not appear to be any statistically difference between the susceptible lines Ambassador and RV31.

The results from the WAC assay were compared to the data provided by the visual assessment method (Figure 3.27). A regression analysis showed a significant positive correlation using Pearson's correlation coefficient ( $R=0.89$ ), which supports the argument that WAC assay is a highly reliable method for determining the disease severity of LLS on *B. napus*, proving to be a more precise and accurate strategy than visual assessment.

qPCR showed ten distinct groups amongst the selected *B. napus* cultivars (Table 3.7). This phenotyping method has the largest distinction ratio with higher statistical difference between cultivars, allowing for an efficient discernment of susceptible and resistant lines. Furthermore, alike the WAC assay, it can statistically distinguish between each group and the non-inoculated control. Additionally, this was the only phenotyping method that was able to establish a significant difference within the phenotypical groups, showing statistical variation between the susceptible cv. Ambassador and line RV31, and resistant cvs Lipid and Nikita.

A comparison between the visual assessment and qPCR methods generated Figure 3.27, that showed positive significant correlation using Pearson's correlation coefficient ( $R=0.97$ ), the

highest R value for any of the quantitative phenotyping methods studied. These comparisons indicate that qPCR is a highly specific method, significantly more accurate, precise and reliable than visual assessment when assessing the severity of LLS on *B. napus*. When compared to the other quantitative phenotyping methods it proved the most efficient, providing the highest statistical differences between cultivars.

**Table 3.7.** Adjusted *P*-values using Tukey's HSD test to compare different phenotyping methods used to analyse the presence and effect of *Pyrenopeziza brassicae* on different *Brassica napus* cultivars. Susceptible (Ambassador, RV31) and resistant (Lipid, Nikita) *B. napus* lines were inoculated with *P. brassicae* and assessed along with a non-inoculated control (cv. Cabriolet).

|                      | Visual<br>assessment | Acervuli<br>quantification | Spore<br>counting | WAC assay   | qPCR        |
|----------------------|----------------------|----------------------------|-------------------|-------------|-------------|
| Cabriolet-Ambassador | 0.0481*              | 0.0816                     | 0.0011**          | < 0.0001*** | < 0.0001*** |
| Lipid-Ambassador     | 0.1058               | 0.0937                     | 0.0017**          | 0.0011**    | 0.0002***   |
| Nikita-Ambassador    | 0.0546               | 0.1176                     | 0.0045**          | < 0.0001*** | < 0.0001*** |
| RV31-Ambassador      | 0.9167               | 0.4221                     | 0.4525            | 0.4140      | 0.0267*     |
| Lipid-Cabriolet      | 0.9167               | 0.9999                     | 0.8815            | < 0.0001*** | 0.0009***   |
| Nikita-Cabriolet     | 0.9999               | 0.9991                     | 0.2157            | < 0.0001*** | 0.0192*     |
| RV31-Cabriolet       | 0.1058               | 0.0052**                   | 0.0029**          | < 0.0001*** | < 0.0001*** |
| Nikita-Lipid         | 0.9537               | 0.9999                     | 0.5338            | 0.6312      | 0.0203*     |
| RV31-Lipid           | 0.2445               | 0.0060***                  | 0.0051**          | < 0.0001*** | 0.0016**    |
| RV31-Nikita          | 0.1214               | 0.0074***                  | 0.0164*           | < 0.0001*** | 0.0002***   |
| Nº distinct groups   | 1                    | 3                          | 6                 | 8           | 10          |

Significant differences between cultivars are indicated by asterisks using ANOVA and Tukey's HSD test.  
 \*\*\**P*-value < 0.001; \*\* *P*-value < 0.01; \* *P*-value < 0.05

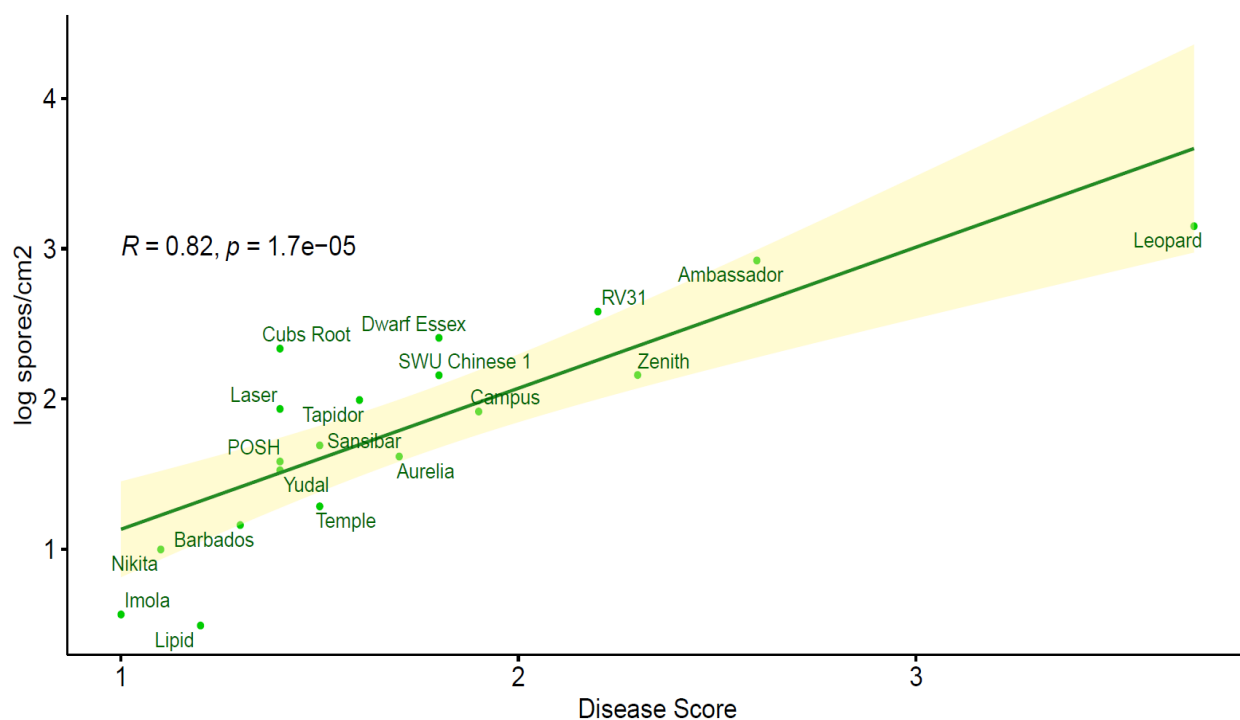


**Figure 3.26.** Regression analysis comparing visual assessment (disease score) and spore counting (log spores/cm<sup>2</sup>) as methods to phenotype *Brassica napus* cultivars inoculated with *Pyrenopeziza brassicae*, grown in a field trial (Thriplow, Cambridgeshire). The severity of the disease was assessed using spore counting. The line represents regression with associated Pearson's correlation. The yellow area represents the 95% confidence interval.

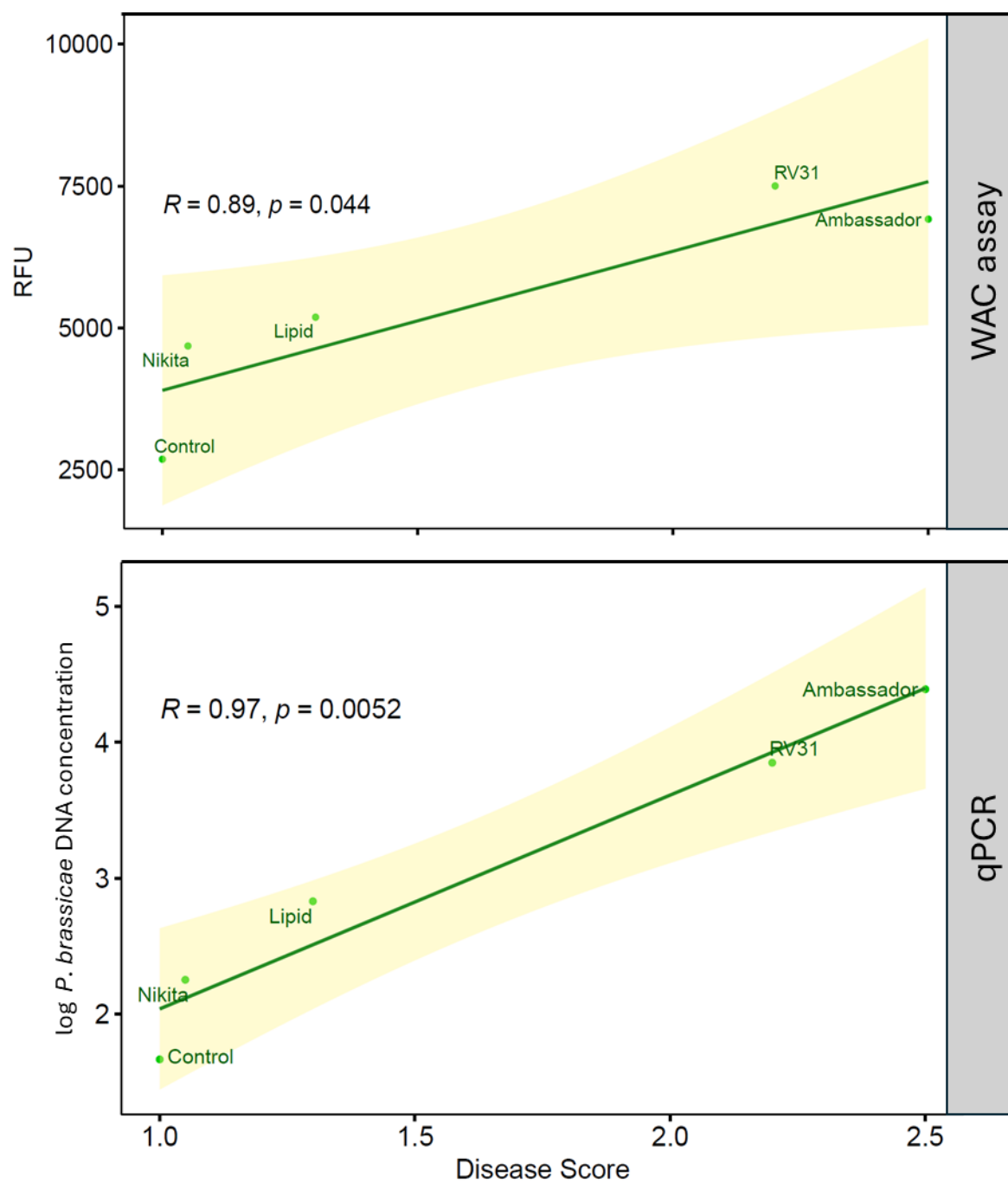


**Figure 3.27.** Regression analysis comparing visual assessment (disease score) and quantitative phenotyping methods WAC assay (RFU) and qPCR (log *P. brassicae* DNA concentration), set at a confidence level of 95% (yellow area).

### **3.4. Discussion**

The comparison between the traditional qualitative visual assessment and the quantitative phenotyping strategies has yielded significant results. All of them have demonstrated to be significantly more reliable and accurate, as well as repeatable, confirming the initial hypothesis that these methods are a better option than the scoring method currently used. Nevertheless, these methods have specific advantages and disadvantages that should be taken into consideration when choosing a method to determine the severity of the disease, depending on the specific requirements and conditions of the experiment in question.

Acervuli quantification performed better than the visual assessment. However, it was unable to significantly differentiate between cv. Ambassador and the resistant cultivars. *B. napus* leaves that were assigned the same disease score by visual assessment showed a greater difference in the number of acervuli counted. These results align with previously documented information about acervuli being misleading. Muthayil-Ali (2023) recorded that it was not uncommon to find *P. brassicae* spores in the water used to wash leaves that did not show any observable signs of sporulation.

Spore counting has been efficiently used in the past (Muthayil-Ali, 2023), as well as throughout this project frequently, tested along with the visual assessment. Results obtained in different environments and at different times have proven its accuracy and reliability, as seen comparing the results from the glasshouse experiment and the field trial, experiments conducted under different conditions. Spore counting could distinguish between resistant and susceptible *B. napus* cultivars, although it lacked strength when differentiating between the resistant cultivars and the non-inoculated controls. An additional compelling argument would be that this method uses readily available laboratory equipment, with no additional expense in chemicals.

In addition, both visual assessment and spore counting showed a significant positive correlation between different environmental conditions (field trial and glasshouse experiment), as seen in Figures 3.11 and 3.17, proving these are reliable methods to determine the severity of the LLS disease on *B. napus*. However, examining the results closely, it can be appreciated that spore counting generated a greater correlation coefficient and a more significant *p*-value ( $R=0.75$ ;  $P=0.00076$ ) than visual assessment ( $R=0.47$ ;  $P=0.037$ ). Furthermore, there was a better correlation between the results from the replicate blocks for spore counting ( $R=0.66$ ;  $P=1.3e-05$ ) than for visual assessment ( $R=0.53$ ;  $P=0.00082$ ). Likewise, the correlation between the data harvested from samples in March and in May is higher when phenotyping using the spore counting method ( $R=0.62$ ;  $P=4.6e-05$ ), than with visual assessment ( $R=0.59$ ;  $P=0.00014$ ). All these comparisons indicate that spore counting has an improved precision and higher reproducibility than visual assessment.

Despite the positive aspects this phenotyping methods offers, it does have disadvantages. It is more labour-intensive than merely assigning the disease scores, as it requires washing the leaves and calculating the spore concentration under the microscope. Nonetheless, the process of spore quantification could be sped up by using a microscope equipped with high-resolution cameras. Pictures of the haemocytometer loaded with the spore suspension could be analysed with image processing software that could automate this step, count the number of spores present in the haemocytometer and calculate the concentration of spores for each sample.

This is the first report on the use of the WAC assay to measure relative fungal biomass of *P. brassicae* in oilseed rape. It was demonstrated that the relationship between the fungal biomass and the fluorescence is directly proportional, and the percentage of pathogen's biomass in *B. napus* leaf tissue can be calculated (Table 3.6). The detection and quantification of fungal biomass *in planta* is rapid, sensitive and reliable (Ayliffe *et al.*, 2013). It uses readily available laboratory equipment, with relatively inexpensive chemicals that are less costly than the qPCR method (around £2.00 per sample for consumables).

One of the many positive aspects of this method is that it can be used prior to the development of visual symptoms, since it does not require the presence of sporulating structures on the leaf surface, nor does it need incubation time for the samples after harvesting. The samples can be freeze-dried right after collecting it and then can be stored at room temperature for long periods before they are processed. This allows for the detection of the pathogen at early stages of its life cycle. Additionally, this method allows the processing of large numbers of tissue at the same time because very little plant material is required, 50 mg of dried leaf tissue are sufficient for quantification.

However, because the WAC method detects the presence of chitin, it cannot discriminate between fungal species that might colonise the host plant; it therefore does not discriminate plant infections with multiple pathogens. This issue is not relevant for qPCR, which targets specific pathogen DNA markers, or spore counting because the morphology of the spores can be differentiated under the microscope.

When using the WAC assay, it was noticed that WGA-FITC bound to GlcNAc, not only present in the chitin wall of fungi but is also present in plant cell walls (Olszewski *et al.*, 2009). For this reason, there is a degree of background fluorescence when processing oilseed rape samples that does not correspond to the pathogen but to the host. Hence, it is crucial when designing a WAC assay experiment to include non-inoculated samples to establish a baseline of fluorescence associated to the sole plant material.

Finally, it is worth mentioning an issue that arose when attempting to follow the method described in the paper by Schnippenkoetter *et al.* (2021). Their method incubated the samples with 5  $\mu$ L of WGA-FITC at a concentration of 1  $\mu$ g/mL, however when those instructions were followed, the results were always inconclusive, with an insignificant level of baseline fluorescence. Reviewing papers that also used WGA-FITC in their methods, we considered the possibility that this concentration was not the correct one for this experiment. Ayliffe *et al.* (2013) and Kovács-Simon & Fones (2024) used volumes of WGA-FITC at a concentration of 1 mg/mL. When the experiment was adapted to fit this change, results became clearer, with expected fluorescence values for the controls, which extended to the samples. Samples that were expected to have less pathogen content had lower RFU values than samples with a higher presence of the pathogen.

The qPCR technique to quantify pathogen DNA proved to be the most efficient quantitative phenotyping method, offering the highest distinction between cultivars. This technique has been reported in multiple occasions, used to quantify *P. brassicae* DNA in the host and determine disease severity (Qi & Yang, 2007; Brunner *et al.*, 2009; Klosterman, 2011). The use of specific primers that target the ITS region reduces probability of detecting a pathogen different from the one of interest, proving that not only is it an accurate and reliable method to detect the presence

of *P. brassicae* colonising *B. napus*, but also precise in discriminating from other pathogens that might be infecting the host at the same time (Huang *et al.*, 2014). Furthermore, similar to the WAC assay, it does not require for the development of visual symptoms on the plants such as the development of sporulating structures, therefore it becomes a useful tool for early-stage detection and speeds-up the process as the incubation period after harvest can be skipped.

Despite the positive aspects associated with this technique, it also has pitfalls. It is more costly than the other methods, estimated at approximately £7.00 per sample for consumables. It is also more labour intensive because it requires an extra step of DNA extraction for every sample, *P. brassicae* mycelium DNA extraction for the standards, and the preparation of the qPCR. Additionally, unlike the WAC assay, qPCR could not be used to accurately calculate the percentage of pathogen's biomass in *B. napus* samples (Appendix 3). This is due to the amplification targeting a multi-copy gene, a portion of rDNA gene ITS, which results in an overestimate of pathogen DNA. Although the results are probably not dissimilar to those obtained by the WAC assay, the values are abnormally high and, therefore, cannot be directly compared to the chitin results from the WAC assay. Because the exact number of ITS copies in the *P. brassicae* genome are unknown, it is challenging to obtain an exact estimate of pathogen DNA. To improve accuracy, a single-copy gene should be used, which would provide strictly linear results that could be compared to the chitin data from the WAC assay.

Besides the analysis of the different quantitative phenotyping methods in comparison to visual assessment, some of the experimental work conducted showed a discrepancy with *B. napus* cv. Ambassador. Traditionally, it has been classified as moderately resistant to LLS, (AHDB Recommendation List), with a score of 7 out of 9 in the 1-9 disease severity scale, which means that 1-5% plants were affected with *P. brassicae* sporulation (Appendix 2), however this classification does not align with the results. Each of the five phenotyping methods used to determine disease severity has confirmed that Ambassador shows susceptibility to *P. brassicae*, both in glasshouse experiments and field trials. The conclusion drawn from these results coincides with previous research by Muthayil-Ali (2023) who also found Ambassador to be significantly less resistant to the pathogen than the literature stated. This suggests that the level of resistance to LLS for *B. napus* cv. Ambassador should be updated accordingly.

### **3.5. Conclusion**

Acervuli quantification, spore counting, WAC assay and qPCR provide a non-subjective, reliable and accurate assessment of the *P. brassicae*-*B. napus* pathosystem interaction. These quantitative phenotyping methods could significantly distinguish between different genotypes or cultivars and can be used to efficiently determine the severity of the light leaf spot disease on oilseed rape. They offer a more rigorous alternative to visual assessment that would reduce the probability of human error by solely relying on quantitative data.

The choice to use these techniques and which one should be selected should always be conditional to the type of experiment being conducted. These assays are not meant to substitute for experienced pathologists, who are able to visually assess large numbers of plant samples in a rapid way, however the quantitative methods do provide for more precise, accurate and repeatable alternatives for determining severity of the light leaf spot disease. They could be used

for studies that require a higher level of accuracy, for example for treatment differentiation or for identification of quantitative host resistance.

## **Chapter 4: Characterisation of the function of a BAHD acyltransferase gene potentially associated with susceptibility to *Pyrenopeziza brassicae***

### **4.1 Introduction**

#### **4.1.1. Transcriptomics**

Transcriptomics is the comprehensive analysis of the transcriptome, formed by the collection of all ribonucleic acid (RNA) produced by the genome of a sample at a given time. This provides insight into gene function, expression patterns and regulation. Associative transcriptomics (AT) is an advanced genomics approach to gene mapping that integrates the study of genetic variation in gene sequence and gene expression, examining Single Nucleotide Polymorphisms (SNPs) and Gene Expression Markers (GEMs). This enables the identification of trait-associated loci, such as pathogen resistance loci, by using genomic and transcriptomic sequences from diversity collections instead of biparental mapping populations (Bartoli & Roux, 2017). Small or intermediate-sized populations are often unable to discern sufficient genetic diversity, which reduces the mapping resolution and statistical power (Mackay *et al.*, 2009). Furthermore, traditional biparental mapping populations may not be a good portrayal of the full spectrum of natural variation in a specific species and might limit findings to specific germplasm or environmental conditions (Mackay *et al.*, 2019). For this reason, AT is favoured using wide diversity collections from natural populations, taking advantage of the accumulation of recombination events, for a better representation of diverse populations, which leads to an improved way of associating genetic polymorphisms with phenotypes of interest (Fell *et al.*, 2023). This is especially useful in crops with polyploid genomes such as *Brassica* vegetables, with complex linkage disequilibrium patterns and high degree of gene redundancy that hinders gene-trait association and accurate marker placement (Harper *et al.*, 2012).

The development of AT platforms can be used for crop improvement, since the identification of genetic markers associated with beneficial alleles can accelerate breeding programs through rapid marker-assisted selection. This approach has been used in polyploid species *Brassica napus* (Harper *et al.*, 2012) and *Brassica juncea* to identify traits of agronomic importance (Harper *et al.*, 2020). It has also been used specifically in *B. napus* to study the genetics behind seed glucosinolate content (Lu *et al.*, 2014) and new sources of resistance against fungal pathogens, which led to the identification of potential *R* genes associated with resistance against *Plasmodiophora brassicae* clubroot (Wu *et al.*, 2016) and resistance loci against *Sclerotinia sclerotiorum* stem rot (Hejna *et al.*, 2019) and *Pyrenopeziza brassicae* (Fell *et al.*, 2023).

Fell *et al.* (2023) screened a diversity set formed by 195 *B. napus* accessions for resistance against *P. brassicae* in an attempt to characterise genomic regions associated with quantitative disease resistance (QDR). This research showed that all resistance against this pathogen was associated with QDR rather than *R* gene-mediated resistance. Further analysis using AT and GWAS (Genome-Wide Association Studies) revealed four associated trait loci and eight GEMs that were significantly associated with QDR in regions showing linkage disequilibrium. The expression of seven of the GEMs was positively correlated with resistance against the pathogen and might contribute partially to host defence. Identified genes included Phosphatidylinositol-

specific phospholipase C4 (*PLC4*), cinnamate-4-hydroxylase (*C4H*), *SNF1* kinase homolog 10 (*KIN10*), universal stress protein (*USP*) and  $\beta$ -*adaptin*, some of which had previously been associated with host-pathogen interactions. The gene expression of some of these GEMs was further studied by Muthayil-Ali (2023). Susceptible and resistant *B. napus* cultivars were inoculated with *P. brassicae* at different inoculation time points. RNA was extracted from inoculated leaves and cDNA was synthesised from it, which was analysed using qPCR, to study the gene expression of the genes identified by Fell *et al.* (2023) at different stages of infection.

The *PLC4* gene, encoding for a phosphatidylinositol-specific phospholipase C4, showed a higher expression in resistant *B. napus* cultivars than in susceptible ones after *P. brassicae* inoculation (Muthayil-Ali, 2023). This gene had previously been associated with resistance against pathogens and hypersensitive response. Its expression in tomatoes contributed to *R* gene-mediated resistance of the apoplastic fungal pathogen *Cladosporium fulvum* (Vossen *et al.* 2010).

The *C4H* gene was significantly more expressed in *B. napus* resistant cultivars and seemed to reduce the branching of *P. brassicae* in the subcuticular space at 4 days post-inoculation (DPI). This gene had previously been associated with host pathogen interactions in *B. napus* with *S. sclerotiorum* and *Leptosphaeria maculans* (Wu *et al.*, 2016; Becker *et al.*, 2017). It encodes for enzyme cinnamate-4-hydroxylase, whose product (4-coumarate) is involved in the biosynthesis of phenylpropanoid, flavonoids and lignins. Targeted mutations of these gene in *Arabidopsis thaliana* resulted in developmental defects (El Houari *et al.*, 2021).

Moreover, the genes for *USP* and  $\beta$ -*adaptin* were significantly expressed in the resistant *B. napus* cultivars at 1 DPI, which plays a role in limiting the germination of the *P. brassicae* spores (Muthayil-Ali, 2023). Regarding the *KIN10* gene, its expression was not further studied but it has been previously identified as a central regulator in response to energy deprivation, which facilitates host survival under stress conditions (Baena-Gonzalez *et al.*, 2007), such as biotic stress. It has been associated with resistance against the clubroot pathogen in *A. thaliana* (Chen *et al.*, 2021).

Out of the eight GEMs identified, one of them was correlated with susceptibility to *P. brassicae*: the *BnHAT1* gene (*BnaA04g20860D*) in chromosome A04, an acyltransferase with an HXXXD motif belonging to a BAHD supergene family. The expression of this gene at different inoculation time points was studied in different cultivars, showing that its expression was significantly up-regulated after *P. brassicae* inoculation in susceptible *B. napus* cultivars but not expressed in the resistant ones (Muthayil-Ali, 2023). These results suggested its potential as a candidate gene for susceptibility to *P. brassicae*, which will be further studied in this chapter to characterise its function.

#### **4.1.2. BAHD acyltransferase family**

*BnHAT1*, the candidate gene, belongs to a super-gene family of enzymes known as the BAHD-acyltransferases, primarily found in the plant kingdom, but also present in algae, bacteria and fungi. Fungal and algal species have shown to only have 1-5 copies of BAHD-encoding genes per genome, whereas diploid angiosperms typically contain 50-200 copies in their genome (Kruse *et al.*, 2022). This research suggested that this drastic expansion of the BAHD super-family is

associated with the adaptation of these organisms to new land environments and BAHD acyltransferases had an important role in adjusting to terrestrial life.

This enzyme super-family gets its name from the first letter of each of the first biochemically characterised enzymes that belonged to this group: benzyl alcohol O-acyltransferase (BEAT), anthocyanin O-hydroxycinnamoyltransferase (AHCT), N-hydroxycinnamoyl/benzoyltransferase (HCBT) and 4-O-acetyltransferase (DAT) (St Pierre & De Luca, 2000). To this date, the BAHD acyltransferases that have been identified are all monomeric enzymes with a molecular mass that ranges from 48 to 55 kD, composed by an average of approximately 445 amino acids, and located in the cytosol since none of them presented transit peptides or any other sequences that would suggest secretion or location in any organelle (Suzuki *et al.*, 2004). Phylogenetic analysis has been used to categorise them into eight clades, classified either by their substrates or by the conditions that lead to the activation of the encoding-genes (D'Auria, 2006; Kruse *et al.*, 2022).

BAHD-acyltransferase enzymes are involved in the acylation of various plant secondary metabolites, playing an important role in biosynthetic pathways. They are coenzyme A (CoA)-dependant enzymes that transfer acyl groups of acyl-activated CoA thioesters to acceptor molecules. Although the catalytic versatility of individual enzymes varies greatly, they can typically acylate a wide variety of substrates; thus, their substrate selectivity is considered low (D'Auria, 2006). Enzyme-catalysed acylation can alter molecule polarity, volatility, chemical stability and even the biological activity of plant metabolites. Some relevant catalytic products include small volatile esters, amides and modified anthocyanins, as well as important defence compounds such as phytoalexins. *In planta*, the relative availability and abundance of substrates can determine the enzymatic products that are formed.

BAHD acyltransferase family members share two highly conserved domains, two strings of consecutive amino acids: motifs HXXXD and DFGWG. Multiple independent experiments showed that site-directed mutagenesis that generated deletions or significant modifications of one or both motifs resulted in a substantial reduction in enzyme activity (Xu *et al.*, 2023). The study of vinorine synthase, the first member of the BAHD acyltransferase super-family whose crystal structure was obtained and analysed (Ma *et al.*, 2005), largely contributed to the understanding of the function of these conserved motifs.

The HXXXD motif is typically located at the centre of the solvent reaction channel, which runs through the enzyme, allowing substrates, co-substrates and donors to bind independently, with the motif being accessible from both sides. The histidine residue (H) works as a general base during catalysis, whereas the aspartic acid residue (D), which points away from the active site of the enzyme, appears to play a structural role (Ma *et al.*, 2005). The function of the residues that contribute to this motif has been further studied. A site-specific mutation affecting the histidine residue of barley's agmatine coumaroyl transferase (HvAC) resulted in severe loss of enzymatic activity (Yamane *et al.*, 2020). Similarly, Morales-Quintana *et al.* (2013) studied *Vasconcellea pubescens* alcohol acyltransferase (VpAAT1) and reported that induced mutagenesis of H166 and D170 in the HXXXD motif resulted in loss of activity. Moreover, when studying the HXXXD motif of *Taxus cuspidata* 10-deacetylbaicatin III-10 $\beta$ -O-acetyltransferase (TcDBAT), Wang *et al.* (2021) concluded that H162 and D166, located in the catalytic pocket of the enzyme, were important for enzymatic activity. Results from these studies confirm the active involvement of histidine and aspartic acid in catalytic activity.

The DFGWG motif, found around the carboxyl terminus of the proteins, has been previously associated with CoA binding (D'Auria, 2006), although some studies suggested that, since their location is far from the active site, it is more likely that it has a structural function, maintaining the integrity of the CoA binding site (Ma *et al.*, 2005). Mutations that affected this motif showed a significant reduction in binding capacity to multiple substrates, which appeared to be related to the differences in electrostatic potential on the surfaces of the protein (Morales-Quintana *et al.*, 2015). This suggested that the DFGWG motif contributes to maintaining the structural integrity of the solvent channel, which is fundamental for catalysis.

Since the first major review of the BAHD-acyltransferases (D'Auria, 2006), more than 2,500 articles studying members of this super-family have been published. Many have identified enzymes involved in the biosynthesis of secondary metabolites that play a fundamental role in processes such as plant growth, reproduction or defence against biotic and abiotic stresses (Mogue *et al.*, 2023). Additionally, the crystal structure of approximately 25 BAHD members have been reported (Xu *et al.*, 2023), which contributed to a better understanding of the conserved motifs and their involvement in catalytic mechanisms. Some studies were also able to elucidate the specific substrates and products of each identified enzyme and characterise their functions. These include enzymes involved in the biosynthetic pathways of anthocyanins (Unno *et al.*, 2007), major chlorogenic acids found in coffee (Lallemand *et al.*, 2012) and phenylpropanoid metabolism (Levsh *et al.*, 2016; Chiang *et al.*, 2018), amongst others.

#### **4.1.3. TILLING**

Targeting Induced Local Lesions in Genomes (TILLING) is a powerful 'reverse genetics' method that can be used for the study and characterisation of genes of interest. TILLING is a powerful and high-throughput molecular biology tool for functional analysis of genes. It allows for the correlation between specific induced genetic mutations and their associated phenotype, aiding in the functional characterisation of genes (Colbert *et al.*, 2001; Comai *et al.*, 2004).

The TILLING method aims to induce random mutations in the genome through chemical mutagenesis. The selection of adequate mutagens and its dose are crucial, since it needs to generate a population with a high mutation frequency above a certain threshold, but not too high that it will lead to a high degree of lethality or sterility of the resulting plants (Szurman-Zubrzycka *et al.*, 2023). The most common ones used are ethylmethanesulphonate (EMS), that causes G/C to A/T transitions, and sodium azide, that causes A/G to T/C changes. These genetic modifications can result in various mutation types (silent, missense, nonsense, splice site...). Chemical mutagenesis is used in combination with high-throughput DNA screening for identification of specific single-base mutations, also known as point mutations, in a gene of interest within a large population (Henikoff *et al.*, 2004). As a result of the screening, mutant lines that contain interesting mutations are selected and seeds are produced, which can then be sown, grown and studied. By comparing their genotype and phenotype, the function of the mutated genes can be characterised.

One of the biggest advantages of this technique is that it does not require genetic transformation or transgenic modifications; thus, the mutants generated are not classified as genetically modified organisms (GMOs) and can be directly used in breeding programs without any of the

regulatory restrictions that are common in many countries for GMO-labelled material (Henikoff *et al.*, 2004). Nevertheless, most mutant lines cannot be used directly in breeding programs. Chemical mutagenesis is a random process that generates mutations across the genome, not only in the sites of interest. This results in TILLING lines that carry background mutations. Several independent studies have reported mutant lines that not only presented a modified gene of interest but also carried several thousand additional SNPs in their genome (Enders *et al.*, 2015; Szurman-Zubrzycka *et al.*, 2018). Even though this appears to be a significant issue, it has also been noted that most of those background mutations were found in noncoding sequences of the genome, or caused mutations that were either silent or their amino acid substitution did not affect the protein activity; thus, background mutations had a low probability of having a significant impact on the phenotype (Szurman-Zubrzycka *et al.*, 2023). Despite them not being relevant alterations of the genetic material, it is convenient to remove them, which is typically done by backcrossing the mutant to the parent variety, reducing the amount of background mutations by half. For a TILLING mutant line to be integrated into a breeding program, it is recommended that it goes through a series of backcrosses, typically 1 to 2 are needed to restore the genetic background, which can be very time-consuming.

The TILLING method was first developed in *A. thaliana* by Colbert *et al.* (2001), and since then more than 3,000 mutations have been identified in *Arabidopsis* populations using this procedure (Henikoff *et al.*, 2004). TILLING has been in continuous modification and substantial improvement, making it a more robust technique, especially regarding the identification of the induced mutations. The development of NGS has led to a variation of the original method known as ‘TILLING by Sequencing’, which allows for a faster detection of genetic mutations (Tsai *et al.*, 2011). This addition, however, is not always implemented since it is limited to species with an available reference genome and it is significantly more expensive than screening without NGS (Szurman-Zubrzycka *et al.*, 2023). Furthermore, in the 20 years since its development it has been successfully transferred to multiple other plants such as maize (Till *et al.*, 2004), wheat and rice (Tsai *et al.*, 2011) and *Brassica* vegetables (Stephenson *et al.*, 2010; Gilchrist *et al.*, 2013); amongst others (Szurman-Zubrzycka *et al.*, 2023). TILLING platforms can be applied to any organism regardless of the size of their genome or their ploidy, which is a significant challenge when working with transgenic techniques because of genetic redundancy. The presence of multiple homoeologous copies of each gene in polyploids can lead to a lower expression of a single transgene insertion, often the result of the masking effect of other copies of the allele that have not been altered (Schaart *et al.*, 2021).

#### **4.1.4. Aims and objectives**

QDR is predominant in natural crop populations and provides more durable resistance to different types of biotic stress than *R* gene-mediated resistance; however, it is not frequently used in breeding programs because it has not been as widely studied due to their higher complexity and poorly characterised QDR loci. Their partial contribution to resistance makes them more challenging to track because the trait does not commonly appear as an “all-or-nothing” inheritance but rather as a continuous range of resistance levels, making it difficult to set strict thresholds for selection (Nelson *et al.*, 2018). Despite its complexity, expanding the understanding of the molecular basis of QDR would improve the chances of introducing durable

disease resistance into crop improvement programs for the development of resistant cultivars, which would lead to a reduction in the use of fungicides.

The study and characterisation of the potential candidate gene for susceptibility to *P. brassicae* discovered in the *B. napus* genome through associative transcriptomics (Fell *et al.*, 2023) could improve the current knowledge on the susceptibility mechanisms to this pathogen. The identification of molecular markers for susceptibility could aid in the development of marker-assisted selection in breeding programs, allowing breeders and farmers to favour those cultivars that do not present this potential susceptibility gene. It could also be eliminated from the genome through selective breeding or gene editing to obtain improved varieties with a significantly lower susceptibility to *P. brassicae*.

The aim of this chapter was to characterise the function of the *BnHAT1* gene, that was associated with susceptibility to *P. brassicae*. The separate objectives contained in this chapter were:

1. To generate a TILLING mutation population targeting the *BnHAT1* gene.
2. To generate a segregating population from the TILLING mutant lines and compare their genotype and their phenotype after inoculation with *P. brassicae*.
3. To study transcriptomics of an infection series and understand possible metabolic signatures through an RNA-seq analysis.
4. To study three-dimensional structural models of the protein encoded by the *BnHAT1* gene and the changes caused by the mutation in TILLING mutant line 2 (D167N).

## **4.2 Materials and methods**

### **4.2.1. Generation and screening of TILLING mutant lines**

Using *B. napus* cv. Cabriolet as the genetic background, the TILLING population was generated at the John Innes Centre (Wells *et al.*, 2013). The TILLING process starts with mutagenesis to create a large population containing many thousands of random mutations per plant. The customary procedure involves treating the seeds with the chemical mutagen EMS (ethylmethanesulphonate) that causes G/C to A/T transitions. The mutagenised seeds produced M1 plants that were selfed to produce the M2 generation. DNA samples are taken from M2 for mutational screening, whilst their seeds are harvested, catalogued and stored in a seed bank. DNA samples are 8-fold pooled and amplified using gene-specific primers that targeted the *BnHAT1* gene. In the presence of a mutant, the amplification products when heated and cooled will form mismatched heteroduplexes between the wild type and mutant DNA. To enable identification of the point mutations the re-annealed amplification products are incubated with a plant endonuclease called CEL I (Oleykowski *et al.*, 1998), which preferentially cleaves at sites of heteroduplex mismatches that occur between wild-type and mutant DNA. Cleavage products are run on a Fragment Analyzer™ (Agilent, UK) which is an automated fluorescence-based Capillary Electrophoresis System that enables sensitive and high-resolution separation of DNA. Wells containing Cel I digested fragments are identified as containing a mutant allele within the pool. Upon detection of a mutation in the pooled DNA, PCR products amplified from the individual DNA samples that make up the pool are sequenced to identify the specific plant carrying the mutation. This rapid screening procedure determines the location of a mutation. The identified M2 individuals that contain the mutation of interest were selfed to generate M3 seeds, which can be grown for genotypic and phenotypic analysis. This process is shown in Figure 4.1.

### **4.2.2. Plant growth of TILLING mutant lines**

#### **4.2.2.1. Growth of M3 seeds**

The resulting M3 seeds from TILLING mutant lines that presented mutations of interest were germinated and grown in glasshouse facilities at Rothamsted Research, following the protocol described in Chapter 2.2 (Growing *Brassica napus* under controlled environment conditions), at 20°C in light ( $250 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for 16 hours and 18°C in dark for 8 hours, with a 60% relative humidity. A minimum of three biological replicates were grown per mutant line. Alongside these, *B. napus* cv. Cabriolet wild type (WT) individuals were grown as a control.

#### **4.2.2.2. Breeding of the M4 generation through selfing**

All plants of each TILLING mutant line from the M3 generation were selfed by covering each plant with a perforated bread bag. This resulted in a progeny corresponding to M4 generation (Figure 4.2), whose seeds were threshed and sown in the glasshouse of the University of Hertfordshire as described in Chapter 2.2 (Growing *Brassica napus* under controlled environment conditions). Each seed from each line of the M4 generation was a different biological replicate. Several replicates of cv. Cabriolet were also sown as WT control. However, they were only grown until first true leaves appeared and harvested with the cotyledons for DNA extraction.

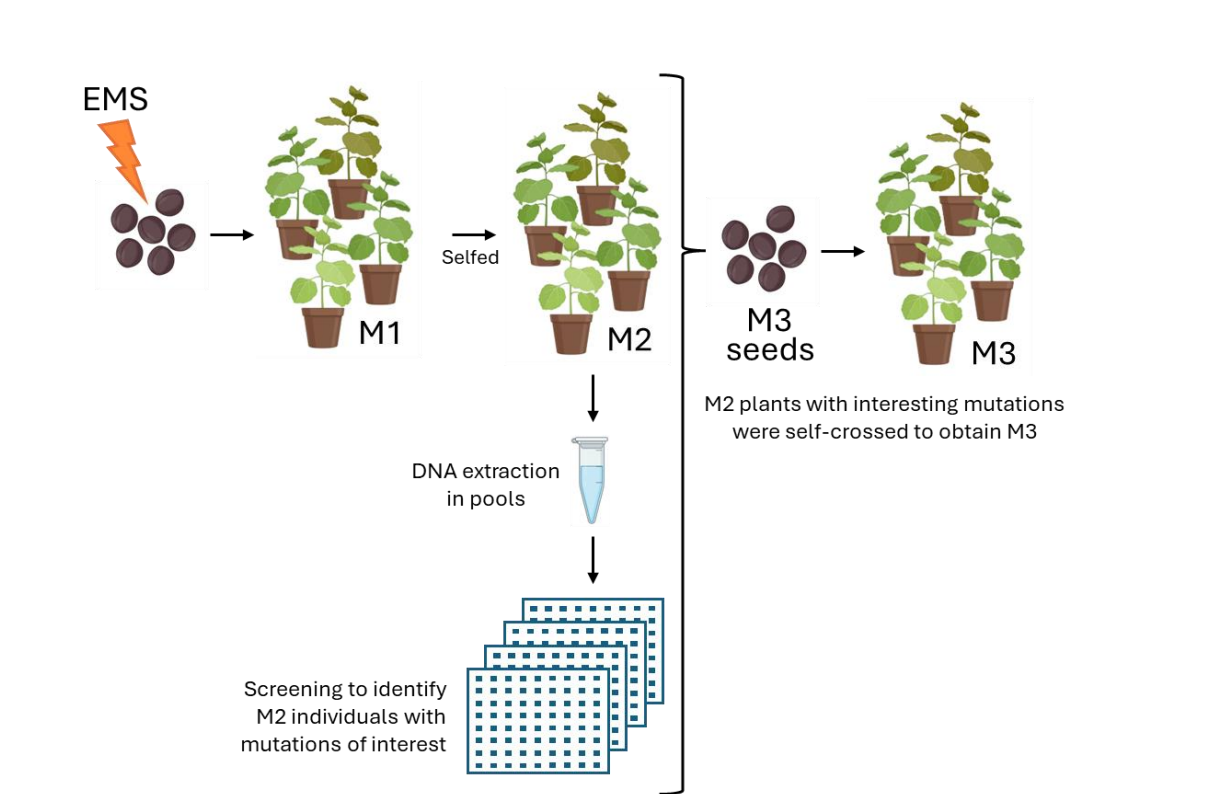


**Figure 4.1.** Outline of the steps for typical TILLING assays. The first is chemical mutagenesis of the seeds using EMS, resulting in mutated M1 plants that are selfed to obtain the M2 generation. DNA is extracted from M2 individuals for screening in pools using PCR to identify mutations in genes of interest, using specific primers. Selected M2 individuals are propagated through selfing, resulting in M3 seeds that will be used for phenotypic characterisation of the genes affected by mutations. (Created with BioRender.com)

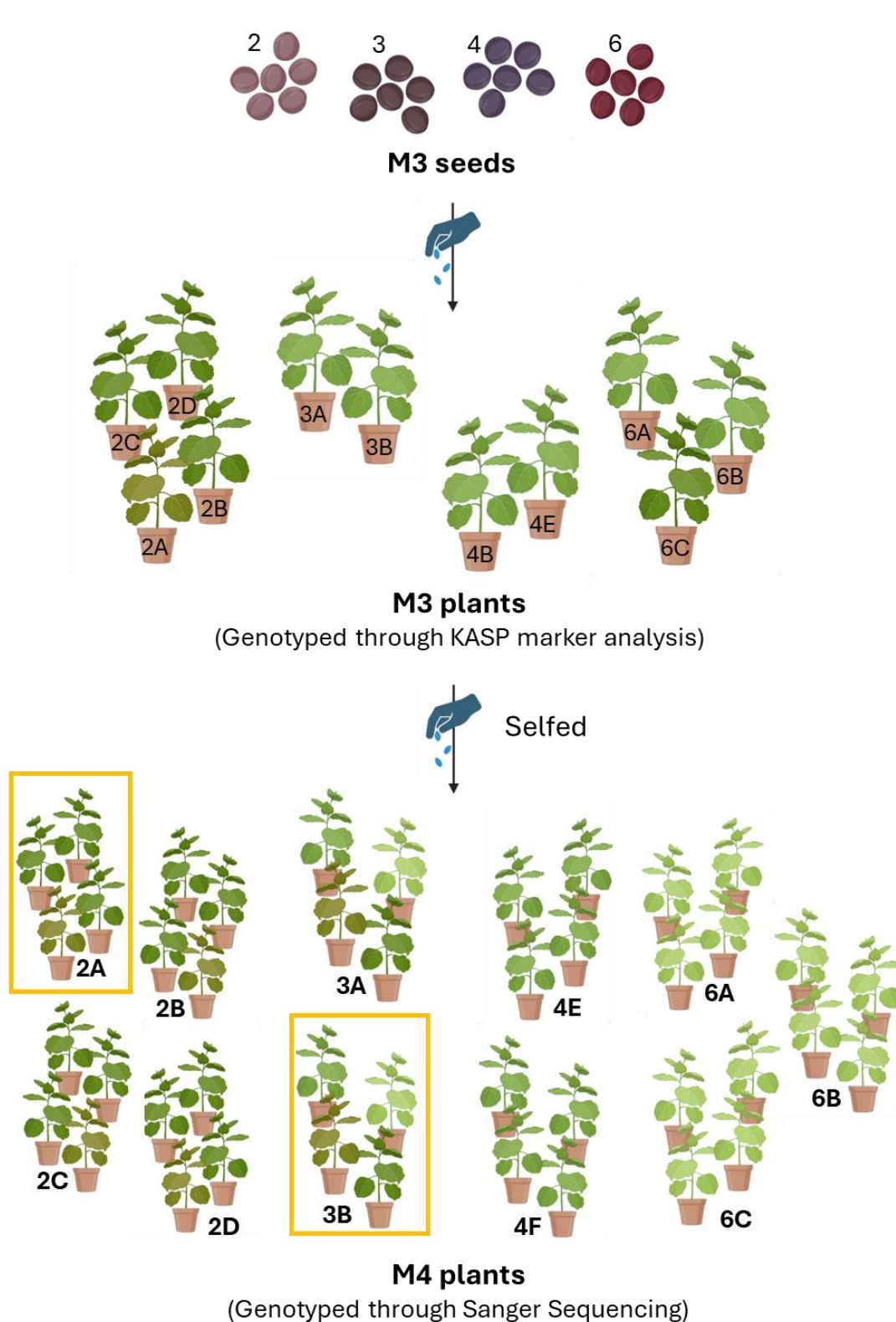


**Figure 4.2.** Breeding scheme of TILLING mutant lines. The John Innes Centre produced the mutated seeds from generation M3, which were grown in glasshouse conditions in Rothamsted Research and genotyped using KASP marker analysis. This was followed by selfing of each of the TILLING mutant plants, resulting in generation M4, whose seeds were grown in glasshouse conditions at the University of Hertfordshire and genotyped by sequencing. TILLING mutant lines highlighted in yellow were further studied (Created with BioRender.com).

#### 4.2.2.3. Plant growth and pathogen inoculation of M4 seedlings

TILLING mutant lines 2A, which was homozygous for the SNP mutated allele (D167N), and 3B, which was heterozygous (W172X), were selected for further study of susceptibility to *P. brassicae*. For the experiment with 2A, ten biological replicates of this line were grown against ten of WT *B. napus* cv. Cabriolet in CE cabinets. For the experiment with 3B, 42 seedlings of the TILLING mutant line were grown in glasshouse conditions at the University of Hertfordshire.

All the plants were grown following the protocol described in Chapter 2.2 (Growing *Brassica napus* under controlled environment conditions). The plants were inoculated with a *P. brassicae* population that originated from glasshouse experiments at Rothamsted Research (Muthayil-Ali, 2023), prepared as described in Chapter 3.2.1 (*Pyrenopeziza brassicae* populations and isolates). The inoculation was done as described in Chapter 2.3. (*Pyrenopeziza brassicae* spray-inoculation method) and the harvested plants were assessed using two different phenotyping strategies: visual assessment and spore counting, as described in Chapters 3.2.3 and 3.2.5, respectively.

#### **4.2.3. Genotyping by KASP marker analysis**

Leaf samples were taken from each plant. The leaves were freeze-dried for 48 hours and the DNA extracted following the protocol described in Chapter 2.5 (DNA extraction). KASP™ (Kompetitive Allele Specific PCR) marker analysis was used to determine whether the mutations of interest in each individual were homozygous or heterozygous. This is a fluorescence-based genotyping variant of PCR that allows for SNP genotyping due to allele-specific primers labelled with different fluorescent dyes (Semagn *et al.*, 2014). This technique requires two forward primers: one of them targets the WT nucleotide and is labelled with fluorophore fluorescein amidite (FAM), whilst the other primer targets the SNP mutation and is labelled with fluorophore hexachlorofluorescein (HEX). Only one reverse primer is needed, which is unlabelled, common to both alleles. For the primer design, sequences spanning 50 nucleotides up and downstream of each specific SNP were considered. It was important to differentiate these sequences from similar ones that can be found across the *B. napus* genome; hence, to ensure that the KASP primers only bound to the specific sequences of the TILLING mutant lines, an anchor was included in the primer: a nucleotide that is only present in the sequence of interest and absent in all the others. The KASP primers for each TILLING mutant line (Table 4.1) were synthesised by LGC Group (UK). They all produced amplicons of approximately 50 bp.

The protocol for KASP™ genotyping provided by the manufacturer (LGC Group, UK) was followed using the primers designed and KASP Master Mix, a buffer solution containing Taq polymerase for the PCR reaction and a FAM and HEX specific FRET (Förster Resonance Energy Transfer) cassette, which is a molecular tool that contains donor and acceptor dyes, designed for sensitive detection of molecular interactions. Individual reaction volumes were set in a 96-well plate in technical triplicates, each containing KASP Master Mix, DNA and their corresponding primers, in the concentrations established in the provided protocol. Three non-template controls (NTCs) were included in the genotyping plate, negative controls to improve the confidence in the validity of the results.

**Table 4.1.** Primers for KASP marker analysis designed for the specific SNPs of each TILLING mutant line.

| TILLING mutant | Targeted sequence*    | Forward primer (WT-FAM)   | Forward primer (SNP-HEX)   | Reverse common primer (unlabelled) |
|----------------|-----------------------|---------------------------|----------------------------|------------------------------------|
| 2              | <C>GTTACA(G/A)ACGGAGC | CTCCAGACCGAAGCTCCGTC      | AACTCCAGACCGAAGCTCCGTT     | TTATCATCGGGTTTGTTATAACCATCTC       |
| 3              | CG<G>TCTG(G/A)AGTTTT  | CTCCGTCCAGGTTTGAAAAAATC   | ATCTCCGTCCAGGTTTGAAAAAATC  | CTCGTTACAGACGGAGCTTCGG             |
| 4              | AAAC<C>TG(G/A)ACGGAGA | GCCGGTAGAGAAAATCTCCGTC    | GGCCGGTAGAGAAAATCTCCGTT    | GCTTCGGTCTGGAGTTTTTCAAACC          |
| 6              | AAGTCTT(C/T)TCTTTC<A> | ACATGATCTCTCAGCTGAAGTCTTC | CACATGATCTCTCAGCTGAAGTCTTT | GTATGGATAGAATATCTTCAATGCGGCT       |

\*SNPs can be seen in red between parenthesis, i.e. (WT/SNP). The anchor can be seen in between brackets.

KASP genotyping was run on an Mx3000P thermal cycler (Agilent, UK), following the specific instructions from the manual for this instrument to set-up, run and read the plate. This included defining the plate setup, selecting the dyes used as FAM, HEX and ROX™ (carboxy-X-rhodamine) as the passive reference dye used for normalisation of signal variation, and setting their respective excitation and emission values. The thermal cycling conditions were programmed in a touchdown 2-step PCR protocol that includes a final read after amplification. The thermal cycling conditions for KASP chemistry required a 15-minute hot-start activation at 94°C, followed by 10 cycles of 94°C for 20 seconds and 61-55°C for 60 seconds (dropping 0.6 °C per cycle). Then, 26 cycles of 94°C for 20 seconds and 55°C for 60 seconds. This was followed by a KASP recycling program to improve results which consisted of 3 cycles of 94°C for 20 seconds and 57°C for 60 seconds. Using the MxPro software, the FAM and HEX fluorescence data were plotted on the X and Y axis, respectively, using the ROX to normalise the data by dividing FAM and HEX values by the passive reference value for each particular well of the genotyping plate.

#### **4.2.4. Genotyping by Sanger sequencing**

##### 4.2.4.1. Genotyping of the M4 generation

DNA from the M4 generation was extracted from each plant following the instructions described in Chapter 2.5 (DNA extraction). The extracted DNA templates were used in a PCR to amplify the sequence in the *BnHAT1* (*BnaA04g20860D*) gene that holds the SNPs of interest. The primers that were used for this were BnaA04g-Forward (5'-CGGGCTAAGACGATCCATCTC-3') and BnaA04g-Reverse (5'-CCAGGCCATTACCAACCATG-3'), with an expected amplicon size of approximately 900 bp. DNA templates were transferred to a 96-well plate along with the PCR reagents, following the instructions from the HotStarTaq Master Mix Kit (Qiagen, UK) that was used. For the cycling conditions, a touchdown PCR programme was used with a melting temperature ( $T_m$ ) of 60°C. HotStarTaq DNA polymerase was activated by a heating step set at 94°C for 15 minutes. This was followed by 10 cycles of 94°C for 30 seconds, 66-60°C for 45 seconds (dropping 0.6 °C per cycle), and an extension step at 72 °C for 45 seconds. Then, 25 cycles of 94°C for 30 seconds, 60°C for 45 seconds and 72 °C for 45 seconds. The last step was a final extension of 10 minutes at 72 °C.

Following PCR, 2 µL of PCR product were diluted in 18 µL of sterile distilled water (SDW) in the wells of a 96-well dimpled tray and mixed by pipetting. The final volume of 20 µL was then transferred to an Invitrogen™ 2% agarose E-Gel™ (ThermoFisher Scientific, UK), a precast 96-well gel with SYBR Safe DNA gel stain, designed for fast electrophoresis. A 1 kb ladder was loaded in the first lane on the top row. The gel was run for 5 minutes in an E-Gel Electrophoresis System (ThermoFisher Scientific, UK) at 60V and visualised in a gel imaging system (Syngene, UK) to confirm the amplification.

The PCR products were processed using Exonuclease I and shrimp alkaline phosphatase (SAP) in an enzymatic cleanup method known as ExoSAP, which removes excess primers and unincorporated nucleotides. In a new PCR plate, 5 µL of PCR products were mixed with 5 µL of ExoSAP master mix containing 0.025 µL of Exonuclease I (New England Biolabs, UK), 0.25 5 µL of SAP (Merck, UK) and 4.725 µL of SDW. The plate was sealed, briefly vortexed and spun before

placing it in a PCR machine. The programme was set to 30 minutes at 37°C, followed by 15 minutes at 80°C.

For sequencing, BigDye® Terminator v3.1 Cycle Sequencing kit (Thermofisher Scientific, UK) was used, this technique enables robust fluorescence-based DNA sequencing. Duplicate reactions were set for each of the PCR products, one using exclusively the forward primer and the other using the reverse. Consequently, two master mixes were prepared using the different primers. These contained, per sample, 1 µL of BigDye, 1.5 µL of 5x Buffer and 5 µL of SDW and 1 µL of the corresponding primer (10 µM). Two new 96-well PCR plates were prepared, one for the forward reactions and the other one for the reverse, and 8.5 µL of the corresponding master mix were pipetted into the plates. In duplicate, 1.5 µL of the PCR products were transferred into both plates. The plates were sealed, briefly vortexed and spun. They were placed in a PCR machine that was set to 95°C for a minute, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for four minutes, and finally lowered to 10°C on hold.

For the final purification step, two 96-well Sephadex plates (Cytiva, UK) were prepared following the instructions provided by the manufacturer, suspending them on in SDW for approximately three hours to allow the resin to rehydrate and swell at room temperature. The Sephadex plates were spun to remove the excess water and later placed on top of a 96-well collection plate. In parallel, 20 µL of SDW were added to the BigDye reactions. Once mixed, the resulting 30 µL of diluted BigDye reactions were transferred to the Sephadex plates and centrifuged at 900×g for 5 minutes. The collection plates containing the purified DNA samples were sealed, sequenced using a 3030XL DNA Analyser (Thermo Fisher Scientific, UK) and visualised with software Geneious Prime (10.0.9) to establish the genotypes of each sample.

#### 4.2.4.2. Genotyping of TILLING mutant line 3B

Leaf samples were taken from each of the 42 individuals from the TILLING mutant 3B segregating population before the pathogen inoculation, when the plants had between three and four true leaves. DNA was extracted following the instructions described in chapter 2.5 (DNA extraction). A PCR was performed as in Chapter 4.2.4.1 to amplify the sequence in the *BnHAT1* gene that holds the SNPs of interest. To confirm the amplification, the resulting PCR products were run in an 0.8% agarose-TBE gel, prepared as described in Chapter 2.7 (Agarose gel electrophoresis) and run at 80V for 90 minutes, using a 1 kb DNA ladder (Sigma Aldrich, UK).

The PCR products were diluted to 200 ng/µL and prepared for sequencing using ExoSAP as an enzymatic cleanup method to remove excess primers and unincorporated nucleotides, as described in Chapter 4.2.4.1. The resulting clean amplified DNA samples were sent to Source BioScience (Cambridge, UK) for Sanger Sequencing, that used the primers from Chapter 4.2.4.1. The resulting sequenced files were visualised using software Geneious Prime (10.0.9) to determine whether the individuals from the TILLING mutant 3B segregating population were homozygous for the WT allele, homozygous for the mutated allele or heterozygous, containing both alleles.

#### 4.2.5. RNA-seq analysis

##### 4.2.5.1. Quality control of the RNA samples

For a gene expression study conducted by Muthayil-Ali (2023), three biological replicates of susceptible *B. napus* cultivars (cvs) Cabriolet and Sansibar, and resistant cvs POSH and Cubs Root were inoculated with *P. brassicae*. Leaf samples were harvested from the plants at different time points of the infection (1, 2, 4 and 8 DPI), plus a sample taken at 0 DPI after a mock inoculation, which was used as a negative control. An E.Z.N.A. Plant RNA Kit (Omega Bio-tek) was used for RNA extraction of the samples. The concentration and quality of the RNA samples were first quantified using Nanodrop ND-1000 spectrophotometer (Labtech International, UK).

As part of this current project, the RNA samples from Muthayil-Ali (2023) were examined for further study using Invitrogen™ Qubit™ 4 Fluorometer (Thermo Fisher Scientific, UK) and the 4150 TapeStation Electrophoresis systems (Agilent, UK) to confirm the quality and concentration of the extracted RNA met the recommended values for RNA sequencing: 25-50 µL containing a minimum of 500 ng of RNA per sample, equivalent to samples with a concentration of 20-50 ng/µL. The RNA ScreenTape assay protocol was followed (G2964-90022 Rev. D, 2014). TapeStation was especially important because it provided the RNA Integrity Number equivalent (RINe), a quality metric that determines the integrity of RNA samples, typically used for sample quality control in NGS workflows. This was calculated by analysing the ratio between the 28S and 18S ribosomal RNA (rRNA) subunits, with an optimal ratio of 2:1 which would correspond to samples with high RINe values. RNA degradation causes the decrease of 28S relative to 18S, which lowers the ratio and results in smaller RINe values. The samples that met the required concentration and quality values were submitted to GENEWIZ (Azenta, UK) for library preparation and strand-specific RNA-seq (IlluminaR NovaSeq™).

##### 4.2.5.2. DESeq2 analysis

Preliminary RNA-seq data analysis was carried out by Zedi Gao. The reference sequence for *B. napus* genome was for Darmor-bzh v10 and was taken from the Genoscope genome at <http://www.genoscope.cns.fr/genomes> (Rousseau-Gueutin *et al.*, 2020). Initial computational analyses were performed using the Linux high-performance computing (HPC) cluster provided by the University of Houston. Using *FastQC* (v0.11.9), the raw paired-end sequencing reads were subjected to quality control checks before and after trimming. To ensure high-quality data for downstream analysis, the raw fastq data files were trimmed using *Trimmomatic* (v0.39), which removed low-quality bases with a quality score below 3, and a sliding window approach was applied to trim reads with an average quality below 15 over a 4-base window. Additionally, the Illumina adapter sequences (PrefixPE/1: TACTCTTTCCCTACACGACGCTCTTCCGATCT and PrefixPE/2: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT) were trimmed allowing maximum of two seed mismatches, a palindrome clip threshold of 30 and a simple clip threshold of 10. After trimming, reads that were shorter than 36 base pairs were also discarded. The trimmed and filtered paired-end reads were aligned to the reference genome with *HISAT2* (v2.2.1) with default parameters. Following alignment, *featureCounts* (Subread package v2.0.6) was used to generate transcript-level read counts, a matrix that represents the number of reads mapped to each gene for all the samples.

The resulting counts table was analysed using BioConductor R package DESeq2 (Love *et al.* 2014). To account for the experimental design, the model formula incorporated the main effects of the cultivars (Cabriolet, Sansibar, POSH and Cubs Root) and the treatment conditions (0, 1, 2, 4 and 8 DPI), as well as their interaction ( $\sim$  Line + DPI + Line:DPI). The differential expression analysis was performed using a negative binomial generalized linear DESeq2 model, which estimated the log<sub>2</sub> fold changes and adjusted *p*-values. The read counts were normalised and transformed using variance stabilizing transformation (vst), adjusted to compensate for the effect of different library sizes, putting the data on the log<sub>2</sub> scale. With this dataset, multiple graphs were plotted to illustrate differential expression across genotypes and treatments.

#### **4.2.6. Gene expression of *BnHAT1* in TILLING mutant 2A**

For this experiment, four plants of TILLING mutant 2A were grown in CE cabinets, along with resistant *B. napus* cv. Lipid and susceptible cvs Cabriolet and Abukuma, as described in Chapter 2.2 (Growing *Brassica napus* under controlled environment conditions). The plants were inoculated with a *P. brassicae* population that originated from glasshouse experiments at Rothamsted Research (Muthayil-Ali, 2023), prepared as described in Chapter 3.2.1 (*Pyrenopeziza brassicae* populations and isolates). The inoculation was done as described in Chapter 2.3. (*Pyrenopeziza brassicae* spray-inoculation method). Infected leaf samples were harvested at 1, 2, 4, 8, 12, 16 and 21 DPI, plus after a mock inoculation at 0 DPI as a negative control.

The mRNA was extracted from the leaf samples using an E.Z.N.A. Plant RNA Kit (Omega Bio-tek), following the manufacturers instructions that included DNase I digestion step. The resulting RNA samples were quantified using Nanodrop ND-1000 spectrophotometer (Labtech International, UK) and Invitrogen™ Qubit™ 4 Fluorometer (Thermo Fisher Scientific, UK) and stored at the -80°C freezer.

The next step would have been an RT-qPCR (Reverse Transcription Quantitative Polymerase Chain Reaction), a two-step technique that combines reverse transcription with real-time fluorescence monitoring. It converts mRNA into complementary DNA and measures the amplification, allowing for precise quantification of gene expression. However, the RT-qPCR was not done during the length of this project, although primers for this were carefully designed. They could not be part of the intron since it would not be present in an RNA sample, therefore the primers were selected in the exon: Forward (5'-GTGACTGTCTCGGTGAGTGA-3') and Reverse (5'-AAACTCCAGACCGAAGCTCC-3'), located in the first exon, with a product size of 197 bp.

#### **4.2.7. Statistical analysis**

The analysis of the data obtained from the experiments with TILLING mutants 2A and 3B was done using RStudio (R Core Team, 2023), which was used to statistically compare the genotypes studied with their corresponding phenotypical results. Normal distribution was tested using Shapiro's test to decide whether to apply a parametric or a non-parametric statistical analysis. For normal distributed data, a one-way analysis of variance (ANOVA) was done, followed by Tukey's Honestly Significant Difference (HSD) to determine significant differences between genotypes. For non-normally distributed data, Mann-Whitney-Wilcoxon was used to compare

two groups. To compare more than two groups, the Kruskal-Wallis statistical test was used, followed by the Dunn test for multiple pairwise comparison to check differences between or within groups.

#### **4.2.8. AlphaFold3 to study the structure of BnHAT1**

The structure of the BnHAT1 acyltransferase was studied using AlphaFold3 (Jumper *et al.*, 2021), an AI model able to predict the 3D structures of proteins from their amino acid sequence with high accuracy. The models generated can be useful in establishing the tertiary structure of the polypeptide chain and to discover the existing interactions within the protein between amino acids or with other biological molecules.

Some useful metrics to determine the validity and accuracy of a model are pTM (Predicted Template Modeling), which measures the global fold similarity to the original structure, evaluating the overall accuracy of the predicted structure, and ipTM (Interface Predicted Template modelling), which measures the quality of the interface between protein chains. Another important value is pLDDT (predicted Local Distance Difference Test), which indicates the local confidence in the accuracy of the predicted atomic position per-residue.

## 4.3 Results

### 4.3.1. Generation and identification of TILLING mutant lines

TILLING mutant seeds (generation M3) were generated at the John Innes Centre using *B. napus* cv. Cabriolet; 28 of these lines targeted the *BnHAT1* gene (*BnaA04g20860D*) (Appendix 4). The specific SNP mutations in lines 2, 3, 4 and 6 were selected for further study (Table 4.2). The SNPs for these four TILLING mutant lines were located in the first exon of the DNA sequence, emphasised in Figure 4.3A, which also included the coding sequence (CDS) of the HXXXD motif. The full DNA sequence of the *BnHAT1* gene is shown in Appendix 4, with the mentioned SNPs of interest highlighted.

SNPs in the gene sequence resulted in changes in the amino acid sequence of the BAHD acyltransferase protein, the residues affected were highlighted in Figure 4.3B, the protein sequence shows both domains that are highly conserved in the BAHD super-gene family members. The HXXXD motif found in the first exon (HLVTD) was targeted by the mutation in TILLING mutant line 2. The DFGWG motif was found in the third exon of the gene (Appendix 5).

TILLING mutant line 6 contained the most upstream SNP mutation of TILLING mutants. It carried a missense mutation caused by a C to T mutation that resulted in the amino acid substitution of serine (S) to phenylalanine (F) at position 64. Line 2 carried a G to A substitution that led to a missense mutation in position 167 of the protein sequence, changing an aspartic acid (D) into an asparagine (N). TILLING mutant lines 3 and 4 both produced a stop codon mutation (X) in positions 172 and 178, respectively, that significantly shortened the polypeptide chain, truncating the protein.

The mutated seeds of these four lines of interest were grown in glasshouse conditions along with *B. napus* cv. Cabriolet individuals as a control. Leaf samples were harvested and their DNA was extracted. KASP marker analysis, a PCR-based technique that is highly accurate for SNP differentiation, was used to determine the genotype of each TILLING plant. Cluster plots were used to determine whether individuals were homozygous or heterozygous (Figures 4.4 and 4.5). Samples close to the X axis, related to FAM fluorescence, identified the WT allele (represented as blue in the graphs), whereas samples that were close to the Y axis, related to HEX fluorescence, identified mutant alleles (represented as red in the graphs). Samples that appeared in the middle of both axes were classified as heterozygotes (represented as green in the graphs), because they had fluorescence associated with both alleles.

TILLING mutants 2A, 2B and 2C were homozygous for the mutant allele with the majority of their technical replicates were clustered together close at the Y axis, presenting with fluorescence intensity corresponding to the HEX label (Figure 4.4). Two technical replicates (2B and 2C) were identified as outliers, clustering in the up-right corner associated with a heterozygous genotype, along with two NTCs. TILLING mutants 3A and 3B were clustered together in the middle of the graph, showing fluorescence signal corresponding both to the HEX and FAM fluorophores, indicating they were heterozygous. However, some of the replicates presented low fluorescence signal, which reduces the reliability of the genotype determination. It is likely that the presence of the unexpected outliers in the up-right corner might have affected the dimensions of the graph.

If they had been removed, resolution would have increased and, potentially, it would have been easier to distinguish the genotypes close to the cluster in the lower left corner.

TILLING mutant 6A clustered with a fluorescence intensity exclusively associated to the HEX level, indicating that it is homozygous for the SNP mutation (Figure 4.5). The other biological replicate for TILLING line 6 (6B and 6C), as well as 4E, showed a fluorescence signal that corresponded to the FAM label, therefore they are homozygous for the WT allele, whilst 4F presented both HEX and FAM fluorescent, suggesting heterozygosity.

The WT samples from cv. Cabriolet primarily showed fluorescence corresponding to FAM, the fluorophore associated with homozygosity for the WT allele, as expected of this positive control, although in Figure 4.5 it was less consistent. The majority of the NTC samples showed very low levels of fluorescence signal, although there were some unexpected exceptions in both graphs.

**Table 4.2.** TILLING mutant lines with their corresponding SNPs, the change in codon and the resulting amino acid changes and type of mutation.

| <b>TILLING mutants</b> | <b>SNP</b> | <b>Change in codon</b>      | <b>Amino acid change</b>               | <b>Type of mutation</b> |
|------------------------|------------|-----------------------------|----------------------------------------|-------------------------|
| 2                      | 499 G → A  | <u>G</u> AC → <u>A</u> AC   | 167 Aspartic acid (D) → Asparagine (N) | Missense                |
| 3                      | 516 G → A  | TG <u>G</u> → TG <u>A</u>   | 172 Tryptophan (W) → Stop codon (X)    | Stop-codon              |
| 4                      | 534 G → A  | TG <u>G</u> → TG <u>A</u>   | 178 Tryptophan (W) → Stop codon (X)    | Stop-codon              |
| 6                      | 191 C → T  | T <u>C</u> T → T <u>T</u> T | 64 Serine (S) → Phenylalanine (F)      | Missense                |

#### A. Fragment of the *BnaA04g20860D* gene

TTGTGTTTTGTAAAGAAAAAATGATGAAATTAGATTAATATAAATAGTTCGCAGATCCC  
TCGCAGTCTATCATACTACTCCCATCCATTGGTGTAAATAAAAAGAAAAAGAAGATA  
ATGGTAGATATCGTAGTAATCTCTGACACCATTGTTTCGACCAGAGAGCTACGAGGATGGA  
TCGGACCGGGCTAAGACGATCCATCTCACTCCATGGGACCTCTCCTTCCTTCGAACCGAA  
TACGCTCAAAGAGCTCTCATCTTTCCTAAACCCGACCCGGAGACCCACATGATCTCTCAG  
CTGAAGTCT**TCT**CTTTCAGCCGCATTGAAGATATTCTATCCATACGCGGGACGTCTAGTC  
AAGACCCACAACGAAGACGACGAAACGGCGTCGTTTTTCATAGATTGCGATGGCTCGGGA  
GTCAGATTTCGTGCACGCAGCGGCCGTGACTGTCTCGGTGAGTGATGTCTTAAGACCGGAC  
AATGATTACTGGAGCGAGTTTTTTCAGCGAACGGAGTTAAAAGCTGGGAAGGGGTTCG  
AAGCCGTTGCTTGCCTTCCAAGTAACGGAGTTAAAAGACGGAGTTATCATCGGGTTTGGT  
TATAAC**CATCTCGTTACAGAC**GGAGCTTCGGTC**TGG**AGTTTTTTCAAACCT**TGG**ACGGAG  
ATTTTCTCTACCGGCCGGAAGAGTTTTCTCCCTTGTCACTCTGTGGATGGTTTCTCGGC  
GGGATTGGTTATCCGGTTCGAGTCCCCATCTCAGAGACGGTCCCATCACCATCACCATCA  
CATGGGCTTGTTGCTGCTTCAACGTTACAAAAGAAGATCTTTCGGTTCACGAGTAGACAC  
GTCTCTGAGCTTAAAGCTAAAGCCAACGGCGAGGTTGGTGGTTCGATAATGTCTCGTCG  
TTTCAAGCCGTGTTGGGGCACACGTGGCGGTCGATAATCAGAAACAGTGGTCTCGGTCCG  
GAAGAAGTGATTCATTGCAAGTTGGCGATGGATATTAGACAGAGGCTAAACCTCCGGTC  
GAGAAAGAGTGTTTTGGAAACATGGTCGGACTAGCGACGACCACCACAACCGCGGGAGAC  
ATGGTTGGTAATGGCCTGGGCTGGGCTGCTTTGCAAATAAACAACCGTGGGGGCGCAG  
ACGAATGAAGAATTAAGACGGTTTGCTGAGGACTGGGTGAAGGATCCGAAAATACCGAAC  
CGTTTGTTGGTGAATAATTGCTCACTGTTGCCGGCTCCCACGCAGGGGCGGATCTACT  
TGCATGGAGGGTGGGGCACATGCCCCATACAAATTTTATAATTTTGTCTGTAAAATAAA

#### B. Protein amino acid sequence

1 MVDIVVISDTIVRPESYEDGSDRAKTIHLTPWDLSFLRTEYAQRALIFPKPDPETHMISQ  
61 **LKSS**LSAALKIFYPYAGRLVKTHNEDDETSFFIDCDGSGVRFVHAAVTVSVSDVLRPD  
121 NDYWSEFFPANGVKSWEVSKPLLAQVTELKDGVIIGFGYN**HLVTD**GASV**WS**FFKT**WTE**  
181 IFSTGRKEFPPLSLCGWFLGGIGYPVRVPISETVPSPSPSHGLVAASTLQKKIFRFTSRH  
241 VSELKAKANGEVGGSDNVSSFQAVLGHTWRSIIRNSGLGPEEVIHCKLAMDIRQLNPPV  
301 EKECFGNMVG LATTTTTTAGDMVGNLGLWAALQINKTVGAQTNEELRRFAEDWVKDPKIPN  
361 RLNIHYFETDGVLFPCPSLSPRFDVFGN**DFGWG**KPIAVRGGPGIAGHGKILAYPGTEKGST  
421 EIHTSLWSHVLDKLLADEEFLQHVDVVVCV

**Figure 4.3.** Modifications in TILLING mutant lines 2, 3, 4 and 6 in **(A)** the DNA sequence of the first exon of the *BnHAT1* gene (*BnaA04g20860D*), which features the SNPs in bold and underlined and highlights the affected codons, and in **(B)** the amino acid sequence of the protein it encodes for, which highlights the affected residues. TILLING mutant line 6 is highlighted in green, 2 is highlighted in orange, line 3 in red and line 4 in blue. Framed by orange rectangles are the sequences corresponding to the HXXXD and DFGWG motifs, although the latter does not appear in the DNA fragment **(A)** because the sequence corresponding to this domain belongs to exon 3.

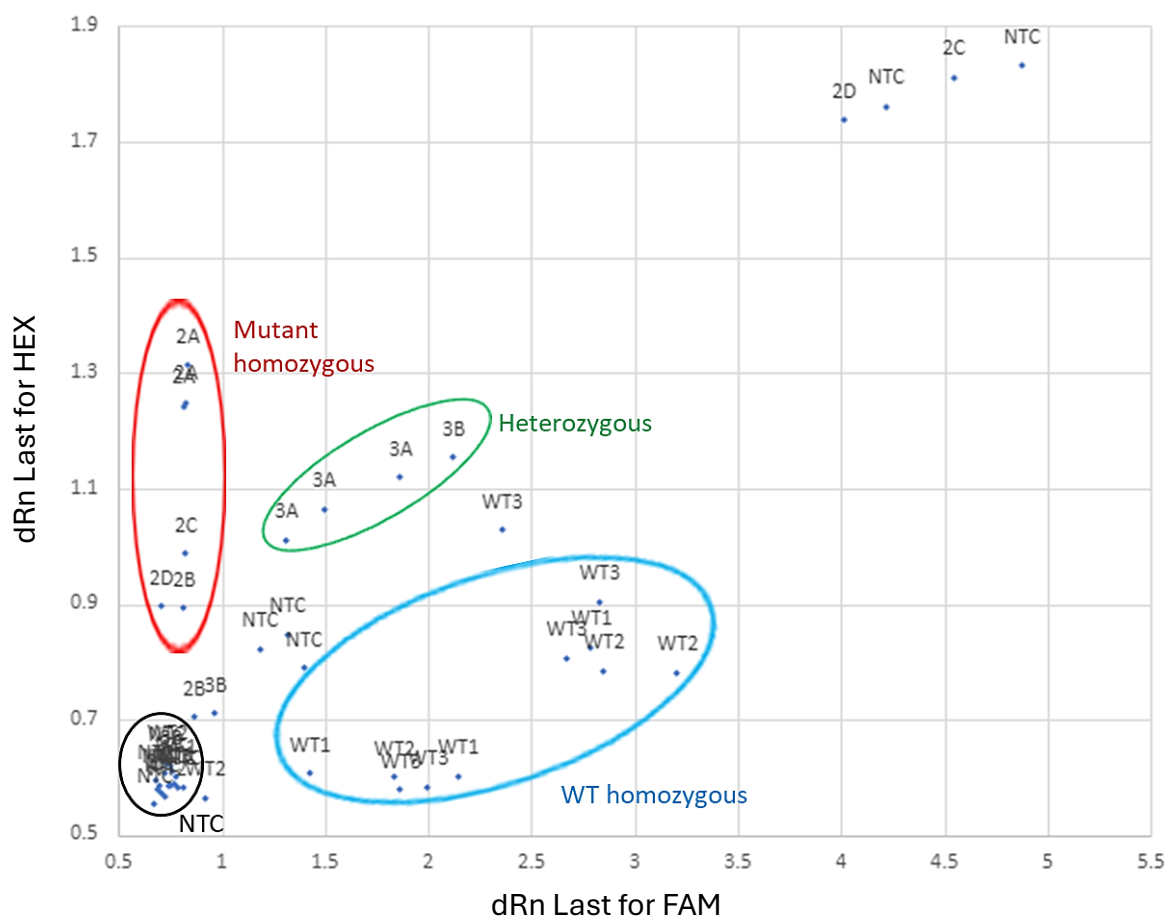


**Figure 4.4.** Cluster plot resulting from the KASP marker analysis of individual plants of TILLING mutant lines 2 (A to D) and 3 (A and B), along with *Brassica napus* cultivar Cabriolet samples as positive control for the wild type allele (WT1, WT2, WT3) and negative non-template controls (NTCs). For each biological replicate there were three technical replicates. Samples rounded in red are homozygous for the mutation allele, samples rounded in green are heterozygous, samples surrounded in blue are homozygous for the WT allele and samples surrounded in black are NTCs.

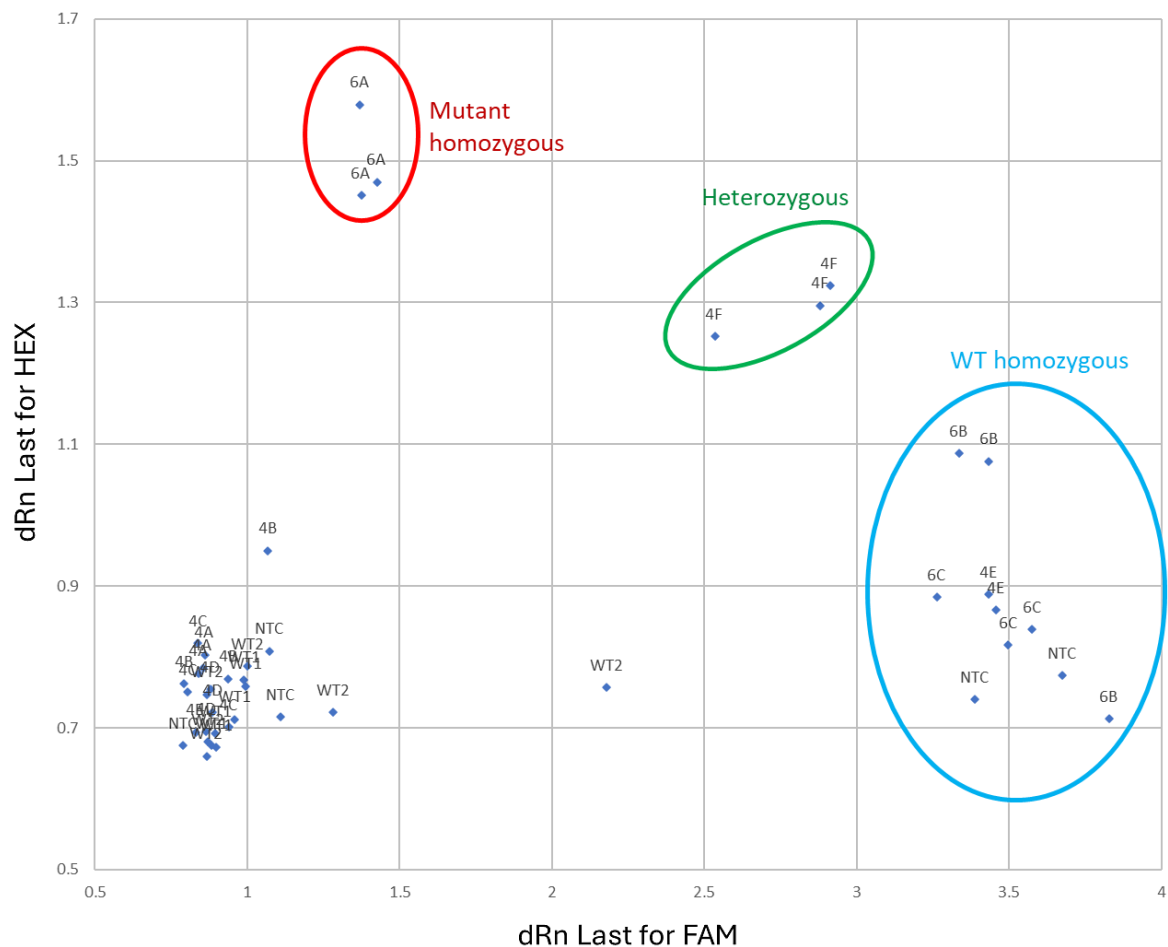


**Figure 4.5.** Cluster plot resulting from the KASP marker analysis of individual plants of TILLING mutant lines 4 (E and F) and 6 (A, B and C), along with *Brassica napus* cultivar Cabriolet samples as positive control for the wild type allele (WT1, WT2, WT3) and negative non-template controls (NTCs). For each plant there were three technical replicates. Samples rounded in red are homozygous for the mutation allele, samples rounded in green are heterozygous, samples surrounded in blue are homozygous for the WT allele and samples surrounded in black are NTCs.

#### 4.3.2. Genotyping of TILLING mutant generation M4 by sequencing

All the plants of the TILLING mutant lines 2, 3, 4 and 6 were selfed to produce generation M4. For the TILLING lines that are homozygous for the SNP mutation (2A, 2B, 2C, 2D and 6A) and for the WT allele (6B, 6C and 4E), the resulting seeds from this self-cross were genetically identical to the parent. For heterozygote TILLING mutants (3A, 3B and 4F), selfing resulted in segregating populations that included all three genotypes. To confirm this, the seeds resulting from these selfs were grown in the glasshouse, along with *B. napus* cv. Cabriolet as the WT control, and samples were taken for DNA extraction in an attempt to repeat the KASP marker analysis previously done. However, this method failed repeatedly; thus, sequencing of the *BnHAT1* gene of interest was used as an alternative approach to determine the genotypes of this generation.

The samples were genotyped using Sanger sequencing. An initial PCR was done, and the PCR products were run in an electrophoresis to confirm the amplification. The first gel run (Figure 4.6A) showed 20 samples that did not produce a band indicating the amplicon was not amplified. These samples were run again (Figure 4.6B) and the second PCR amplification was mostly successful except for TILLING mutants 3A\_3 and 2C\_3, which showed a faint amplification in comparison to the rest, and mutant 6A\_2 and 2C\_2, which failed and did not amplify the sequence of interest, potentially due to a higher degree of contamination in the DNA template. A 1 kb ladder was loaded in the first lane on the top row, although it should have been added to every row.

Sequencing resolved the genotypes of each of the individuals of the multiple selected TILLING mutant lines from the M4 generation (Table 4.3). All 10 cv. Cabriolet individuals were homozygous for the WT allele, as was expected from the control. All the individuals from TILLING mutant lines 2A, 2B, 2C and 6A presented a homozygous genotype for the mutated allele. As for lines 3A and 3B, the genotype of the different individuals was diverse, presenting one of the three possible genotypes: homozygous for the WT allele, homozygous for the mutated SNP or heterozygous. These results aligned with what was expected from the progeny of heterozygous parents that were selfed, resulting in segregating populations. Even though the sample size to confirm this should be bigger, the proportions of the genotypes from lines 3A and 3B suggested that these are, in fact, segregating populations. The 3A population had 3 heterozygote individuals and 3 homozygotes, 2 of which with the mutated SNP allele and 1 with the WT allele. The 3B population had 3 heterozygotes and 4 homozygotes that included 3 individuals with the mutated SNP allele and 1 with the WT allele. Seeing this, heterozygotes appeared to constitute half of the population, whereas the other half was formed by the sum of the homozygote options (WT or SNP mutation). This aligns with typical segregating populations after selfing a heterozygote parent.

Finally, mutant 4F only represented heterozygous genotypes. It could also be a segregating population and due to the small sample size, the other homozygous genotypes have not been detected, since only four individuals reached the sequencing stage. This could be confirmed by increasing the number of individuals assessed and sequenced. The lack of homozygous individuals could also be due to the SNP mutation (W178X) being lethal when homozygous.

These sequencing results further confirmed and validated the results from the KASP marker analysis, since some of the plants were challenging the expected genotype in these graphs (Figures 4.4 and 4.5). The 2C and 2D outliers that appeared as heterozygous in Figure 4.4 were

found to be homozygous when sequencing, clearing this discrepancy in establishing their genotype.

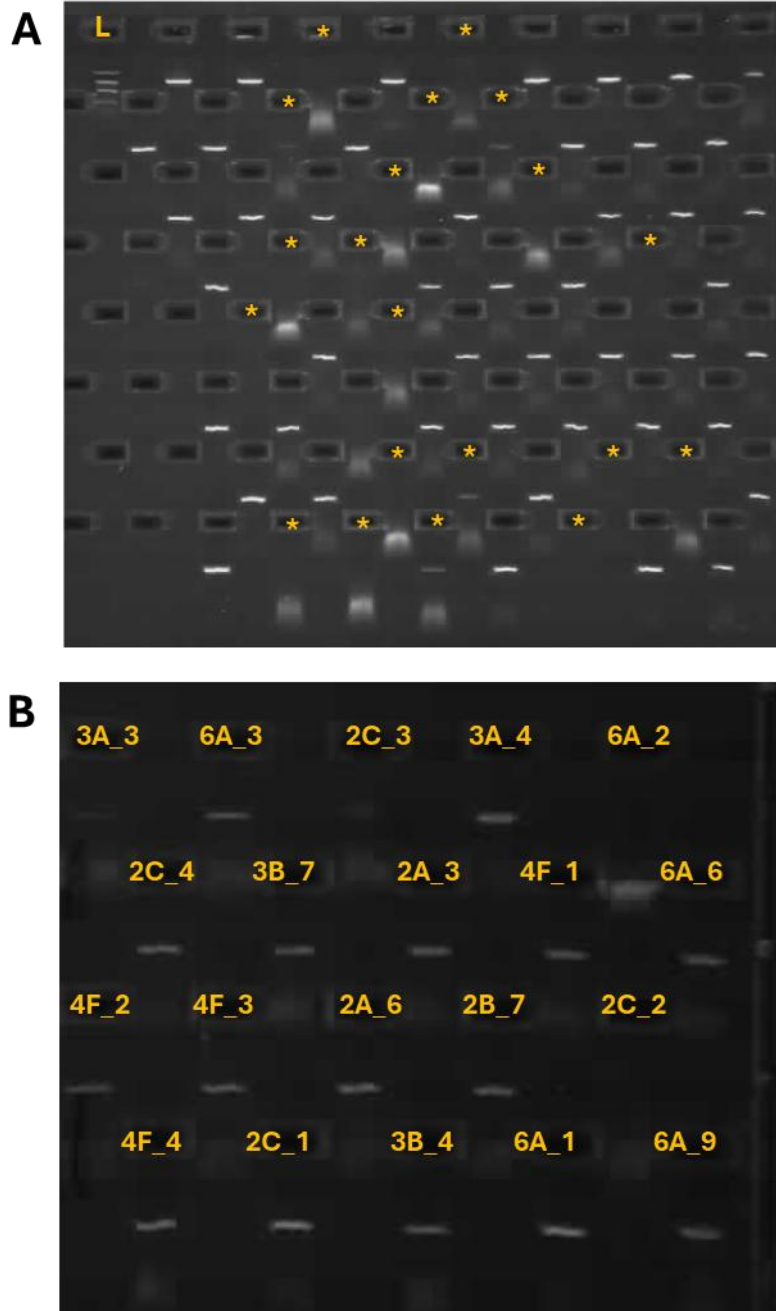


**Figure 4.6.** Electrophoresis of PCR products run in a 2% agarose E-Gel, where bands represent the sequence amplified in the *BnHAT1* gene. **(A)** Gel run with the PCR products of the first amplification, using a 1 kb ladder marked with a yellow L. The amplifications that failed were marked with yellow asterisks. **(B)** The PCR was repeated for the failed amplifications, and the PCR products run in a second gel.

**Table 4.3.** Genotypes of the individuals of selected TILLING mutant lines (M4 generation) that were sequenced.

| <b>TILLING mutant</b> | <b>Number of individuals</b> | <b>Genotype</b>                        |
|-----------------------|------------------------------|----------------------------------------|
| 2A                    | 7                            | Homozygous for the SNP mutation allele |
| 2B                    | 7                            | Homozygous for the SNP mutation allele |
| 2C                    | 5                            | Homozygous for the SNP mutation allele |
|                       | 3                            | Heterozygous                           |
| 3A                    | 2                            | Homozygous for the SNP mutation allele |
|                       | 1                            | Homozygous for the WT allele           |
|                       | 3                            | Heterozygous                           |
| 3B                    | 3                            | Homozygous for the SNP mutation allele |
|                       | 1                            | Homozygous for the WT allele           |
| 4F                    | 4                            | Heterozygous                           |
| 6A                    | 6                            | Homozygous for the SNP mutation allele |
| WT Cabriolet          | 10                           | Homozygous for the WT allele           |

#### 4.3.3. TILLING mutant 2A confirms reduced susceptibility to LLS

Homozygous TILLING mutant 2A, that carried a SNP in the conserved HXXXD motif, changing aspartic acid to asparagine, was selected to characterise the function of the mutated *BnHAT1* gene. The susceptibility to *P. brassicae* was evaluated in TILLING mutant 2A and WT *B. napus* cv. Cabriolet individuals through visual assessment and spore counting. A non-parametric Mann-Whitney-Wilcoxon Test established there was a statistically significant difference between the susceptibility of cv. Cabriolet (WT) and the 2A TILLING mutant ( $P < 0.001$ ) according to both visual assessment (Figure 4.7A) and spore counting (Figure 4.7B). In both cases, it was observed that TILLING mutant 2A individuals were significantly less susceptible to the pathogen than WT Cabriolet, suggesting that the SNP mutation resulted in an increase of resistance against the pathogen; hence, it is likely that the function of the *BnHAT1* gene is associated with susceptibility to *P. brassicae*.

#### 4.3.4. Study of segregating population (TILLING mutant 3B)

Heterozygote TILLING mutants of the M3 generation that were selfed produced a segregating population in the M4 generation. The TILLING mutant 3B segregating population would include individuals with three different genotypes: homozygous for the SNP mutation in the *BnHAT1* gene (T172X), individuals that were homozygous for the WT allele and heterozygote individuals that represented both the mutant and the WT allele.

To further study and characterise the *BnHAT1* gene, 42 individuals of this segregating population were grown under glasshouse conditions at the University of Hertfordshire. To determine the genotypes of each individual from the segregating population, leaf samples were taken for DNA extraction, and the gene of interest was amplified using PCR. The amplification of each sample was confirmed using electrophoresis (Figure 4.8). All the samples from the TILLING mutant 3B segregating population presented bands around 900 bp, which was the approximate size of the amplicons. Sample 3B\_20 showed no amplification in Figure 4.8B, so the PCR and electrophoresis were repeated successfully, as seen in Figure 4.8D, where it does present the expected band. Additionally, the NTCs in Figure 4.8C-E showed no amplification, as expected from a negative control, confirming the reliability of these results.

The PCR products were sequenced using Sanger Sequencing and the resulting data confirmed that the different individuals from the TILLING mutant 3B segregating population presented one of the three possible genotypes: homozygous for the WT allele, homozygous for the mutated SNP or heterozygous (Figure 4.9). The number of individuals corresponding to each genotype were calculated (Table 4.4). There were 20 heterozygous individuals, which represented 48% of the total population, and 22 homozygous ones, of which 13 presented the SNP mutation allele and 9 presented the WT allele which, respectively, represented 31% and 21% of the total population. These results appeared to align with the expected percentages of the segregating population, formed by 50% heterozygotes and 25% of each of the two homozygous genotypes. This was confirmed by a chi-square test ( $P > 0.05$ ) that showed there was no significant differences between the observed and the expected frequencies.

Due to an unfortunate infection with powdery mildew in the glasshouse, only 30 out of the 42 plants forming the segregating population reached the stage at which *B. napus* plants are ready for pathogen inoculation. These 30 plants were inoculated with *P. brassicae*, and their susceptibility phenotypes were evaluated using both visual assessment and spore counting. The genotypes and phenotypes of the segregating population were compared, and the disease scores and spore counts (log spores/cm<sup>2</sup>) were plotted in Figure 4.10. Individuals homozygous for the WT allele were most susceptible to *P. brassicae*, whilst individuals homozygous for the mutant allele were most resistant. The heterozygous individuals were intermediate for disease severity. The visual assessment data was assessed with the parametric ANOVA statistical test, which proved there was a significant difference between the three genotypes ( $P < 0.001$ ). A *post-hoc* Tukey's HSD test for a multiple pairwise comparison showed a discernible difference between each of the genotypes ( $P < 0.001$ ), with a higher contrast between the phenotypes of the two homozygous genotypes (mutated SNP and WT). Regarding the spore counting data, the data was not normally distributed, therefore a non-parametric Kruskal Wallis test was done, which proved there was a significant difference between genotypes ( $P < 0.001$ ). *Post-hoc* Dunn test further confirmed that each of the genotypes were significantly different from each other ( $P < 0.001$ ), with the biggest difference being, as was the case in visual assessment, between the two homozygotes.

These results showed that the susceptibility to *P. brassicae* was drastically reduced in plants with the mutant genotype, suggesting that the disruption of the *BnHAT1* gene significantly affected host resistance. The specific mutation in TILLING mutant line 3 (G516A) generated a stop codon in place of a tryptophan residue at position 172 of the protein sequence, and these kinds of mutations interrupt the protein sequence, fundamentally altering the protein structure and function. Accordingly, the BAHD acyltransferase that the *BnHAT1* gene encodes was likely not synthesised properly. Therefore, it could be asserted that the *BnHAT1* gene and the protein it produces were associated with susceptibility to *P. brassicae*, since its disruption resulted in more resistant individuals.



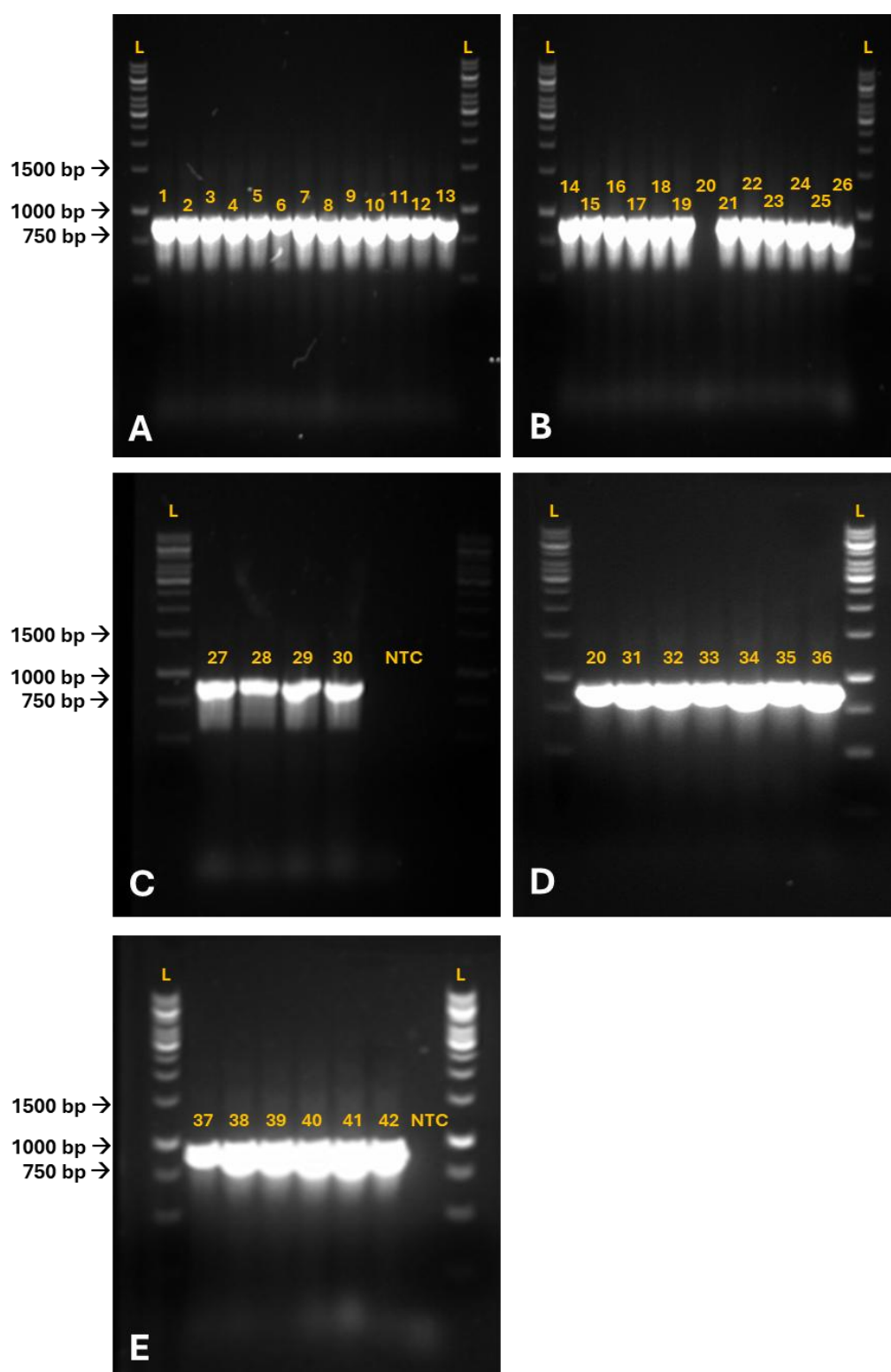


**Figure 4.8.** Electrophoresis of PCR products run in 5 separate 0.8% agarose-TBE gels (A-E), where bands represent the sequence amplified in the *BnaA04g20860D* gene from the 42 samples of the TILLING mutant 3B segregating population. The amplicon has a size of approximately 900 bp. Non template controls (NTCs) were run in gels C and E. A 1 kb ladder was used for each gel (yellow L).

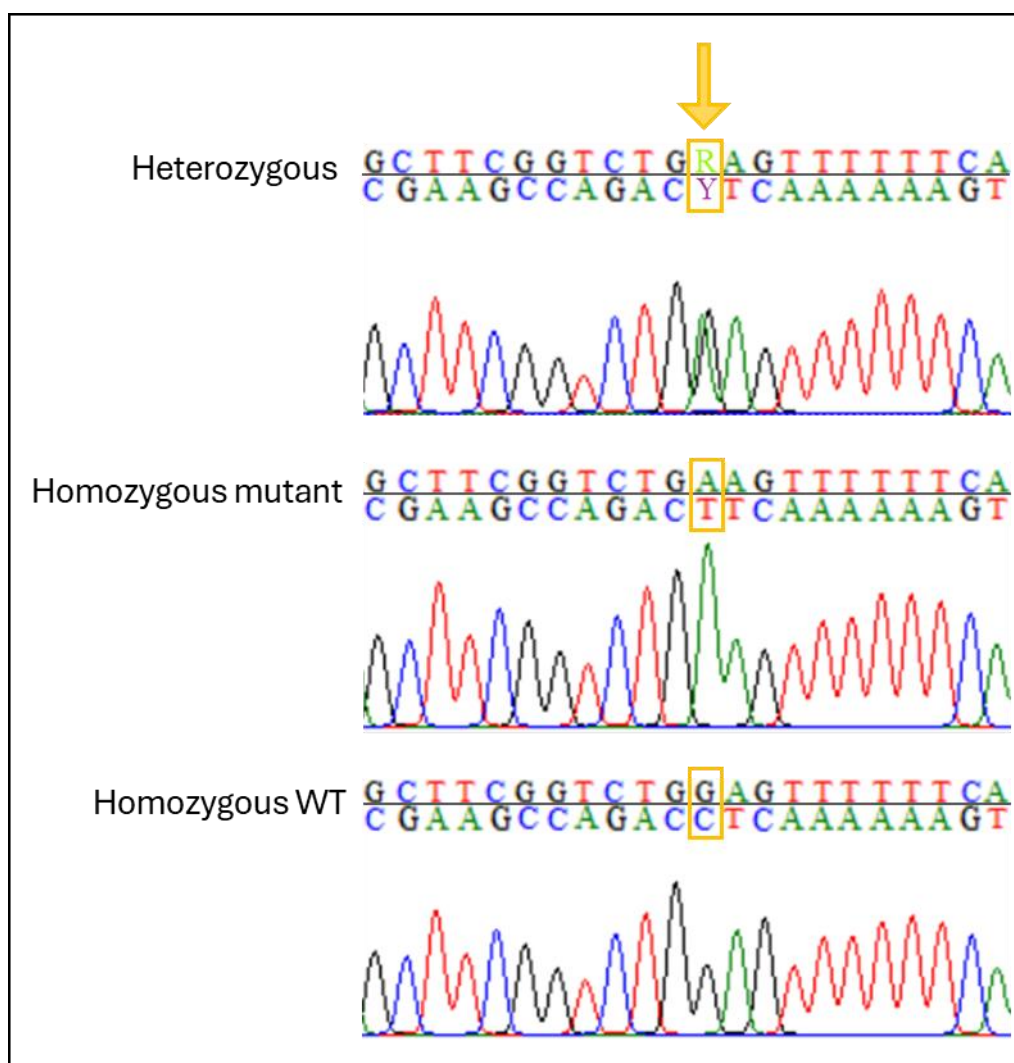


**Figure 4.9.** Sanger sequencing electropherograms of different genotypes of the TILLING mutant 3B segregating population, emphasising the position of interest in the amplified sequence. Heterozygous individuals contained both possible nucleotides G and A (represented with an R). Homozygotes for the mutant allele have an A nucleotide, whereas homozygotes for the wild type (WT) show a G nucleotide.

**Table 4.4.** Genotypes of the individuals of the TILLING mutant 3B segregating population through Sanger sequencing.

| Genotype                         | Nucleotide  | Expected individuals | Observed individuals |
|----------------------------------|-------------|----------------------|----------------------|
| Heterozygous                     | R (G and A) | 21                   | 20                   |
| Homozygous for the mutant allele | A           | 10.5                 | 13                   |
| Homozygous for the WT allele     | G           | 10.5                 | 9                    |

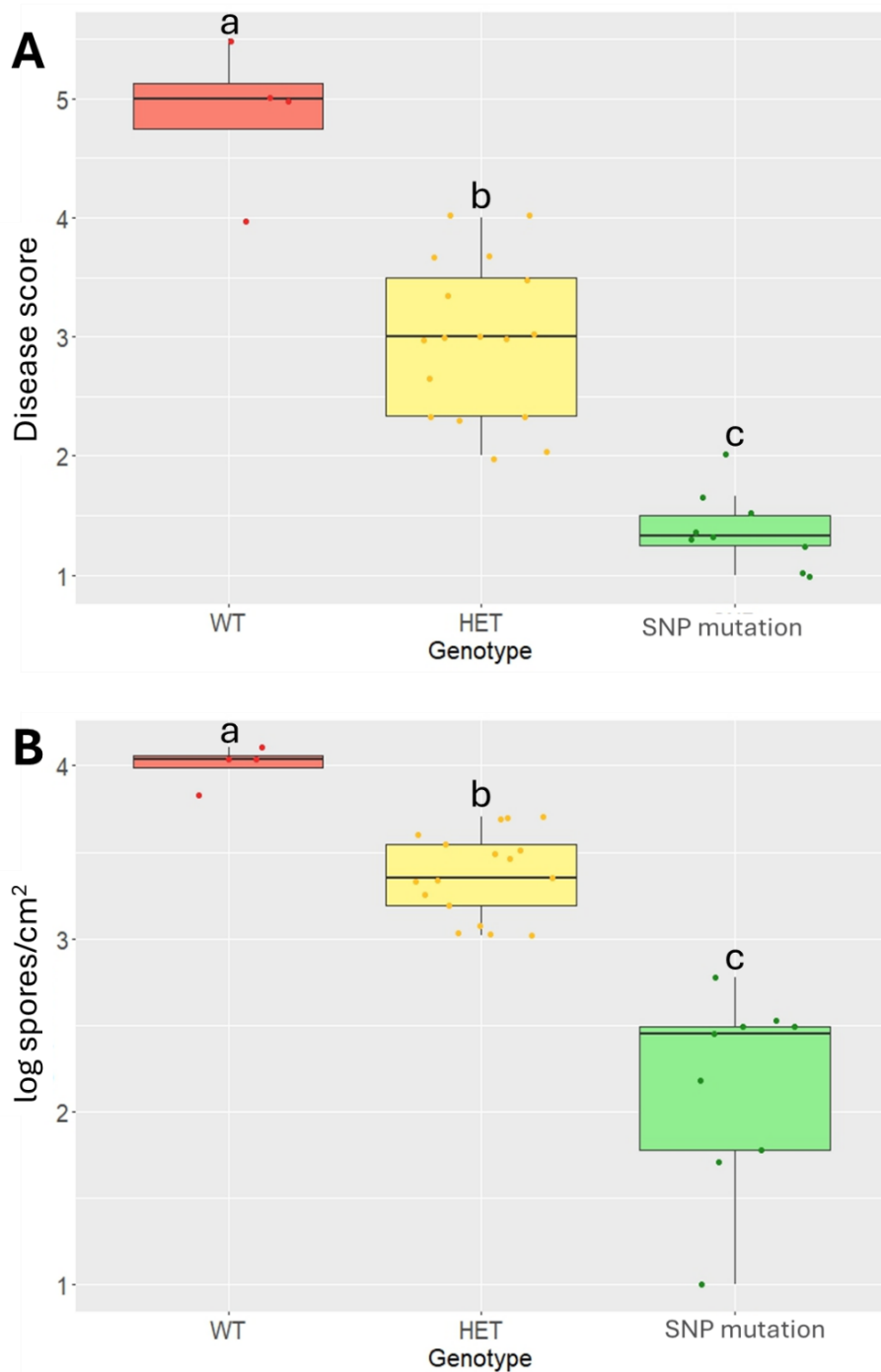


**Figure 4.10.** Comparison of the susceptibility of *Brassica napus* TILLING mutant 3B segregating population inoculated with a *Pyrenopeziza brassicae* population from Rothamsted Research (Muthayil-Ali, 2023). The severity of the disease was evaluated with **A**) visual assessment and **B**) spore counting. The genotypes compared were homozygous for the SNP mutation, homozygous for the wild type (WT) allele and heterozygous. Same alphabetic letter bars do not differ significantly. Boxplots represent the median, first and third quartile of 30 biological replicates. Whiskers represent variability outside upper and lower quartiles, with some outlier samples beyond them. Significant differences between genotypes were determined using **(A)** the ANOVA and *post-hoc* Tukey's HSD statistical test for visual assessment and **(B)** the Kruskal-Wallis and *post-hoc* Dunn test for spore counting.

#### 4.3.5. RNA-seq analysis

Muthayil-Ali (2023) did a gene expression experiment to study the changes in gene expression for resistant and susceptible hosts during the early stages of *P. brassicae* infection: germination (1 DPI), penetration (2 DPI), hyphal branching (4 DPI) and early colonisation (8 DPI). *B. napus* cultivars were inoculated with *P. brassicae* and RNA was extracted at different stages of the infection. As part of this current project, the concentration and quality of the RNA samples were assessed, the results from the TapeStation assessment were shown in Figure 4.11. RNA samples that met the required concentration and RNA quality were sent for library preparation and RNA-seq.

RNA-seq analysis started with the alignment of the sequenced reads to the reference genome of *B. napus* Darmor-bzh (Figure 4.12A), showing an average alignment rate of 93.87%. Then sample library sizes were determined by counting the total number of raw reads mapped to each gene in every sample (Figure 4.12B), revealing differences in sequencing depth. Additionally, using Cook's distance, 0.18% of the total reads were detected as outliers that were not statistically significant (Appendix 6). Directly comparing the raw data between the samples could lead to distorted conclusions, for example, a gene may appear to be more strongly expressed due to a higher read count. For this reason, the read counts were normalised and transformed using variance stabilizing transformation (vst), adjusted to compensate for the effect of different library sizes, putting the data on the  $\log_2$  scale.

##### 4.3.5.1 Gene discovery

To identify genes of interest, a volcano plot was used to visualise the results of the differential expression analysis, using all data points from all the *B. napus* lines and time points (Figure 4.13). This was done by plotting statistical significance, represented as the negative  $\log_{10}$  of the  $p$ -value in the Y axis ( $-\log_{10}p$ ), against magnitude of change in expression between different conditions, represented as a  $\log_2$  Fold Change in the X axis ( $\log_2FC$ ). Genes with  $\log_2FC$  values above 0 in the X axis were upregulated whereas genes with negative  $\log_2FC$  values were downregulated. On the Y axis, the higher the  $-\log_{10}p$ , the higher the statistical significance because the  $p$ -value would be closer to 0. A total of 5107 genes met the combined criteria of statistical significance and magnitude change (in red in the graph), which amounted for 5.05% of the total aligned genes.

Eleven of the most significant genes were annotated in the reference genome (EnsemblPlant and UniProt). These and the *Arabidopsis* ortholog genes were examined to provide some insight into their molecular and biological function. Seven of them were upregulated, shown in Table 4.5, and the function of four of these were associated with host defence response and stress response. This could be linked to the response of *B. napus* after *P. brassicae* inoculation in this experiment.

The *A. thaliana* ortholog of the *BnaA01g03020D* gene, (*At4g34131*), encoded an enzyme with UDP-glucosyltransferase (Lim *et al.*, 2002) and quercetin glucosyltransferase (Lim *et al.*, 2004) activity *in vitro*. Gene expression was induced after treatment with salicylic acid (SA). It was also induced following infection with *Pseudomonas syringae* pv *tomato*, which showed its involvement with host resistance (Langlois-Meurinne *et al.*, 2005). The *BnaA09g13870D* gene was associated with the ortholog gene *At1g61120*, which was identified as a terpene synthase, a

lyase involved in terpenoid biosynthesis. Its expression was induced by jasmonic acid (JA), independent of SA or ethylene (Herde *et al.*, 2008). It was also induced as a response to *Pseudomonas syringae* (Attaran *et al.*, 2008) and *Plutella xylostella* (Ehlting *et al.*, 2008), showing it plays a role in host resistance. *BnaA08g16100D* gene encoded an oxidoreductase with monooxygenase activity in *B. napus*. In the *A. thaliana* ortholog (*At4g39950*) it encoded cytochrome P450, a monooxygenase enzyme that has been associated with induced systemic resistance. Oxidoreductases act as the primary agents against oxidative stress, which is common during pathogen infections. This gene is also involved in the biosynthesis of camalexin, a secondary metabolite associated with the host resistance against necrotrophic fungal pathogen *Sclerotinia sclerotiorum* (Stotz *et al.*, 2011).

The *BnaA02g10980D* gene was associated with the ortholog gene *At5g52300*, whose gene expression in *A. thaliana* was induced by abscisic acid (ABA) in stress responses to cold, salt stress and water deprivation (Nordin *et al.*, 1999; Uno *et al.*, 2000). The *BnaA01g18990D* and the *BnaA01g28440D* genes did not have an ortholog in *A. thaliana* but in *Arabidopsis lyrata*, and their role was inferred from electronic annotation. The first one encoded a Cytochrome P450, an oxidoreductase enzyme with monooxygenase activity. These enzymes are primary agents against oxidative stress, which is highly common during pathogen infections. Thus, the upregulation of this gene could be associated with the response during host defence. The *BnaA01g28440D* gene also had an ortholog in *A. lyrata*, although the gene was not defined, it appeared to be involved in RNA modification. Similarly, the annotation of the *BnaA01g28930D* gene, that had no orthologues in *Arabidopsis*, associated it with the regulation of DNA-template transcription. These last two genes, novel genes with undefined orthologues in *Arabidopsis*, could be having an effect on the host on a molecular level, potentially regulating gene expression.

There were four highly significant downregulated genes (Table 4.6). Plant Defensin 1.2 (*BnaC02g23620D*) is a well-known protein associated with host defence and antifungal activity. Its ortholog (*At5g44420*) has also been associated with host defence response to pathogens. Systemic expression of *PDF1.2* is induced and enhanced by both JA and ethylene signalling pathways, triggered simultaneously, and independent of salicylic acid (Penninckx *et al.*, 1996; 1998). Due to its link to host resistance, it was unexpected to see its gene expression downregulated in this experiment with *B. napus*, after *P. brassicae* inoculation. This protein was studied further in Figure 4.22.

The *A. thaliana* ortholog of the *BnaA05g33410D* gene (*At3g02720*) was identified as a glyoxalase III, an enzyme that converts methylglyoxal to D-lactate, providing a rapid detoxification pathway against glycation and oxidative stress (Kwon *et al.*, 2013). The ortholog of *BnaA07g26670D* (*At1g67730*) was identified as a  $\beta$ -ketoacyl-CoA reductase involved in the elongation of fatty acid precursors, crucial for lipid metabolism (Beaudoin *et al.*, 2009). Finally, the *BnaA01g01670D* gene was associated with ortholog *At4g35860*, which encodes for a GTP-binding protein with GTPase activity, potentially involved in intracellular vesicle trafficking and protein transport (Biermann *et al.*, 1996).

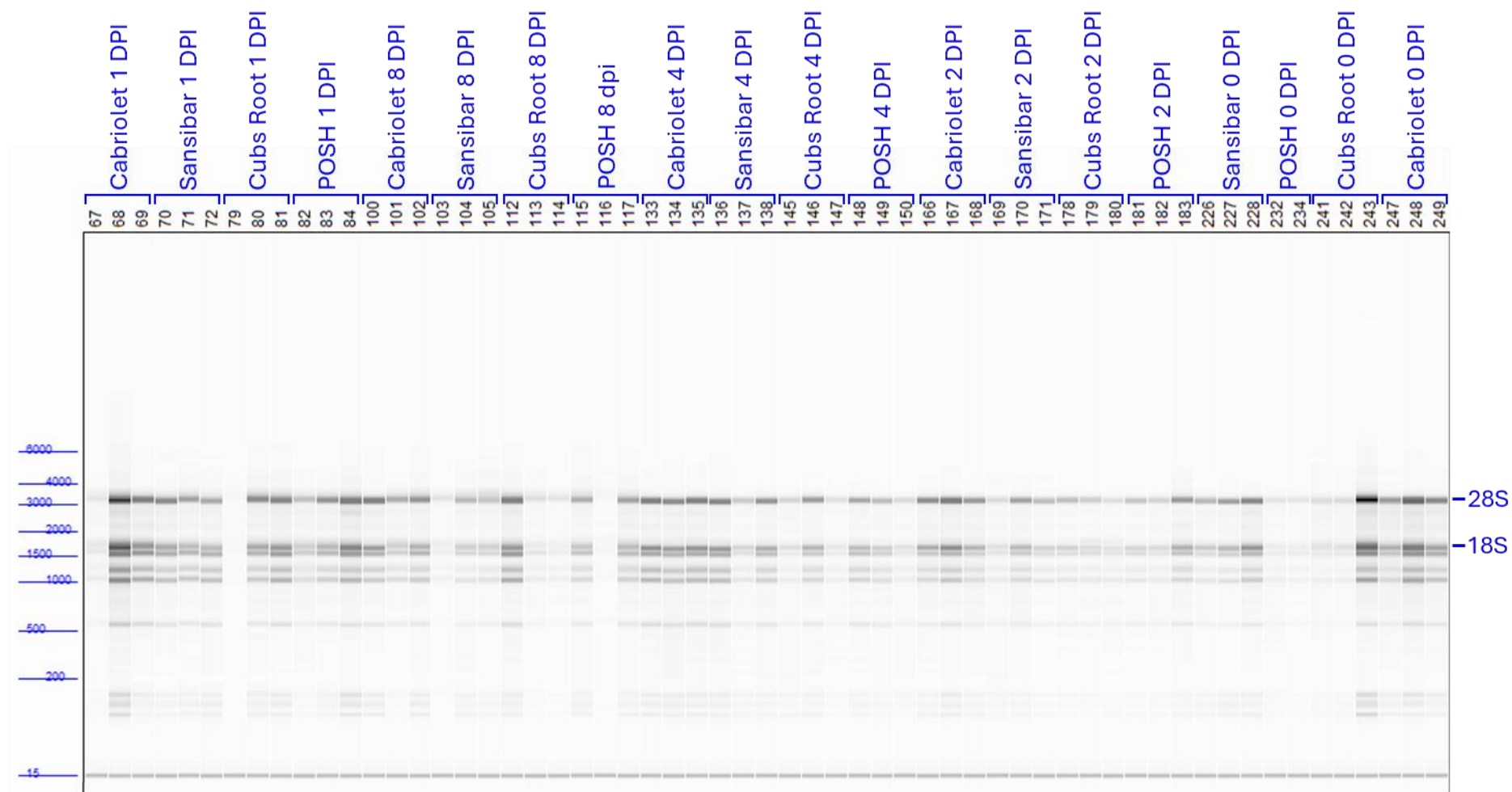


**Figure 4.11.** TapeStation 4150 electropherogram of the RNA samples for quality control, with 3 biological replicates per cultivar and time point. The first and second band, highlighted at the right, represent the 28S and 18S ribosomal RNA subunits, used to determine the quality of the RNA sample. Markers at the left shows the size of the standards.

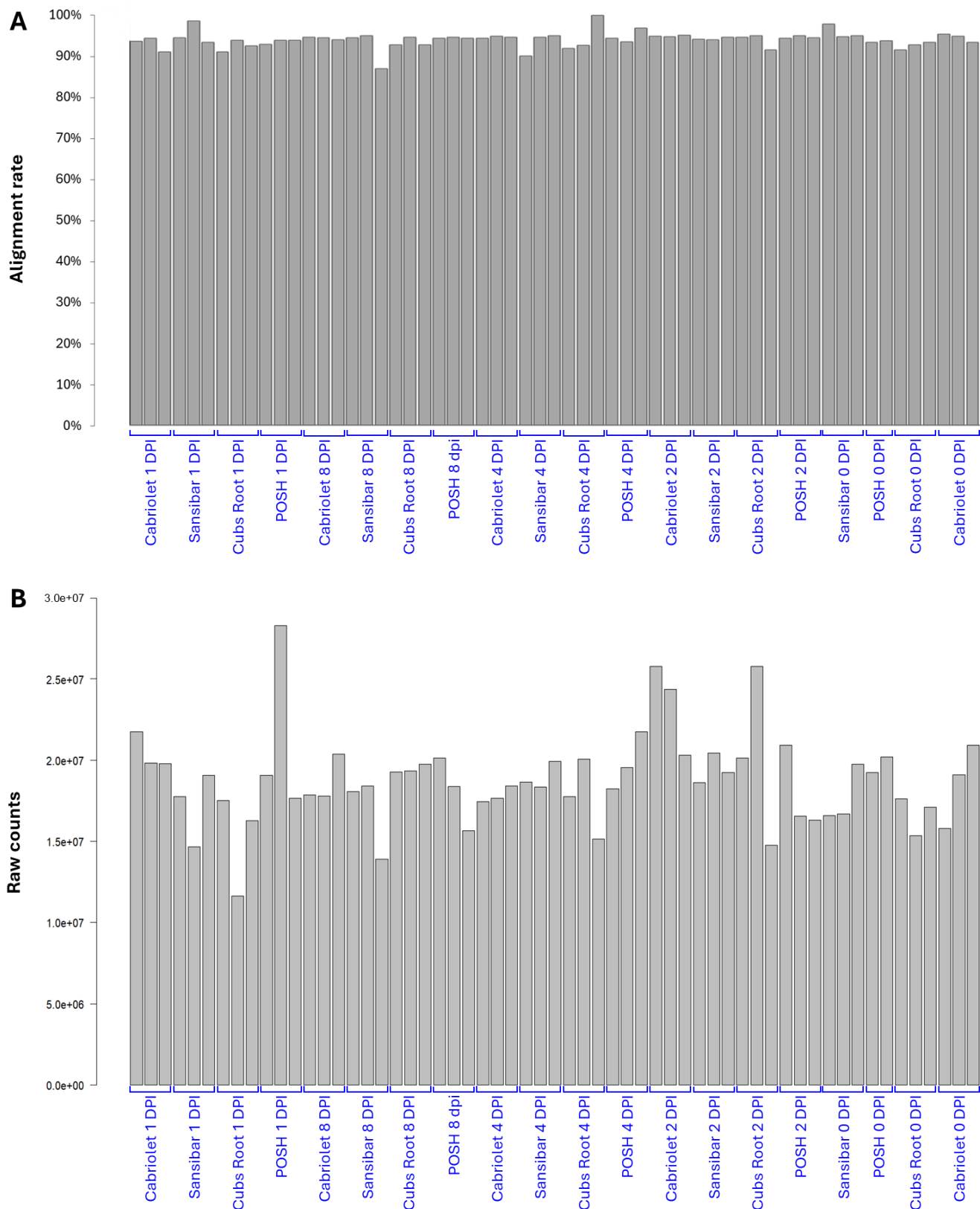


**Figure 4.12. A).** Alignment rates as percentages and **B)** library sizes measured in number of raw counts mapped to each gene in every sample. Different *Brassica napus* cultivars were inoculated with a *Pyrenopeziza brassicae* population from Rothamsted Research (Muthayil-Ali, 2023) at different days post-inoculation (DPI).

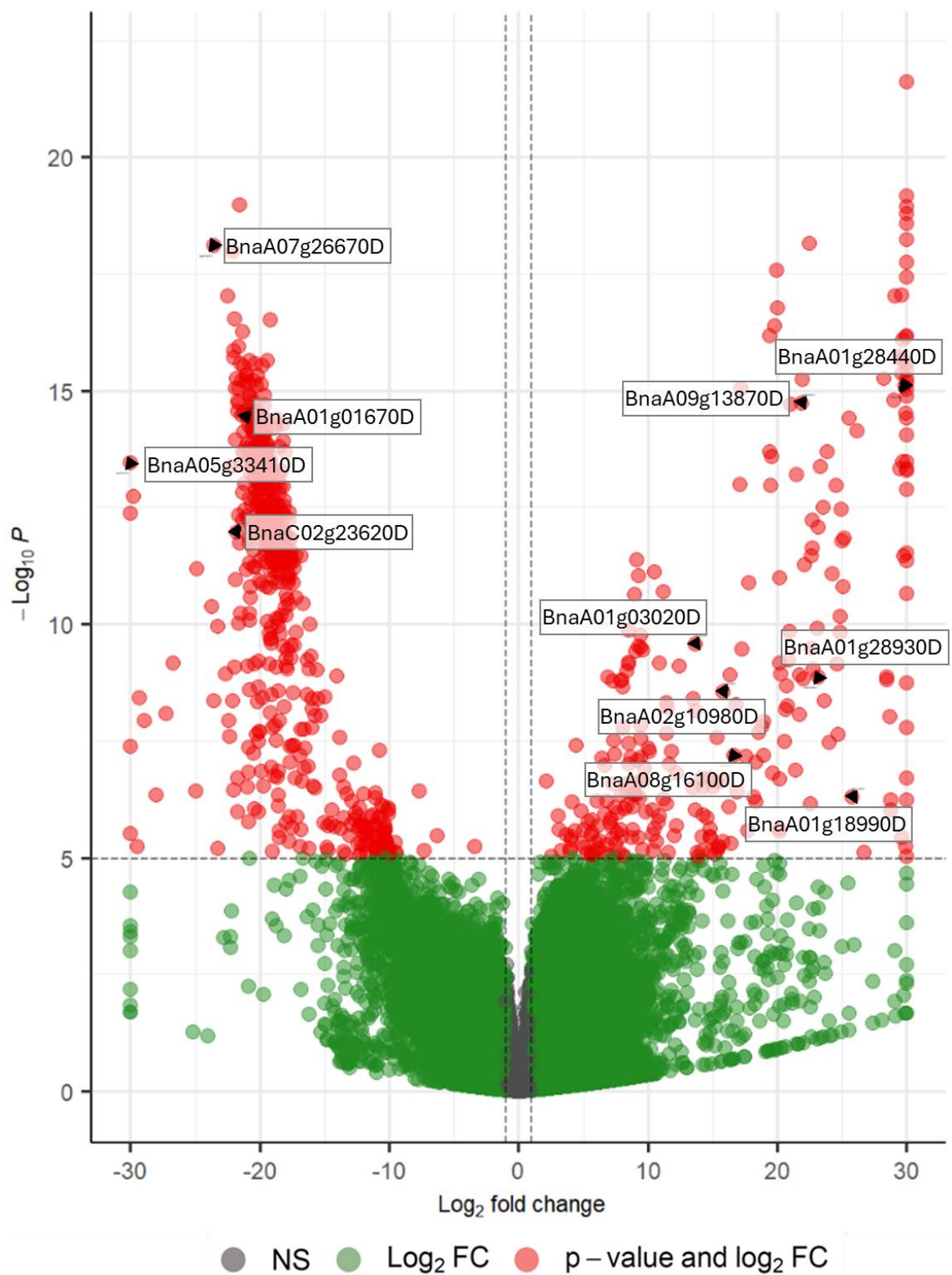


**Figure 4.13.** Visualisation of RNA-seq results with a volcano plot that illustrates differential gene expression. Vertical dotted lines indicate the thresholds, set at  $\log_2 \text{FC} > 1$  (upregulated) and  $\log_2 \text{FC} < -1$  (downregulated), and  $-\log_{10} P > 5$  (statistically significant). Green dots represent genes that do not meet the significance thresholds for either  $p$ -value or  $\log_2 \text{FC}$  fold change. Red dots are genes that do meet the criteria. The specific genes featured were annotated in the reference genome (*Brassica napus* Darmor-bzh)

**Table 4.5.** Differentially expressed *Brassica napus* upregulated genes that were annotated in the reference genome (*Brassica napus* Darmor-bzh) and their gene description.

| <i>Brassica napus</i> genes | Ortholog genes in <i>Arabidopsis</i>                                                        | Gene description                                                                                                                                                                                                                                                                            | Evidence for annotation                                                                                                               |
|-----------------------------|---------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------|
| <i>BnaA01g03020D</i>        | UDP-glycosyltransferase 73<br>(UGT73B3; At4g34131)<br>( <i>Arabidopsis thaliana</i> )       | UDP-glucosyltransferase <sup>1</sup> .<br>Quercetin glucosyltransferase <sup>2</sup> .<br>Involved in host resistance against <i>Pseudomonas syringae</i> pv <i>tomato</i> <sup>3</sup> .<br>Gene expression induced by salicylic acid <sup>1,3</sup> .                                     | <sup>1</sup> Lim <i>et al.</i> , 2002<br><sup>2</sup> Lim <i>et al.</i> , 2004<br><sup>3</sup> Langlois-Meurinne <i>et al.</i> , 2005 |
| <i>BnaA09g13870D</i>        | Terpene Synthase 04<br>(TPS04, At1g61120)<br>( <i>Arabidopsis thaliana</i> )                | Lyase involved in terpenoid biosynthesis <sup>1</sup> .<br>Involved in host resistance against <i>Pseudomonas syringae</i> <sup>2</sup> and <i>Plutella xylostella</i> <sup>3</sup> .<br>Gene expression induced by jasmonic acid, independent of salicylic acid or ethylene <sup>1</sup> . | <sup>1</sup> Herde <i>et al.</i> , 2008<br><sup>2</sup> Attaran <i>et al.</i> , 2008<br><sup>3</sup> Ehlting <i>et al.</i> , 2008     |
| <i>BnaA08g16100D</i>        | Cytochrome P450<br>(CYP79B2; At4g39950)<br>( <i>Arabidopsis thaliana</i> )                  | Oxidoreductase with monooxygenase activity.<br>Associated with induced systemic resistance.<br>Involved in the biosynthesis of camalexin, associated with host resistance against <i>Sclerotinia sclerotiorum</i> .                                                                         | Stotz, <i>et al.</i> , 2011                                                                                                           |
| <i>BnaA02g10980D</i>        | Low-temperature-induced protein 65<br>(LTI65, At5g52300)<br>( <i>Arabidopsis thaliana</i> ) | Gene expression induced by abscisic acid in stress responses to cold, salt stress and water deprivation.                                                                                                                                                                                    | Nordin <i>et al.</i> , 1999<br>Uno <i>et al.</i> , 2000                                                                               |
| <i>BnaA01g18990D</i>        | Cytochrome P450<br>(fgenes1_pm.C_scaffold_8000297)<br>( <i>Arabidopsis lyrata</i> )         | Oxidoreductase with monooxygenase activity.<br>Involved in the metabolism of endogenous and exogenous compounds.                                                                                                                                                                            | Inferred from electronic annotation                                                                                                   |
| <i>BnaA01g28440D</i>        | scaffold_702828.1<br>( <i>Arabidopsis lyrata</i> )                                          | Modification of RNA.                                                                                                                                                                                                                                                                        | Inferred from electronic annotation                                                                                                   |
| <i>BnaA01g28930D</i>        | No <i>Arabidopsis</i> orthologue                                                            | Regulation of DNA-templated transcription.                                                                                                                                                                                                                                                  | Inferred from electronic annotation                                                                                                   |

**Table 4.6.** Differentially expressed *Brassica napus* downregulated genes that were annotated in the reference genome (*Brassica napus* Darmor-bzh) and their gene description.

| <b><i>Brassica napus</i><br/>genes</b> | <b>Ortholog genes in<br/><i>Arabidopsis</i></b>                                                  | <b>Gene description</b>                                                                                                                           | <b>Evidence for<br/>annotation</b>      |
|----------------------------------------|--------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------|
| <i>BnaC02g23620D</i>                   | Plant Defensin 1.2<br>( <i>PDF1.2</i> ; At5g44420)<br>( <i>Arabidopsis thaliana</i> )            | Involved in host resistance against fungal pathogens.<br>Gene expression induced by jasmonic acid and ethylene,<br>independent of salicylic acid. | Penninckx <i>et al.</i> ,<br>1996; 1998 |
| <i>BnaA05g33410D</i>                   | DJ-1 homolog D<br>( <i>DJ1D</i> ; At3g02720)<br>( <i>Arabidopsis thaliana</i> )                  | Glyoxalase III that converts methylglyoxal to D-lactate.<br>Involved in response to oxidative stress and glycation.                               | Kwon <i>et al.</i> , 2013               |
| <i>BnaA07g26670D</i>                   | $\beta$ -ketoacyl-CoA reductase<br>( <i>KCR1</i> ; At1g67730)<br>( <i>Arabidopsis thaliana</i> ) | Ketoacyl-Co A reductase activity.<br>Involved in the elongation of fatty acid precursors.                                                         | Beaudoin <i>et al.</i> , 2009           |
| <i>BnaA01g01670D</i>                   | GTP-binding<br>( <i>GB2</i> ; At4g35860)<br>( <i>Arabidopsis thaliana</i> )                      | GTP-binding protein with GTPase activity.<br>Potentially involved in intracellular vesicle trafficking and<br>protein transport.                  | Biermann <i>et al.</i> , 1996           |

#### 4.3.5.2 Study of GEMs

A principal coordinates analysis (PCA) of the transformed data was plotted to reveal clustering of replicates and differences between treatments. The plot in Figure 4.14 showed susceptible cvs Cabriolet and Sansibar clustering together; there was no significant differences between their gene expressions. The gene expression at 1 DPI for both cultivars clustered together, slightly separate from the rest of the inoculation time points. Resistant cvs POSH and Cubs Root were both clearly separated from the other cultivars with a susceptible phenotype, but did not cluster together, suggesting different mechanisms of resistance between both.

The gene expression of the eight GEMs identified by Fell *et al.* (2023) was examined together with the gene for the pathogenesis-related protein 1 (*PR1*) and plant defensin 1.2 (*PDF1.2*) as controls (Appendix 7). The differential expression of these selected genes across the different treatments was plotted in a heatmap (Figure 4.15) and individually assessed to determine statistically significant differences between genotypes and pathogen inoculation time points (Figures 4.16 to 4.25). The expression of the *BnHAT1* gene (Figure 4.16) was significantly lower in the resistant cv. Cubs Root in comparison to susceptible cvs Cabriolet and Sansibar and, unexpectedly, to resistant cv. POSH ( $P<0.001$ ). Additionally, there was a statistical decrease in the gene expression at 1 DPI in susceptible cvs Cabriolet ( $P<0.01$ ) and Sansibar ( $P<0.05$ ).

The *PR1* gene showed higher levels of expression in comparison to the rest of the selected genes (Figure 4.15), especially at 1 DPI in resistant line Cubs Root, and at 2 DPI in susceptible cvs Cabriolet and Sansibar, and in resistant line POSH. However, these differences were not statistically significant (Figure 4.17). Cinnamate-4-hydroxylase (*C4H*) expression (Figure 4.18) was significantly higher in POSH than the other genotypes ( $P<0.05$ ). Although not statistically significant from 0 DPI, expression of the *C4H* gene was the highest at 2 DPI for all genotypes except for resistant line Cubs Root, which showed the lowest expression values at this time point. The  *$\beta$ -adaptin* gene was mildly and consistently expressed across treatments with no statistical differences between genotypes (Figures 4.15 and 4.19). There was a decrease in gene expression between 0 DPI and 2 DPI in Cubs Root ( $P<0.05$ ). The gene for the 40s Ribosomal subunit protein *S24e* (Figure 4.20) was significantly more expressed in Cubs Root ( $P<0.05$ ) in comparison to the other genotypes. Moreover, there was a significant decrease between control 0 DPI and 1 DPI in the Cabriolet and Sansibar cultivars ( $P<0.01$  and  $P<0.001$ , respectively), and between 0 DPI and 2 DPI in Sansibar ( $P<0.05$ ) and Cubs Root ( $P<0.001$ ).

The *SNF1* kinase homolog 10 (*KIN10*) and *PDF1.2* genes showed similar patterns of expression (Figures 4.21 and 4.22, respectively). In both, POSH showed significantly lower levels of expression ( $P<0.01$ ) compared to the other cultivars. Additionally, the expression of *KIN10* was significantly higher in resistant cv. Cubs Root than in susceptible cv Cabriolet and Sansibar ( $P<0.05$ ). Within Cubs Root, the expression of *KIN10* was statistically higher at 2 DPI in comparison to the 0 DPI control ( $P<0.05$ ).

Phosphatidylinositol-specific phospholipase C4 (*PLC4*), polyadenylate-binding protein (*PBP1*) and universal stress protein (*USP*) genes showed low expression in comparison to the rest of the selected genes (Figure 4.15). The expression of *PLC4* was significantly higher in cv. POSH ( $P<0.01$ ) with no observable differences between inoculation time points within the genotypes (Figure 4.23). There was a statistical difference between the expression of *PBP1* between lines

POSH and Cubs Root ( $P<0.05$ ), more highly expressed in the latter (Figure 4.24). However, expression in Cubs Root was downregulated at 1 and 2 DPI. Statistical differences in *PBP1* expression occurred between the control (0 DPI) and 1 DPI ( $P<0.05$ ) or 2 DPI ( $P<0.01$ ). As for the *USP* gene (Figure 4.25), there was no significant difference in gene expression between the different cultivars, although a statistical difference occurred between the control (0 DPI) and time point 4 DPI in cv. Cabriolet ( $P<0.05$ ).

#### **4.3.6. Gene expression of the *BnHAT1* gene in TILLING mutant 2A**

A gene expression experiment was supposed to further characterise the function of the *BnHAT1* gene, but it was not possible to finish it completely and obtain conclusive results from it in the duration of this project. This experiment intended to analyse the expression of the *BnHAT1* gene in the TILLING mutant line 2A (D167N) at different stages of the *P. brassicae* infection.

For this experiment, samples were harvested from TILLING mutant 2A plants and from *B. napus* cvs Lipid, Cabriolet and Abukuma at different time points after inoculation with *P. brassicae*. From these infected leaf samples mRNA was extracted, and its concentration in ng/ $\mu$ L, as well as the A260/280 ratio, were quantified (Table 4.7). An RNA concentration above 55 ng/ $\mu$ L is typically considered a high-quality yield for RT-qPCR, and only three samples did not meet this requirement. A biological replicate of Cabriolet 1 DPI and 16 DPI yielded concentration values of 53.2 and 50.8 ng/ $\mu$ L, respectively, which were slightly lower than 55 ng/ $\mu$ L. In addition, biological replicate B of TILLING mutant 2A (4 DPI) had a significantly low value, 10.4 ng/ $\mu$ L, considered too small since it is too close to the detection limit of the NanoDrop instrument (approximately 10 ng/ $\mu$ L) and readings become unreliable. The RNA extraction failed for biological replicates B and C of Lipid (16 DPI) and for biological replicate A of Cabriolet (12 DPI), thus no RNA concentration was obtained for these. The RNA samples would have been used to do an RT-qPCR. Unfortunately, the experiment could not be finished, and this last step was not done.

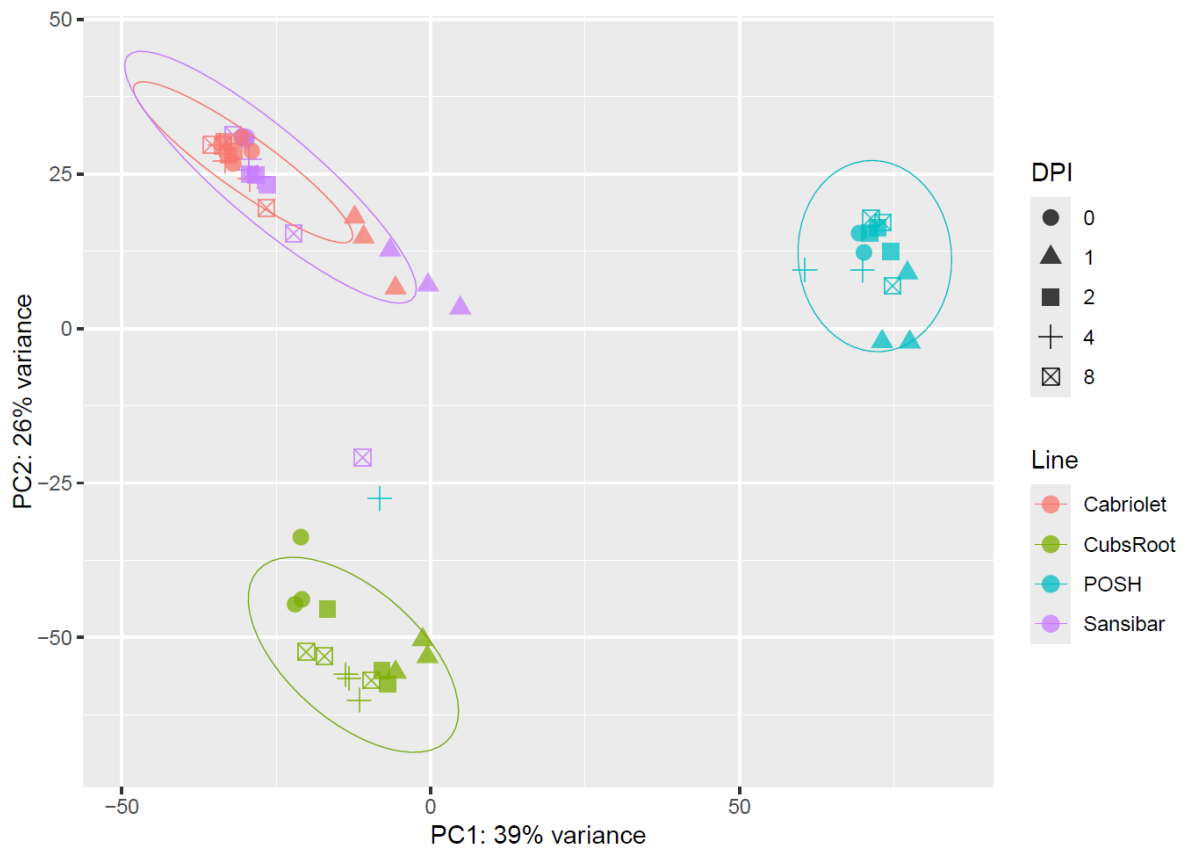


**Figure 4.14.** Principal component (PC) analysis of samples from resistant *Brassica napus* lines Cubs Root and POSH, or susceptible cvs Cabriolet and Sansibar. *B. napus* inoculated with a *Pyrenopeziza brassicae* population from Rothamsted Research (Muthayil-Ali, 2023). Samples were taken at different days post-inoculation (DPI). Cluster ellipses were set at a 95% confidence interval.

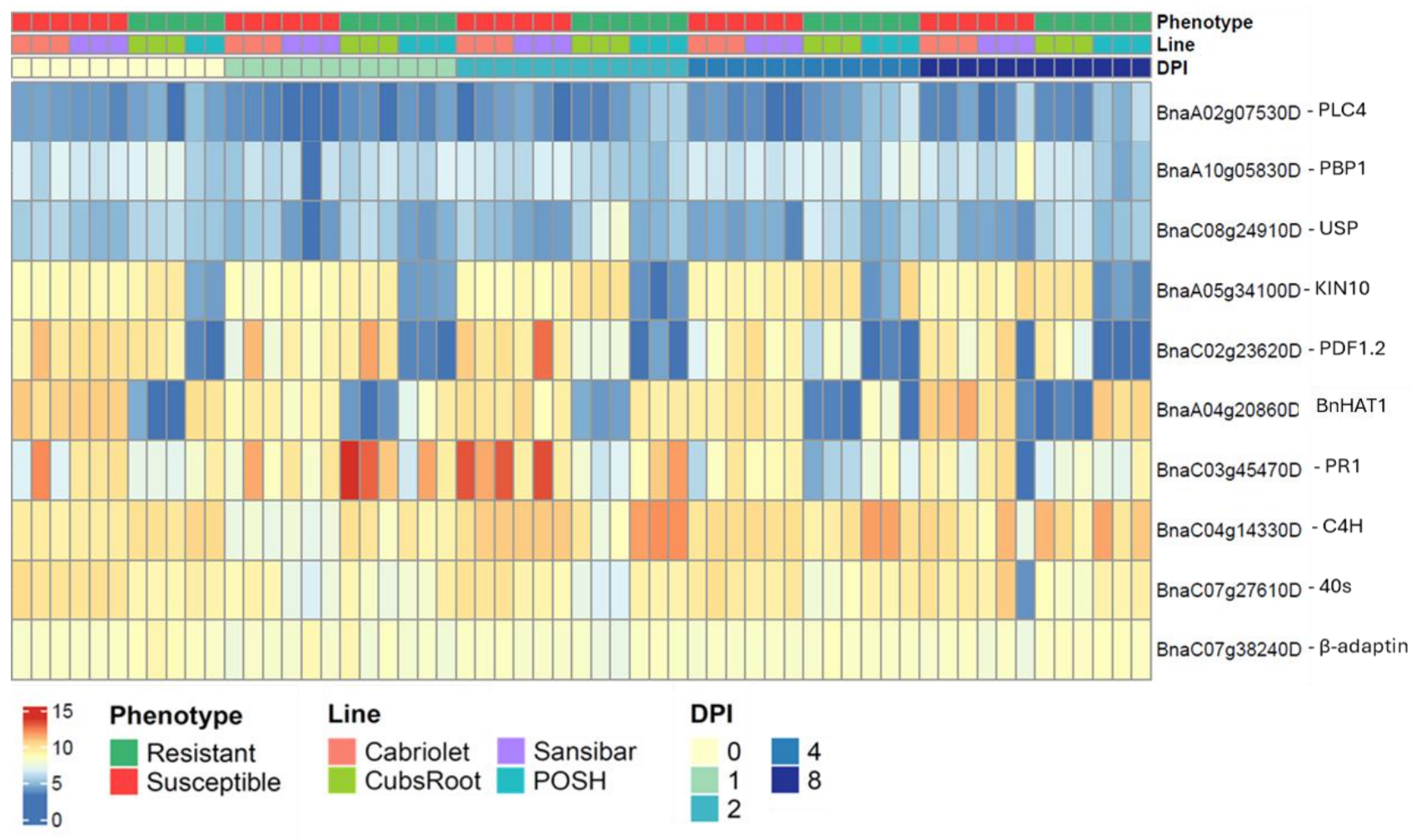


**Figure 4.15.** Heatmap representing the differential gene expression analysis of the 8 gene expression markers (GEMs) associated with *Brassica napus* host resistance to *Pyrenopeziza brassicae* (Fell *et al.*, 2023; Muthayil-Ali, 2023), and *PR1* (Pathogenesis related protein) and *PDF1.2* (Plant defensin 1.2) genes used as controls. Different *B. napus* lines/cultivars were inoculated with a *P. brassicae* population from Rothamsted Research (Muthayil-Ali, 2023) and samples were taken at different days post-inoculation (DPI). GEMs: Phosphatidylinositol-specific phospholipase C4 (*PLC4*), Polyadenylate-binding protein 1 (*PBP1*), Universal stress protein (*USP*), *SNF1* kinase homolog 10 (*KIN10*), BAHD acyltransferase (*BnHAT1*), Cinnamate-4-hydroxylase (*C4H*), 40s Ribosomal subunit protein S24e (40s).

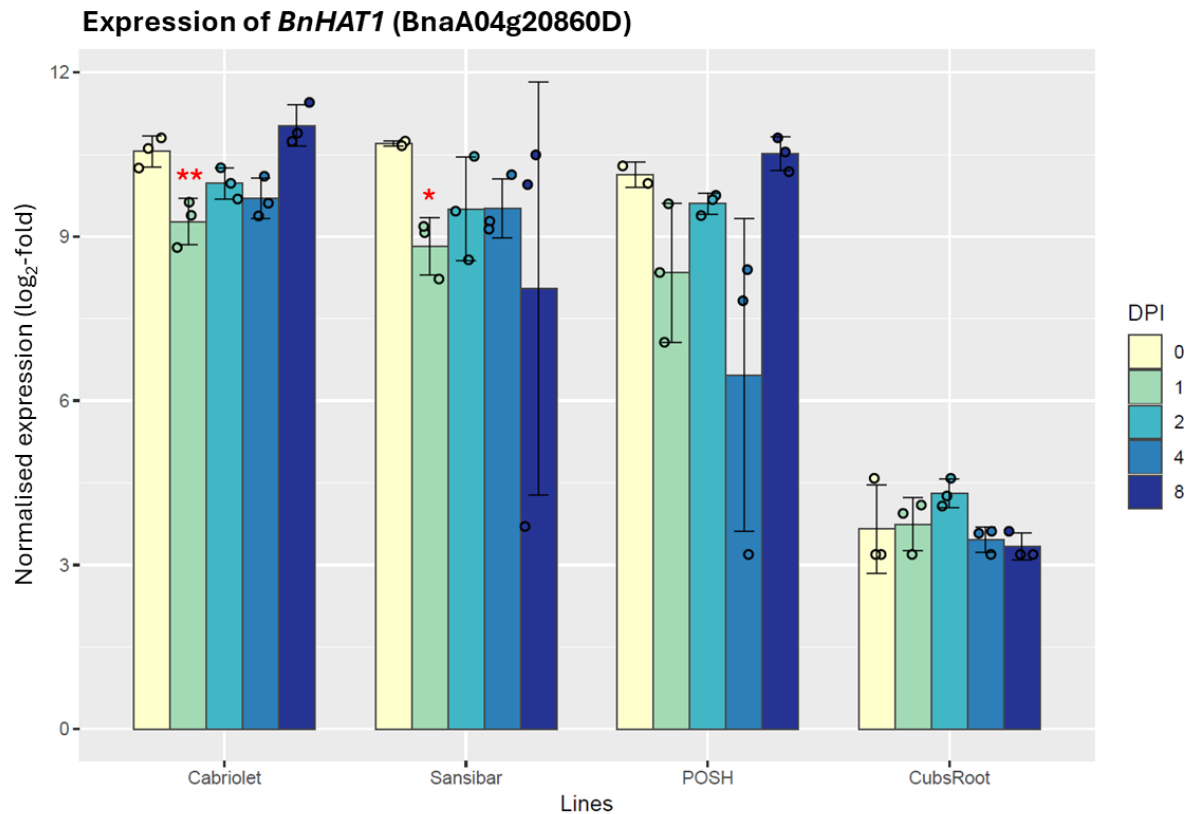


**Figure 4.16.** Regulated expression of *BnHAT1* (*BnaA04g20860D*) as evidenced using RNA-seq. Normalised expression was determined using DESeq2 and expressed as log<sub>2</sub>-fold. Susceptible (Cabriolet and Sansibar) and resistant (POSH and Cubs Root) cultivars/lines were inoculated with a *Pyrenopeziza brassicae* population from Rothamsted Research (Muthayil-Ali, 2023). At 0 days-post inoculation DPI (mock-inoculated control), 1 DPI, 2 DPI, 4 DPI and 8 DPI, total RNA was extracted from inoculated leaves and analysed using RNA-seq. Means and standard deviations of three biological replicates are shown. Significant differences between mean values of 0 DPI control and other inoculation time points within each genotype are indicated by red asterisks using statistical ANOVA and Tukey's HSD test results. \*\* $P$ -value  $\leq 0.01$ ; \* $P$ -value  $\leq 0.05$ .

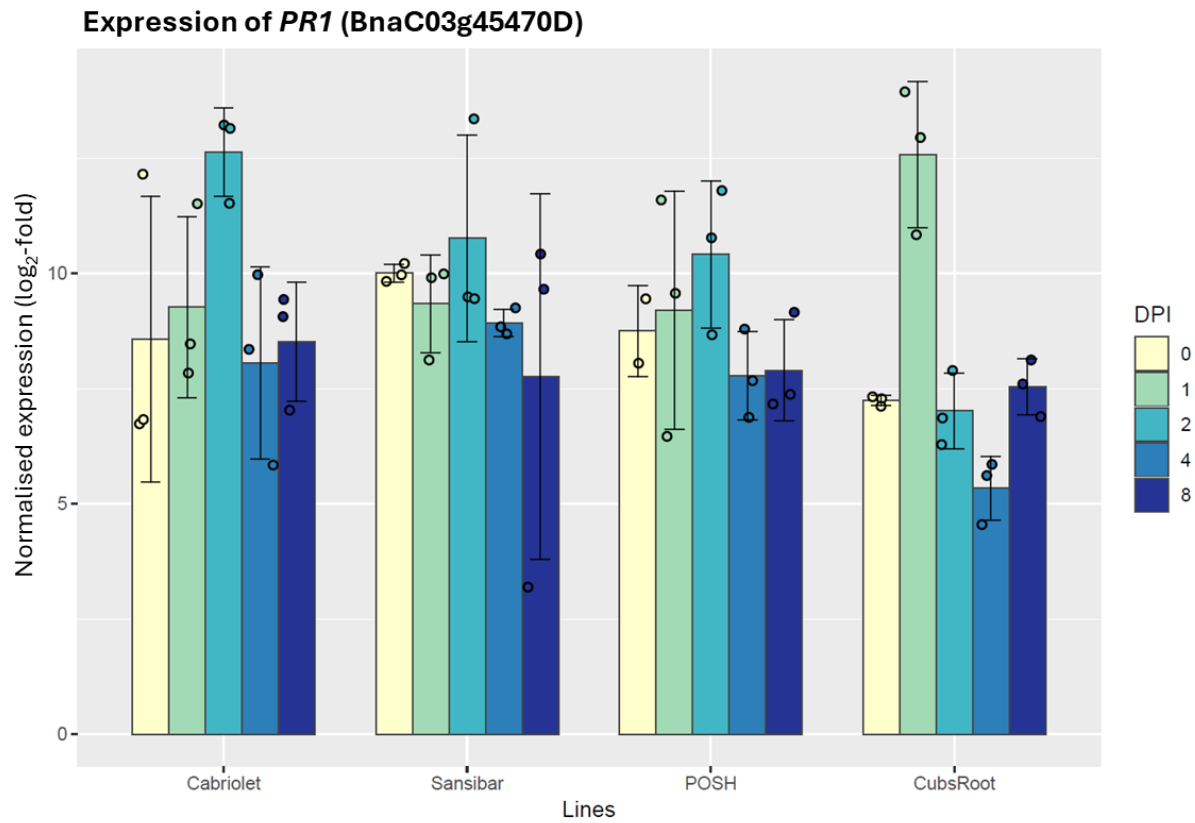


**Figure 4.17.** Regulated expression of *PR1* (BnaC03g45470D) as evidenced using RNA-seq. Normalised expression was determined using DESeq2 and expressed as log<sub>2</sub>-fold. Susceptible (Cabriolet and Sansibar) and resistant (POSH and Cubs Root) cultivars/lines were inoculated with a *Pyrenopeziza brassicae* population from Rothamsted Research (Muthayil-Ali, 2023). At 0 days-post inoculation DPI (mock-inoculated control), 1 DPI, 2 DPI, 4 DPI and 8 DPI, total RNA was extracted from inoculated leaves and analysed using RNA-seq. Means and standard deviations of three biological replicates are shown.

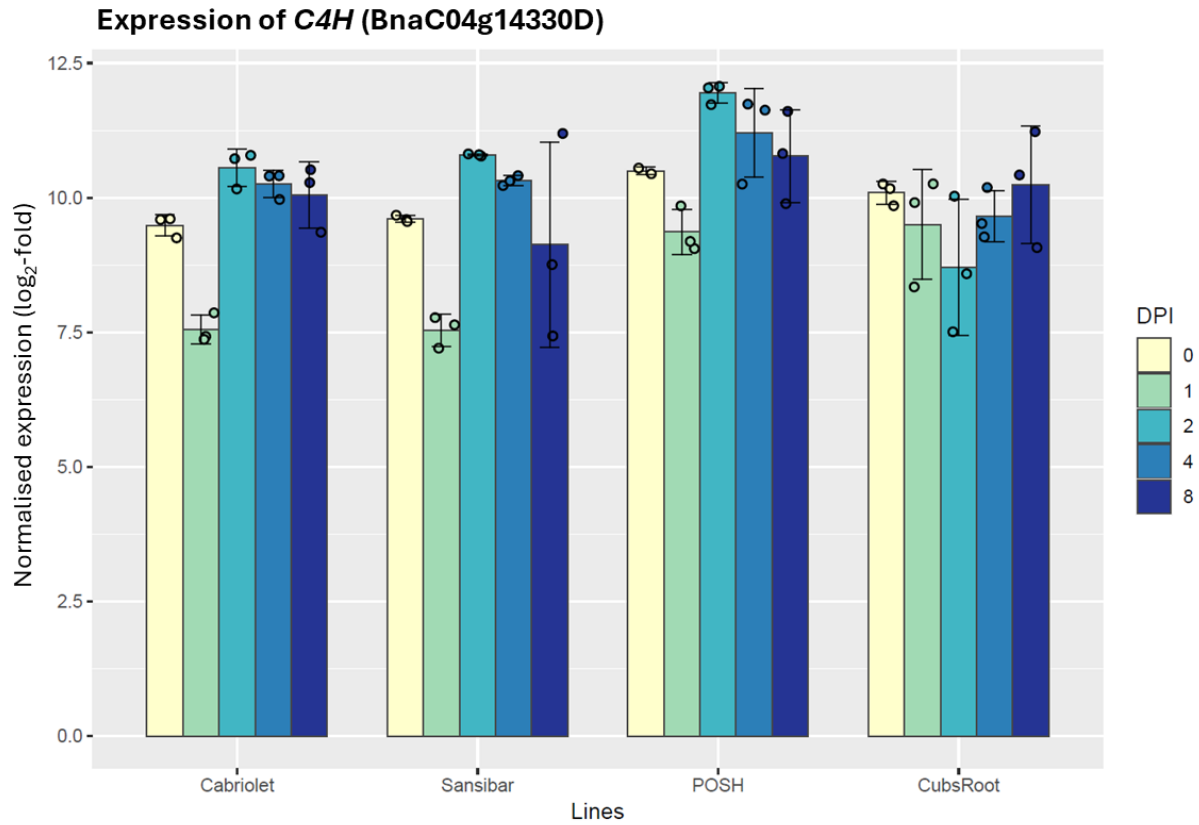


**Figure 4.18.** Regulated expression of *C4H* (BnaC04g14330D) as evidenced using RNA-seq. Normalised expression was determined using DESeq2 and expressed as  $\log_2$ -fold. Susceptible (Cabriolet and Sansibar) and resistant (POSH and Cubs Root) cultivars/lines were inoculated with a *Pyrenopeziza brassicae* population from Rothamsted Research (Muthayil-Ali, 2023). At 0 days-post inoculation DPI (mock-inoculated control), 1 DPI, 2 DPI, 4 DPI and 8 DPI, total RNA was extracted from inoculated leaves and analysed using RNA-seq. Means and standard deviations of three biological replicates are shown.

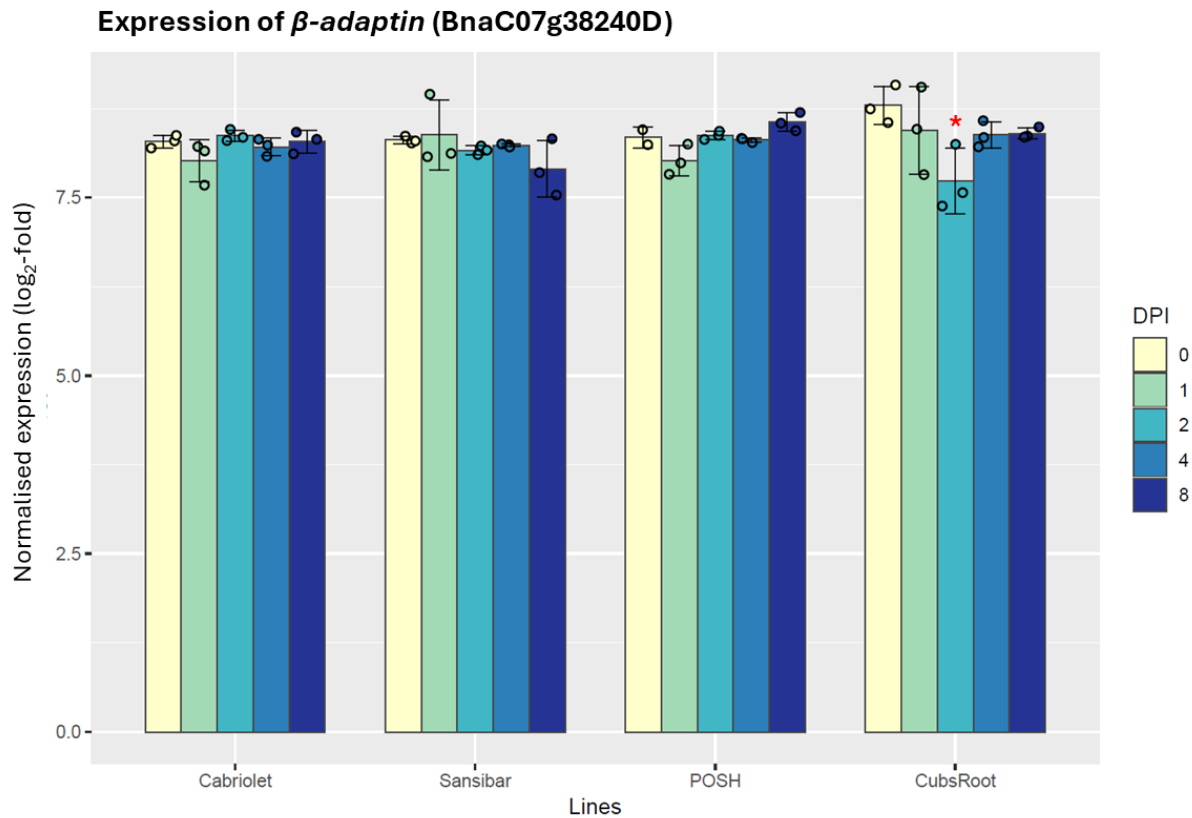


**Figure 4.19.** Regulated expression of  $\beta$ -adaptin (BnaC07g38240D) as evidenced using RNA-seq. Normalised expression was determined using DESeq2 and expressed as log<sub>2</sub>-fold. Susceptible (Cabriolet and Sansibar) and resistant (POSH and Cubs Root) cultivars/lines were inoculated with a *Pyrenopeziza brassicae* population from Rothamsted Research (Muthayil-Ali, 2023). At 0 days-post inoculation DPI (mock-inoculated control), 1 DPI, 2 DPI, 4 DPI and 8 DPI, total RNA was extracted from inoculated leaves and analysed using RNA-seq. Means and standard deviations of three biological replicates are shown. Significant differences between mean values of 0 DPI control and other inoculation time points within each genotype are indicated by red asterisks using statistical ANOVA and Tukey's HSD test results. \* $P$ -value $\leq$ 0.05.

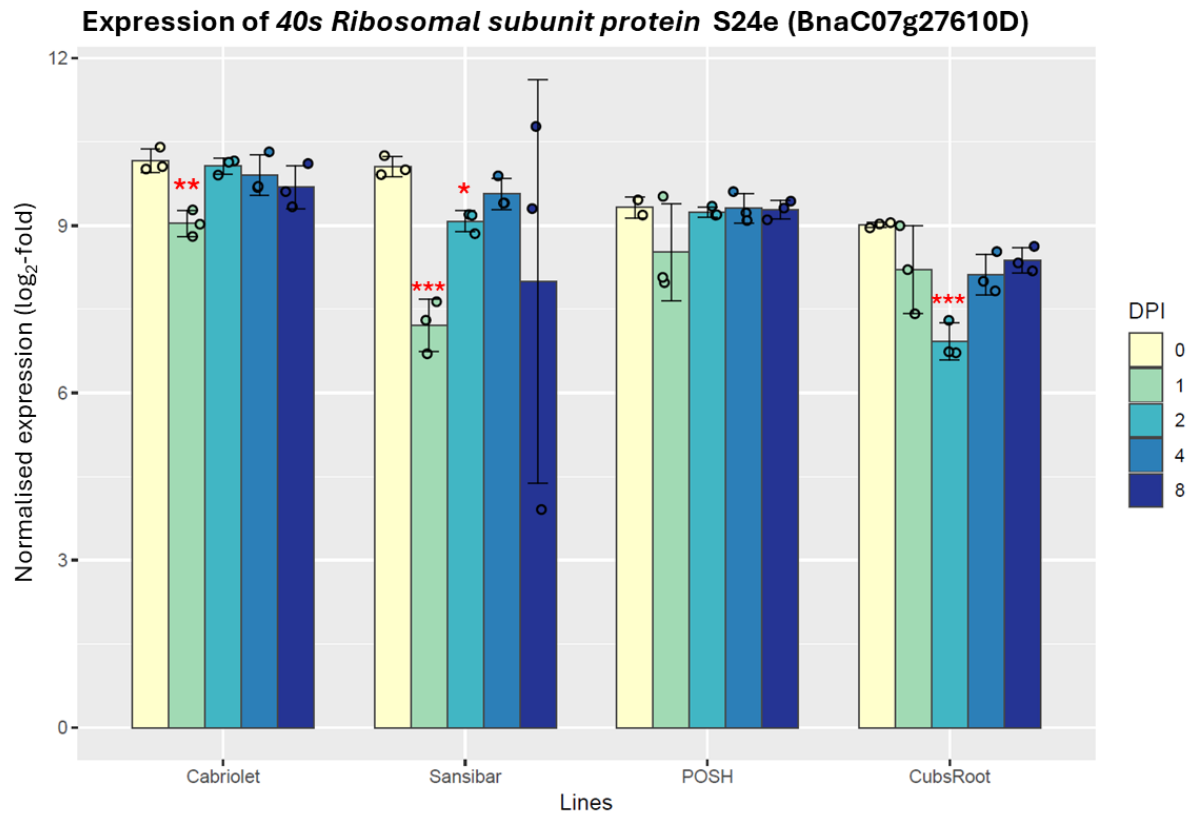


**Figure 4.20.** Regulated expression of 40S Ribosomal subunit protein S24e (BnaC07g27610D) as evidenced using RNA-seq. Normalised expression was determined using DESeq2 and expressed as log<sub>2</sub>-fold. Susceptible (Cabriolet and Sansibar) and resistant (POSH and Cubs Root) cultivars/lines were inoculated with a *Pyrenopeziza brassicae* population from Rothamsted Research (Muthayil-Ali, 2023). At 0 days-post inoculation DPI (mock-inoculated control), 1 DPI, 2 DPI, 4 DPI and 8 DPI, total RNA was extracted from inoculated leaves and analysed using RNA-seq. Means and standard deviations of three biological replicates are shown. Significant differences between mean values of 0 DPI control and other inoculation time points within each genotype are indicated by red asterisks using statistical ANOVA and Tukey's HSD test results. \*\*\* $P$ -value $\leq$ 0.001; \*\* $P$ -value $\leq$ 0.01; \* $P$ -value $\leq$ 0.05.

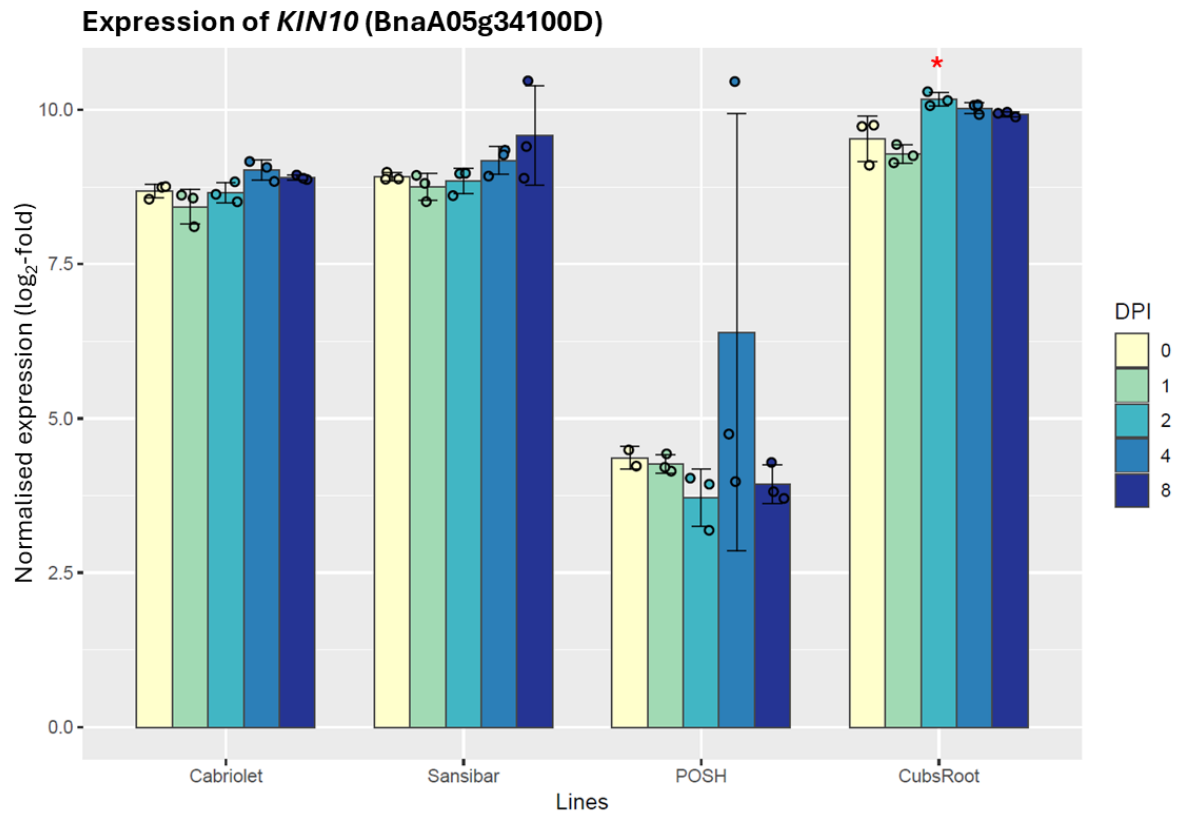


**Figure 4.21.** Regulated expression of *KIN10* (BnaA05g34100D) as evidenced using RNA-seq. Normalised expression was determined using DESeq2 and expressed as  $\log_2$ -fold. Susceptible (Cabriolet and Sansibar) and resistant (POSH and Cubs Root) cultivars/lines were inoculated with a *Pyrenopeziza brassicae* population from Rothamsted Research (Muthayil-Ali, 2023). At 0 days-post inoculation DPI (mock-inoculated control), 1 DPI, 2 DPI, 4 DPI and 8 DPI, total RNA was extracted from inoculated leaves and analysed using RNA-seq. Means and standard deviations of three biological replicates are shown. Significant differences between mean values of 0 DPI control and other inoculation time points within each genotype are indicated by red asterisks using statistical ANOVA and Tukey's HSD test results. \* $P$ -value $\leq$ 0.05.

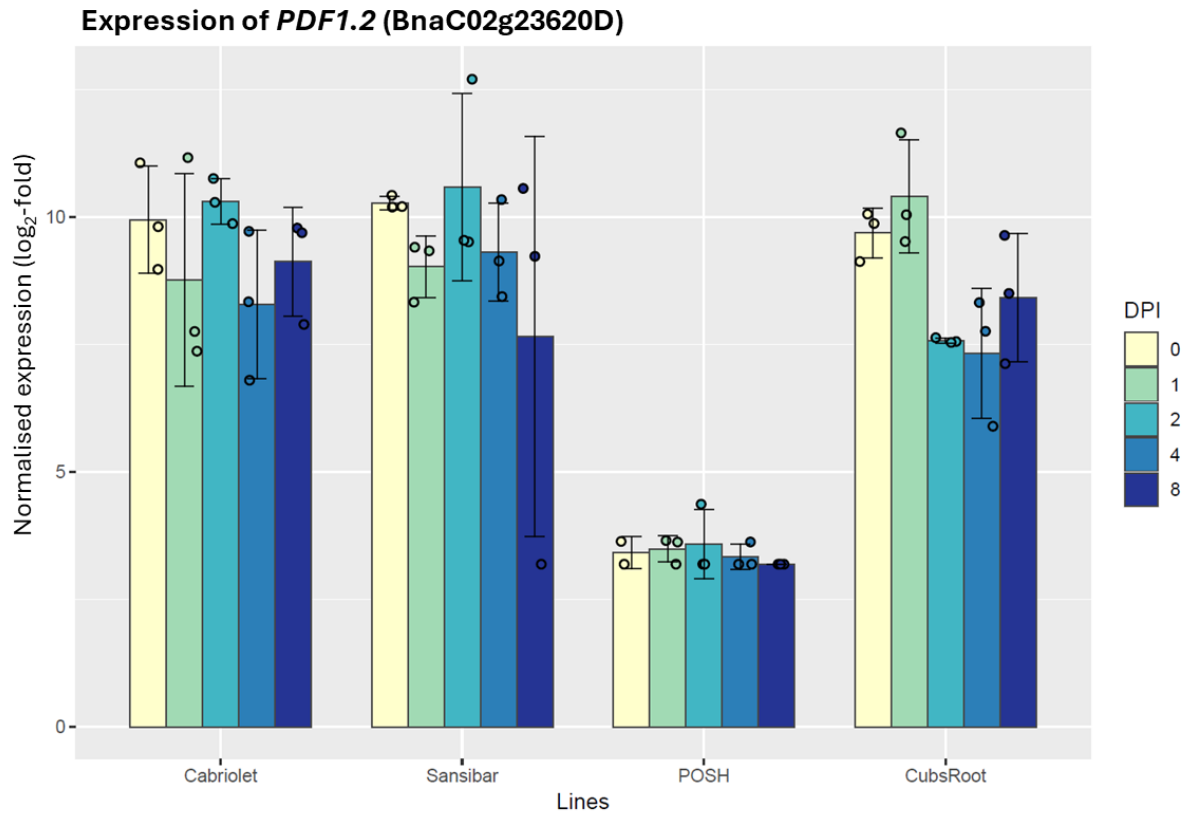


**Figure 4.22.** Regulated expression of *PDF 1.2* (BnaC02g23620D) as evidenced using RNA-seq. Normalised expression was determined using DESeq2 and expressed as log<sub>2</sub>-fold. Susceptible (Cabriolet and Sansibar) and resistant (POSH and Cubs Root) cultivars/lines were inoculated with a *Pyrenopeziza brassicae* population from Rothamsted Research (Muthayil-Ali, 2023). At 0 days-post inoculation DPI (mock-inoculated control), 1 DPI, 2 DPI, 4 DPI and 8 DPI, total RNA was extracted from inoculated leaves and analysed using RNA-seq. Means and standard deviations of three biological replicates are shown.

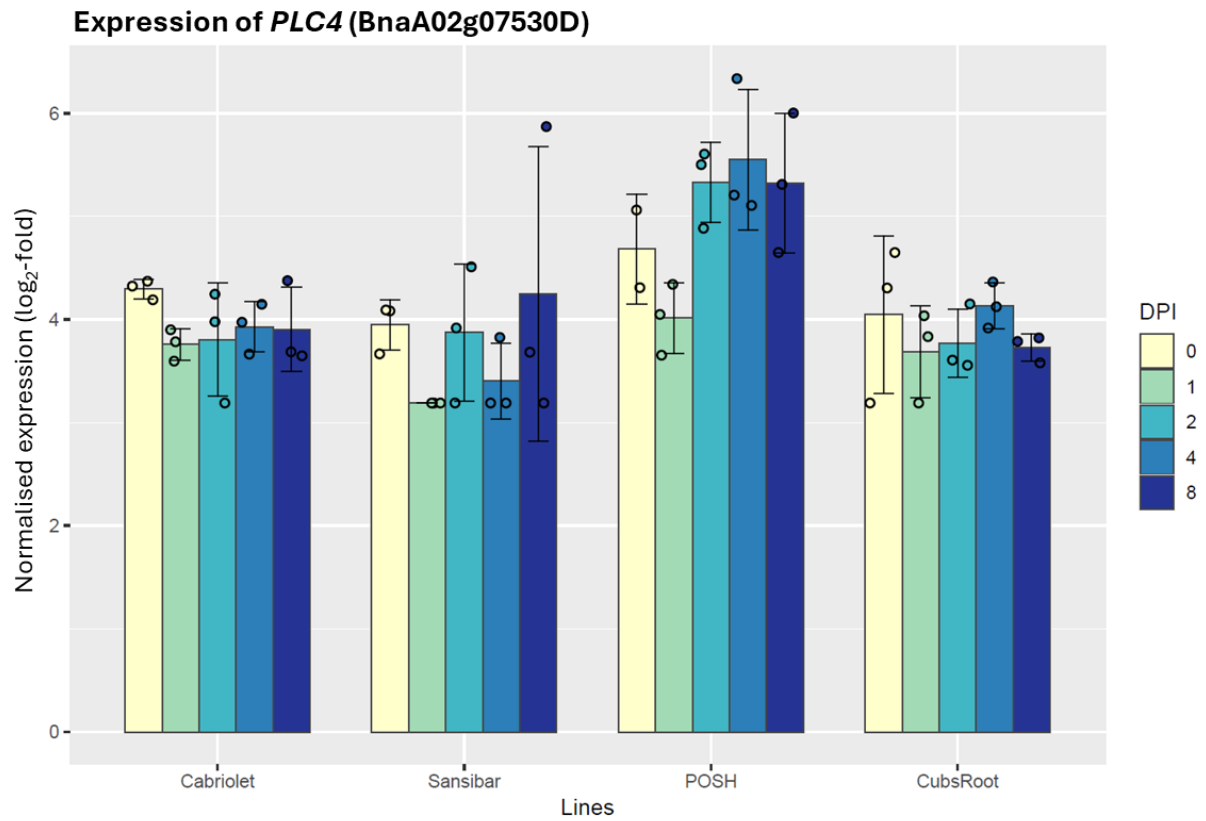


**Figure 4.23.** Regulated expression of *PLC4* (BnaA02g07530D) as evidenced using RNA-seq. Normalised expression was determined using DESeq2 and expressed as log<sub>2</sub>-fold. Susceptible (Cabriolet and Sansibar) and resistant (POSH and Cubs Root) cultivars/lines were inoculated with a *Pyrenopeziza brassicae* population from Rothamsted Research (Muthayil-Ali, 2023). At 0 days-post inoculation DPI (mock-inoculated control), 1 DPI, 2 DPI, 4 DPI and 8 DPI, total RNA was extracted from inoculated leaves and analysed using RNA-seq. Means and standard deviations of three biological replicates are shown.

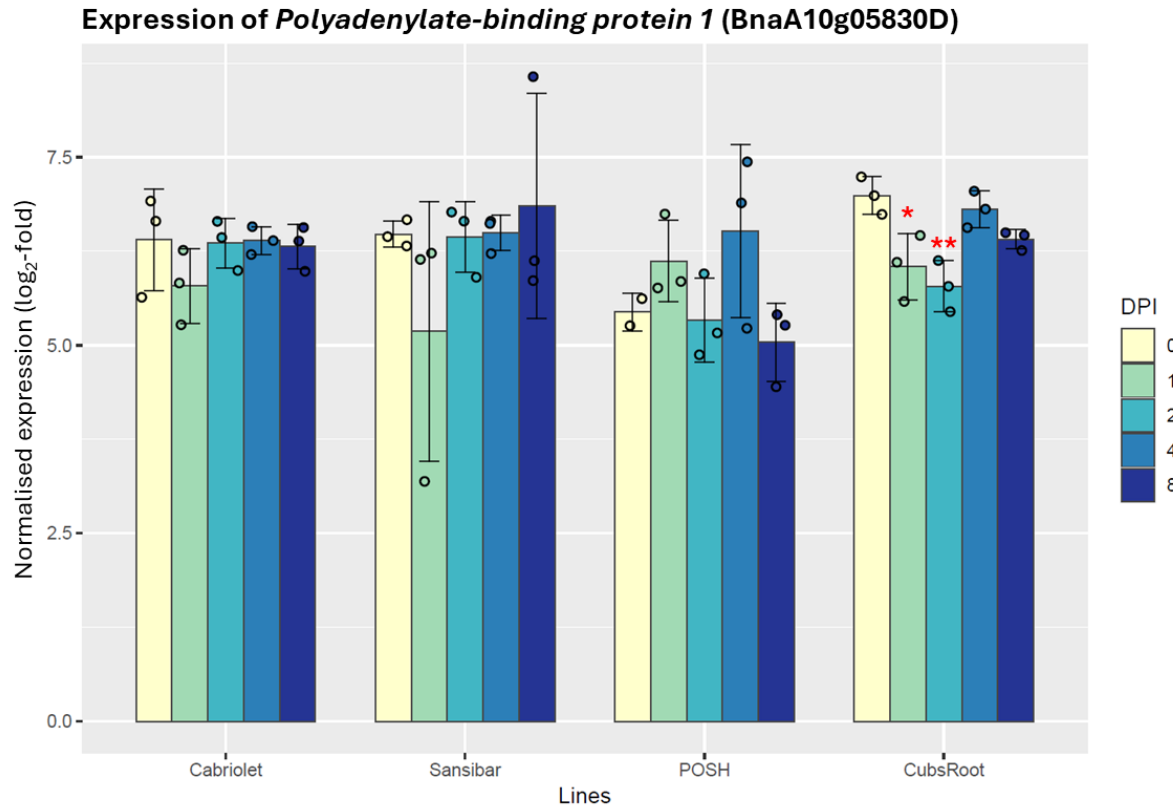


**Figure 4.24.** Regulated expression of *PBP 1* (BnaA10g05830D) as evidenced using RNA-seq. Normalised expression was determined using DESeq2 and expressed as  $\log_2$ -fold. Susceptible (Cabriolet and Sansibar) and resistant (POSH and Cubs Root) cultivars/lines were inoculated with a *Pyrenopeziza brassicae* population from Rothamsted Research (Muthayil-Ali, 2023). At 0 days-post inoculation DPI (mock-inoculated control), 1 DPI, 2 DPI, 4 DPI and 8 DPI, total RNA was extracted from inoculated leaves and analysed using RNA-seq. Means and standard deviations of three biological replicates are shown. Significant differences between mean values of 0 DPI control and other inoculation time points within each genotype are indicated by red asterisks using statistical ANOVA and Tukey's HSD test results. \*\* $P$ -value $\leq$ 0.01; \* $P$ -value $\leq$ 0.05.

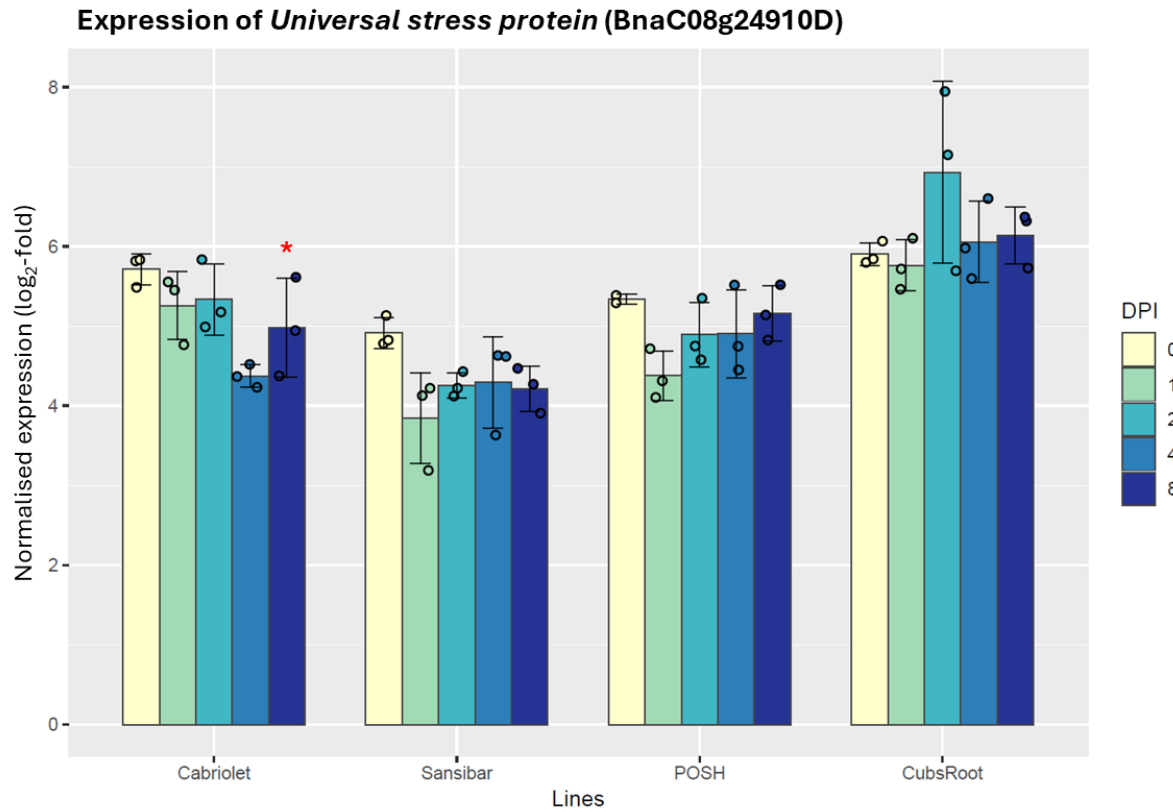


**Figure 4.25.** Regulated expression of *Universal stress protein* (BnaC08g24910D) as evidenced using RNA-seq. Normalised expression was determined using DESeq2 and expressed as log<sub>2</sub>-fold. Susceptible (Cabriolet and Sansibar) and resistant (POSH and Cubs Root) cultivars/lines were inoculated with a *Pyrenopeziza brassicae* population from Rothamsted Research (Muthayil-Ali, 2023). At 0 days-post inoculation DPI (mock-inoculated control), 1 DPI, 2 DPI, 4 DPI and 8 DPI, total RNA was extracted from inoculated leaves and analysed using RNA-seq. Means and standard deviations of three biological replicates are shown. Significant differences between mean values of 0 DPI control and other inoculation time points within each genotype are indicated by red asterisks using statistical ANOVA and Tukey's HSD test results. \* $P$ -value $\leq$ 0.05.

**Table 4.7.** Measurement of the concentration (conc.) and A260/280 ratio of the RNA samples extracted from different *Brassica napus* genotypes (cvs Abukuma, Lipid and Cabriolet, and TILLING mutant line 2A). The plants were inoculated with a *Pyrenopeziza brassicae* population from Rothamsted Research (Muthayil-Ali, 2023) and samples were taken at different inoculation time points or days post-inoculation (DPI). Each treatment had four biological replicates (A, B, C and D). Nanodrop ND-1000 spectrophotometer was used for the quantification.

| Genotype             | DPI | Bio replicate A  |              | Bio replicate B  |              | Bio replicate C  |              | Bio replicate D  |              |
|----------------------|-----|------------------|--------------|------------------|--------------|------------------|--------------|------------------|--------------|
|                      |     | Conc.<br>(ng/μL) | A260/<br>280 | Conc.<br>(ng/μL) | A260/<br>280 | Conc.<br>(ng/μL) | A260/<br>280 | Conc.<br>(ng/μL) | A260/<br>280 |
| Abukuma              | 0   | 155.0            | 2.14         | 140.0            | 2.13         | 93.1             | 2.13         | 230.8            | 2.15         |
|                      | 1   | 235.1            | 2.15         | 72.0             | 2.12         | 229.5            | 2.13         | 117.3            | 2.15         |
|                      | 2   | 168.9            | 2.14         | 188.7            | 2.14         | 230.1            | 2.14         | 94.8             | 2.15         |
|                      | 4   | 57.8             | 2.13         | 73.5             | 2.12         | 78.4             | 2.24         | 110.1            | 2.08         |
|                      | 8   | 75.8             | 2.15         | 56.4             | 2.18         | 62.0             | 2.15         | 95.0             | 2.21         |
|                      | 12  | 130.5            | 2.14         | 99.6             | 2.10         | 218.5            | 2.14         | 210.3            | 2.13         |
|                      | 16  | 124.1            | 2.16         | 65.3             | 2.14         | 135.4            | 2.11         | 177.6            | 2.15         |
|                      | 21  | 97.0             | 2.11         | 112.5            | 2.12         | 125.7            | 2.15         | 157.4            | 2.16         |
| Lipid                | 0   | 151.2            | 2.15         | 80.0             | 2.13         | 122.5            | 2.15         | 177.8            | 2.17         |
|                      | 1   | 197.9            | 2.15         | 56.7             | 2.13         | 84.2             | 2.12         | 201.2            | 2.16         |
|                      | 2   | 150.6            | 2.13         | 88.6             | 2.17         | 140.6            | 2.13         | 163.5            | 2.15         |
|                      | 4   | 200.0            | 2.14         | 127.6            | 2.15         | 120.9            | 2.09         | 158.7            | 2.17         |
|                      | 8   | 156.7            | 2.12         | 113.9            | 2.15         | 118.0            | 2.11         | 146.0            | 2.17         |
|                      | 12  | 100.4            | 2.15         | 108.6            | 2.14         | 98.0             | 2.11         | 203.1            | 2.16         |
|                      | 16  | 132.3            | 2.14         | 98.4             | 2.14         | N/A              | N/A          | N/A              | N/A          |
|                      | 21  | 118.6            | 2.11         | 55.9             | 2.20         | 118.2            | 2.10         | 124.4            | 2.16         |
| Cabriolet            | 0   | 84.4             | 2.09         | 143.2            | 2.13         | 150.6            | 2.14         | 110.0            | 2.11         |
|                      | 1   | 53.2             | 2.16         | 60.6             | 2.12         | 110.3            | 2.14         | 120.6            | 2.11         |
|                      | 2   | 80.3             | 1.99         | 100.0            | 2.15         | 95.6             | 2.14         | 130.8            | 2.12         |
|                      | 4   | 80.0             | 2.16         | 90.5             | 2.13         | 120.4            | 2.12         | 78.9             | 2.12         |
|                      | 8   | 70.6             | 2.17         | 108.8            | 2.12         | 70.8             | 2.07         | 100.5            | 2.10         |
|                      | 12  | N/A              | N/A          | 200.7            | 2.14         | 150.6            | 2.13         | 170.6            | 2.17         |
|                      | 16  | 50.8             | 2.13         | 110.2            | 2.12         | 100.3            | 2.07         | 90.5             | 2.08         |
|                      | 21  | 101.6            | 2.19         | 100.4            | 2.14         | 107.4            | 2.09         | 70.3             | 2.12         |
| TILLING<br>mutant 2A | 0   | 116.1            | 2.15         | 190.2            | 2.14         | 125.0            | 2.09         | 151.0            | 2.13         |
|                      | 1   | 280.3            | 2.14         | 193.7            | 2.14         | 214.9            | 2.12         | 286.9            | 2.16         |
|                      | 2   | 192.4            | 2.15         | 134.0            | 2.13         | 209.7            | 2.14         | 163.1            | 2.16         |
|                      | 4   | 138.2            | 2.11         | 10.4             | 2.17         | 200.1            | 2.14         | 246.5            | 2.18         |
|                      | 8   | 208.9            | 2.13         | 116.6            | 2.13         | 120.7            | 2.12         | 179.5            | 2.17         |
|                      | 12  | 185.8            | 2.11         | 189.6            | 2.15         | 218.2            | 2.13         | 208.6            | 2.18         |
|                      | 16  | 133.6            | 2.11         | 118.1            | 2.14         | 132.2            | 2.15         | 140.3            | 2.17         |
|                      | 21  | 173.3            | 2.15         | 141.1            | 2.14         | 91.8             | 2.04         | 127.8            | 2.17         |

N/A: not available.

#### 4.3.7. Structural modelling of the BnHAT1 protein

The BnHAT1 acyltransferase protein of interest was further studied. According to the UniProt and the AlphaFold Protein Structure databases, the *BnaA04g20860D* protein (A0A078HRT5) has a length of 450 amino acids, with a mass of 49,544 Daltons. The three-dimensional structure of this protein was predicted in the AlphaFold database, with a pLDDT of 88.5 out of 100, which indicates a high confidence in the model. The protein contains two domains, which can be seen in Figure 4.26A in the predicted 3D structure of the protein, with its corresponding amino acid sequences (Figure 4.26B). The N-terminal domain, displayed in green, is formed of 208 amino acids, from positions 6 to 213, and has a pLDDT of 91.83. The HXXXD motif can be found in this domain as HLVTD, as well as all the SNP mutations in the TILLING mutants of interest (lines 2, 3, 4 and 6). The second domain, in orange, is 218 amino acids long, from position 230 to 447, with a pLDDT of 88.63. The DFGWG motif associated with BAHD enzymes can be found in this domain.

The protein has a channel that runs through the enzyme. The HXXXD motif, highlighted in pink in Figure 4.26A, was located inside this channel as part of a short turn segment between a  $\beta$ -sheet and an  $\alpha$ -helix, reversing the direction of the polypeptide chain. The histidine residue (H) of the motif is close to the potential active site, whereas the aspartic acid residue (D) points in the opposite direction. This coincided with the literature that proposed this as the reaction channel that allows access from both sides for substrates and donors (Ma *et al.*, 2005) and proposed the catalytic role of the histidine residue and the structural role of the aspartic acid residue (Ma *et al.*, 2005; Levsh *et al.*, 2016).

Closer examination of the 3D structure revealed that the aspartic acid residue (D) from the HXXXD motif at position 167 was interacting with an arginine residue (R) in position 293 (Figure 4.27A). This interaction constitutes a salt bridge between the two amino acids, a strong non-covalent interaction that combines hydrogen bonding and electrostatic attraction and is commonly associated with protein structure stability. The salt bridge between 167D and 293R is established between the negatively charged carboxylate group ( $\text{COO}^-$ ) of the aspartic acid and the positively charged guanidinium group ( $\text{NHC}(\text{NH}_2)^{+2}$ ) of the arginine (Figure 4.27C). This also coincided with Ma *et al.* (2005), who suggested this interaction is most likely to be important for maintaining the geometry of the active site.

AlphaFold3 was used to predict the structure that would result from TILLING mutant 2, with an asparagine in place of the aspartic acid at position 167. The scores for pTM and ipTM were 0.92 for both in a 0-1 range, a very high value that showed the high confidence of the predicted model. The model showed that the salt bridge between 167D and 293R in the WT protein no longer existed in TILLING mutant line 2 due to the substitution of the aspartic acid for an asparagine (D167N). The chemical group of the asparagine that would be close enough to arginine to establish a potential interaction is a carboxamide group ( $\text{CONH}_2$ ), which is neutrally charged (Figure 4.27C). Thus, the residues cannot establish hydrogen bonds or electrostatic attraction between them, which are necessary for salt bridges.

This suggested that the change in amino acid (D167N) and consequent lack of a salt bridge connecting to 293R had a structural impact that altered the geometry of the active site. This could have resulted in the enzyme activity being affected which, in turn, may relate to the phenotypic difference between WT *B. napus* and TILLING mutant line 2 that have been previously explored in

Chapter 4.3.3. This supports the hypothesis that the *BnHAT1* gene and protein are, in fact, linked to susceptibility to fungal pathogen *P. brassicae* because mutant plants were more resistant against the pathogen than WT ones. Furthermore, this supported the idea of the aspartic acid residue playing a structural role for the active site of the enzyme as part of the HXXXD motif.

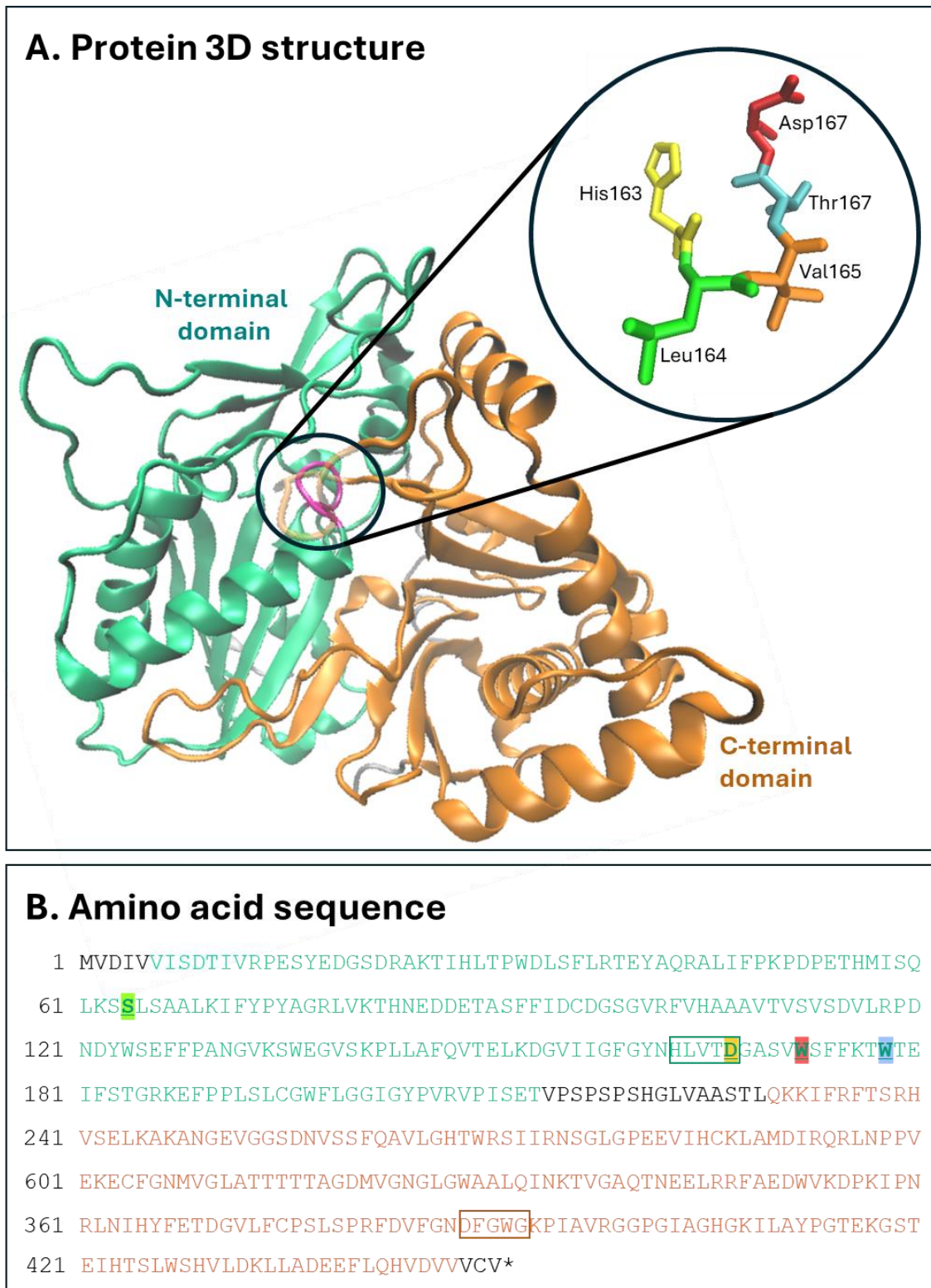


**Figure 4.26.** BAHD acyltransferase (BnHAT1) protein encoded by the *BnHAT1* gene (*BnaA04g20860D*), showing the N-terminal domain in green and the C-terminal domain in orange. **A)** 3D structure of the protein predicted by AlphaFold3. The HXXXD motif is displayed in pink, highlighting its residues. **B)** Amino acid sequence of the protein, showing the conserved domains HXXXD and DFGWG, as well as all the mutations in TILLING mutant lines (line 2 is displayed in orange, line 3 in red, line 4 in blue and line 6 in green).

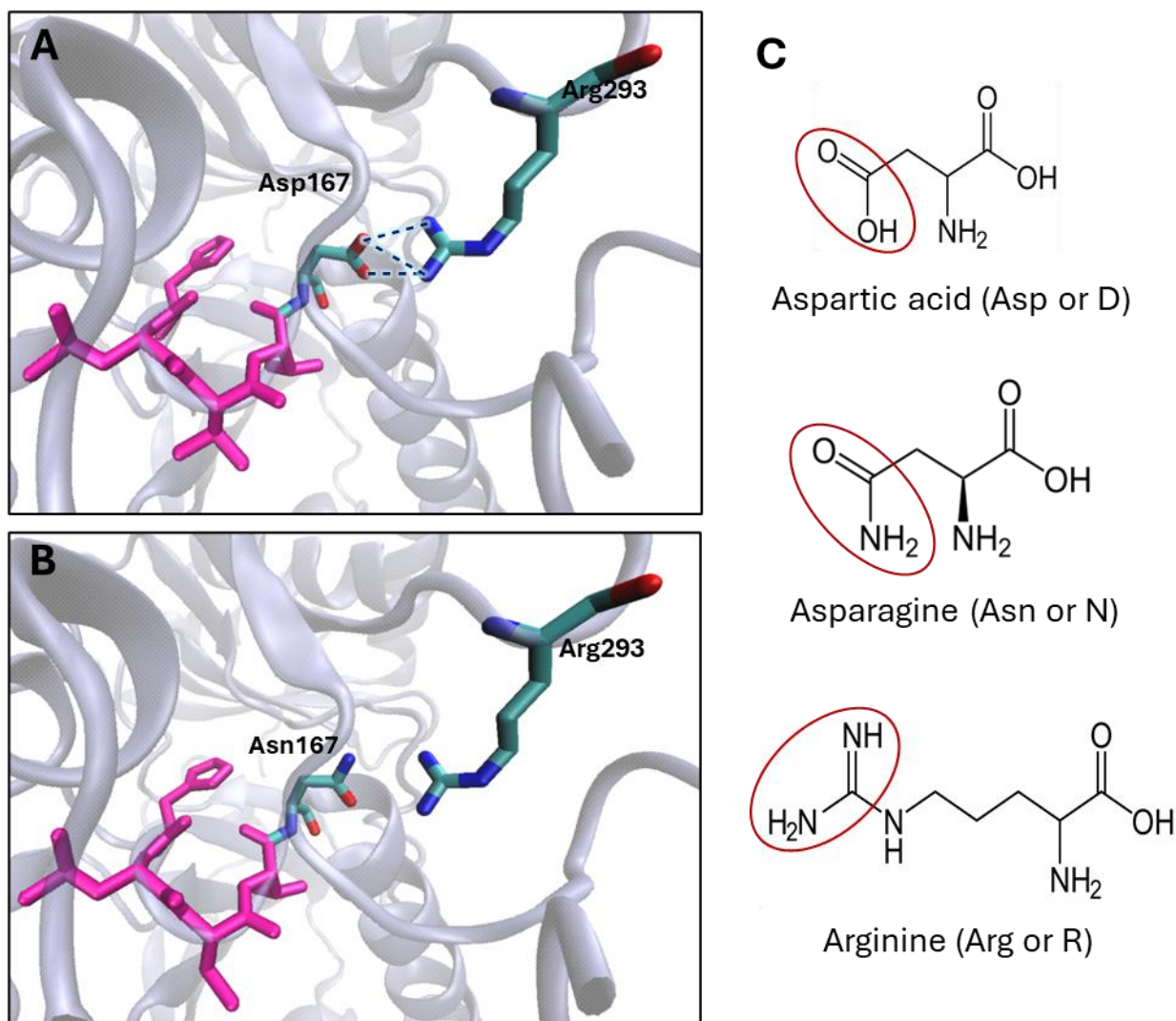


**Figure 4.27.** Details of the molecular structure of BnHAT1. **A)** Molecular interaction between the aspartic acid residue in position 167 that forms part of the HXXXD motif (displayed in pink) and the arginine residue in position 293. The dotted lines represent hydrogen bonds between the carboxylate group of 167D and the guanidinium group of the arginine. **B)** The asparagine residue that substituted the aspartic acid in TILLING mutant line 2 does not form hydrogen bonds that constitute the salt bridge with the Arg293. **C)** Skeletal formula of the three amino acids of interest, emphasising the chemical groups that could interact with other residues: a carboxylate group for the aspartic acid, a carboxamide group for asparagine and a guanidinium group for arginine.

## **4.4. Discussion**

### **4.4.1 Characterisation of the *BnHAT1* gene**

The *BnHAT1* gene, which was originally identified through associative transcriptomics as a GEM that appeared to be associated with susceptibility to *P. brassicae* (Fell *et al.*, 2023; Muthayil-Ali, 2023), was thoroughly studied in this chapter from different approaches to characterise its function and study host susceptibility.

The genotype of *B. napus* TILLING mutant lines 2A and 3B from the M4 generation, carrying SNP mutations in the first exon of the *BnHAT1* gene, was compared to its susceptibility phenotype after *P. brassicae* inoculation in controlled environment conditions. TILLING mutant 2A individuals, homozygous for a SNP mutation (D167N), were significantly more resistant than cv. Cabriolet individuals, that carried the WT allele for the *BnHAT1* gene. This suggested that the function of this gene was linked to susceptibility to the pathogen because its mutation made the host more resistant to the disease.

TILLING mutant line 3B heterozygous for a SNP mutation (W172X), producing a stop codon that significantly disrupted and shortened the protein encoded by the *BnHAT1* gene. The segregating population obtained from selfing this line produced individuals with three different genotypes: homozygous for the mutant allele, homozygous for the WT allele and heterozygous. When comparing the genotype of these plants and the susceptibility phenotype after *P. brassicae* inoculation, there was a clear pattern of segregation. Homozygotes for the mutant allele were significantly more resistant than homozygotes for the WT allele, whereas heterozygotes showed an intermediate phenotype of disease severity. It is likely that the SNP mutation, that drastically interrupted the amino acid sequence, substantially altered the BnHAT1 protein structure and, consequently, its function. Therefore, it could be asserted that the *BnHAT1* gene plays a role in susceptibility to the fungal pathogen because the intact gene produced individuals with lower resistance than those with the mutant allele.

The study of these two *B. napus* TILLING mutant lines carrying different SNP mutations supported the proposed hypothesis that the function of the *BnHAT1* gene is associated to susceptibility to *P. brassicae*. To further investigate TILLING mutants, the M4 plants were selfed to obtain the M5 generation in glasshouse facilities at the John Innes Centre. Additionally, TILLING mutant lines 2A and 6A from the M4 generation, that were homozygous for the mutant allele, were selected to be backcrossed with cv. Cabriolet to eliminate background mutations. Dr Rachel Wells from the John Innes Centre will continue this gene characterisation study.

### **4.4.2 Gene discovery**

The transcriptome of four *B. napus* cultivars with different resistance phenotypes, inoculated at different time points with a *P. brassicae* population (Muthayil-Ali, 2023), was analysed using RNA-seq analysis. A volcano plot that used all data points from all four *B. napus* lines and time points revealed multiple genes that were differentially expressed across the genotypes and treatments (Tables 4.5 and 4.6). The biological function of three of the upregulated genes (*BnaA01g03020D*, *BnaA09g13870D* and *BnaA08g16100D*) and their *A. thaliana* orthologues (*UGT73B3*, *TPS04* and *CYP79B2*) was associated with host resistance to different pests and

pathogens, which suggested that these genes in *B. napus* could potentially be linked to resistance against *P. brassicae*.

The UDP-glycosyltransferase activity of the *UGT73B3* gene in *A. thaliana* proved to catalyse the glycosylation of pinoresinol to form pinoresinol diglucosides (PDG), which plays an important role in promoting plant immunity by increasing callose deposition at the cell wall, which seals off intercellular junction points. This enhances the effect of the physical barrier against pathogens, restricting pathogen movement (Zhao *et al.*, 2025). The expression of this gene is induced by SA (Langlois-Meurinne *et al.*, 2005).

*CYP79B2* was particularly relevant due to its monooxygenase activity, that has been associated with managing the intense oxidative stress often caused by pathogen infections, controlling the levels of ROS and maintaining redox balance. In addition, this gene was linked with the synthesis of camalexin in *A. thaliana*, a phytoalexin significantly associated with resistance against *S. sclerotiorum* (Stotz *et al.*, 2011). Its expression was induced by JA, independent from SA signalling pathways.

The *TPS04* gene encoded a lyase enzyme associated with terpenoid biosynthesis (Herde *et al.*, 2008). Terpenoids are the largest group of secondary metabolites in plants and have been well-studied, defined as crucial for plant defence against multiple pathogens (Li *et al.*, 2023). Upon pathogen attack, its expression is induced by JA signalling pathways. The *TPS04* enzyme is responsible for the synthesis of diterpene (E,E)-geranylinalool, which is a precursor for homoterpene TMTT (4,8,12-trimethyltrideca-1,3,7,11-tetraene). It was reported that volatile TMTT was emitted as an indirect defence mechanism to attract the natural predators of the herbivores attacking the host. This was observed in *Plutella xylostella* larvae feeding on *A. thaliana*, which seemed to trigger this resistance pathway (Herde *et al.*, 2008).

Interestingly, gene expression regulated by jasmonic acid seemed to have opposite effects in the *TPS04* and the *PDF1.2* gene. Reports have demonstrated that these two genes are induced by JA (Herde *et al.*, 2008; Penninckx *et al.*, 1996); however, in this experiment, the expression of *TPS04* was upregulated whereas *PDF1.2* was downregulated (Figure 4.13). These contrasting gene expression patterns could be explained by the study that demonstrated that, for *PDF1.2* activation in *A. thaliana* upon pathogen infection, JA did not suffice. It required both JA and ethylene signalling pathways, triggered concomitantly, not sequentially, for the activation of the gene (Penninckx *et al.*, 1998). This would resolve why, even though the expression of both *TPS04* and *PDF1.2* are induced by JA, the first gene was upregulated whereas the second one was downregulated. Alternatively, this differential gene expression could be a result of the conflict between JA and SA, a well-documented antagonistic interaction. Some of the genes found in this analysis were induced by SA (*UGT73B3*) and some by JA (*TPS04*, *PDF1.2*), therefore the presence of both hormones could have had a complex effect in gene regulation, resulting in unexpected expression patterns. This could be further confirmed in *B. napus* by performing a metabolic assay that could measure the concentration of hormones after *P. brassicae* inoculation over time, as potential future research beyond this current project.

Additionally, the *A. thaliana* *LTI65* gene, ortholog of *BnaA02g10980D* gene the *B. napus*, was associated with stress response to abiotic factors, dehydration amongst them. The expression of this gene is induced by ABA, a vital plant hormone well-known for regulating stomatal closure to reduce water loss. This may relate to *P. brassicae* infection because, to enter the host, the

pathogen's ascospores penetrate the leaf cuticle with the aid of extracellular cutinases through enzymatic degradation (Li *et al.*, 2003). Since the cuticle is an essential barrier against dehydration that controls cuticular transpiration, its degradation during infection could activate the ABA signalling pathways that would result in the expression of the *LT165* gene, explaining why it is upregulated after *P. brassicae* inoculation.

Three novel genes were identified with no orthologues in *A. thaliana*, inferred from electronic annotations. They had not been reported in the literature before. The *BnaA01g18990D* gene had an *A. lyrata* ortholog that encodes a Cytochrome P450 enzyme that could be contributing to the response to the oxidative stress associated with pathogen infection, similarly to *CYP79B2*. The *BnaA01g28440D*, with an ortholog in *A. lyrata*, and the *BnaA01g28930D* gene, with no *Arabidopsis* ortholog, were functionally, linked to DNA modification and DNA regulation, respectively. This suggested that these novel genes could be playing a significant role in gene regulation and expression during pathogen infection, potentially involved in regulating host defence on a molecular level. The expression of these genes should be compared between the resistant and susceptible cultivars specifically, at the different time points considered in this chapter. Further study and characterisation of these novel genes could reveal their role in resistance against *P. brassicae* and contribute to the understanding of the mechanisms that influence and regulate gene expression in *B. napus* after pathogen inoculation.

#### 4.4.3 Study of the GEMs

The PCA (Figure 4.14) done as part of the RNA-seq analysis showed a clear difference in gene expression between susceptible and resistant phenotypes, susceptible cvs Cabriolet and Sansibar clustered together, separate from resistant lines POSH and Cubs Root. Interestingly, these two resistant lines clustered separately, which suggested different mechanisms of resistance. More in depth RNA-seq results of *B. napus* line POSH showed a unique gene expression pattern, statistically different from the other three genotypes. For some of the selected genes, the expression was more similar to the susceptible genotypes (cvs Cabriolet and Sansibar), in stark contrast with its associated resistant phenotype. POSH is a resynthesized line that was bred to increase pod-shattering resistance. It originated from an artificial cross between wild *Brassica oleracea* and *Brassica rapa*, which generated a synthetic line that was crossed with natural *B. napus* to increase genome stability. The offsprings of this cross were used to create a doubled-haploid line from which POSH derived. It is a fairly novel germplasm; thus, it is likely that it presents novel resistance mechanisms that differ from other resistant cultivars such as Cubs Root. This is a potential knowledge gap that would be interesting to explore.

Further analysis of the expression of the GEMs of interest revealed that the expression of the *BnHAT1* gene was significantly higher in susceptible cultivars in comparison to the resistant cv. Cubs Root. The heightened expression of *BnHAT1* in both Cabriolet and Sansibar supported the hypothesis that the *BnHAT1* gene is a factor in susceptibility to the pathogen.

A closer examination of the controls (*PDF1.2* and *PR1*) showed that their expression in Cubs Root reached its highest value at 1 DPI, whilst in susceptible cvs Cabriolet and Sansibar, the gene expression at this inoculation time point was not particularly high; instead, it reached its peaks at 2 DPI. This suggested that, in the resistant cultivar, these resistance genes were induced more rapidly upon pathogen infection, whereas in the susceptible cultivars they were expressed later.

This phenomenon has been observed in other pathosystems (Yang *et al.*, 2022), the expression of resistance-related genes is often faster and more intense in resistant varieties than in susceptible ones, activating a “fast-acting” response that enables the host to detect and fight the infection at early stages. Furthermore, the *PDF1.2* gene, associated with host defence response, was statistically less expressed in the resistant lines than in the susceptible ones (Figure 4.22), which aligned with its overall downregulated gene expression across genotypes and treatments (Figure 4.13).

The RNA-seq results were compared with the results from an RT-qPCR that used cDNA sampled from the same plants (Muthayil-Ali, 2023). The results obtained from the RT-qPCR showed that the selected GEMs associated with resistance to the pathogen showed significantly low expression values in the three susceptible cultivars examined (Cabriolet, Sansibar and Laser). On the contrary, the expression of the *BnHAT1* gene was highly induced in all susceptible cultivars, especially in Cabriolet and Sansibar. The expression pattern of the *BnHAT1* gene obtained using the RT-qPCR method coincided with the results from RNA-seq, supporting the hypothesis that this gene is associated with susceptibility to *P. brassicae*.

Despite this, some discrepancies between the RT-qPCR and the RNA-seq results were discovered with the other seven GEMs. Some of these genes were evidently associated with resistance against the pathogen when analysing the RT-qPCR results because they showed a statistically higher expression in resistant cultivars in comparison to the susceptible ones. However, this contrast was less clear when analysing the RNA-seq results. Many of the GEMs did not show a significant difference between phenotypes or, when they did, the distinction was not consistent because it affected POSH or Cubs Root but not both. This could be due to *B. napus* being a polyploid with two subgenomes, which often leads to a gene having multiple homeologs and homologs that share high sequence similarity. This can be a challenge because it could interfere in the RNA-seq alignment algorithms. A significant amount of RNA-seq short reads could map to multiple locations, assigning the short reads to the incorrect subgenome. This would lead to inaccurate quantification and alignment mismatches. Alternatively, the gene-specific primers used in RT-qPCR potentially amplified not only the targeted gene but might have also cross-reacted with nearly identical homeologs, resulting in an amplified combined signal. This, however, is unlikely since probes were used along with specific primers for RT-qPCR, making the reaction highly specific and sensitive.

The RNA-seq analysis needs to be further investigated to adjust for all the complexities and challenges that allopolyploids genomes introduce into the study. It would be crucial to consider the multiple homeologs of the genes of interest and how the data processing and analysis needs to be accommodated for it to minimise misalignment and optimise the reliable results.

#### **4.4.4 Study of 3D structural model of BnHAT1**

To expand the research into the *BnHAT1* gene and its biological and molecular function, the three-dimensional structural model of the protein encoded by it was studied. The changes to the protein structure caused by SNP mutation in TILLING mutant line 2A (D167N) were analysed and compared to the WT structure. The change in amino acid in the HXXXD motif resulted in the lack of a salt bridge that would have connected the aspartic acid to an arginine residue at position

293. This significantly altered the geometry of the protein and, more specifically, the active site, which could have affected enzyme activity. If this protein is associated with susceptibility to *P. brassicae*, as hypothesised, a decrease in its enzymatic activity would explain the significant phenotypic difference between WT Cabriolet individuals and TILLING mutant 2A individuals that was observed. Moreover, it would uphold the theory of the HXXXD motif playing a crucial role in the functionality of the *BnHAT1* protein, with the aspartic acid residue contributing to the structural integrity of the protein (Ma *et al.*, 2005).

The 3D structure of the BnHAT1 protein was compared to that of other molecules, and high similarities were found in the *A. thaliana* protein AtHCT (Hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase), also part of the BAHD super-family of enzymes. Its specific sequence for the HXXXD motif is HHAAD. It is involved in plant phenylpropanoid metabolism, the histidine residue at position 153 interacted with the substrate p-coumaroylshikimate (Levsh *et al.*, 2016). A similar protein identified was EPS1 (Enhanced Pseudomonas Susceptibility 1), another BAHD acyltransferase-family protein found in *Arabidopsis*. It is associated with SA biosynthesis and plays a role enhancing host resistance against *Pseudomonas* (Chen *et al.*, 2009; Torrens-Spence *et al.*, 2023). However, this protein had a modified HXXXD motif, the characteristic histidine residue was substituted by a serine residue at position 160 with unconventional lyase catalytic activity. EPS1 and BnHAT1 have opposite effects on host resistance, the first one boosting resistance and the latter supporting susceptibility to the pathogen. Thus, although structurally alike, they are not related in terms of functionality.

#### 4.4.5 Gene expression of TILLING mutant line 2A

Finally, for the gene expression experiment of the *BnHAT1* gene in TILLING mutant 2A described in Chapter 4.3.6, the main focus of future work would be using the samples of extracted mRNA for the RT-qPCR that had been planned. The results from an RT-qPCR would have allowed for the study of the changes in the expression of the *BnHAT1* gene during the infection of *P. brassicae*. Moreover, it would have been significant to compare the gene expression of this gene in the TILLING mutant 2A, that presents a SNP affecting the HXXXD motif, with cv. Cabriolet, since Cabriolet is the background genome of the TILLING mutants and thus, would present the WT allele and an intact HXXXD motif. The expressions of these two lines could have also been compared to resistant cv. Lipid and susceptible cv. Abukuma and establish differences between the expression of the resistance phenotypes.

Additionally, another RT-qPCR could be done targeting the  $\beta$ -*adaplin* gene (BnaC07g38240), which was studied by Muthayil-Ali (2023) and showed a contrasting expression to the *BnHAT1*. It showed a significantly higher expression in resistant cultivars than in susceptible ones, hence the analysis of the expression of  $\beta$ -*adaplin* could be used as a control to test the reliability of the results.

#### 4.5. Conclusion

The function of the *BnHAT1* gene in *B. napus* was characterised using different approaches. From this research, it was inferred that it plays a role as a susceptibility factor to the fungal pathogen

*P. brassicae*. This was supported by comparing the genotype and phenotype of TILLING mutant lines with SNP mutations targeting the gene of interest and showing higher resistance levels in the mutated individuals than in the WT. Furthermore, the RNA-seq analysis of a gene expression experiment showed a differential higher expression of the *BnHAT1* gene in susceptible cultivars in comparison to a resistant genotype. Finally, the structure of the BnHAT1 protein was studied through 3D models. This acyltransferase belonging to the BAHD super-family of proteins had a highly conserved HXXXD motif that was significant for the catalytic activity of the enzyme. The study of the model revealed that the aspartic acid residue played a structural role in the integrity of the protein by forming a salt bridge with an arginine. The mutation of this aspartic acid resulted in an increase in resistance against the pathogen, which supported the hypothesis that the *BnHAT1* gene and its encoded protein are involved in susceptibility to *P. brassicae*. Additionally, multiple genes were discovered through the RNA-seq analysis that could be associated with resistance and susceptibility to *P. brassicae*.

## Chapter 5: Identification of QTLs in *Brassica napus* for resistance against *Pyrenopeziza brassicae*

### 5.1 Introduction

#### 5.1.1. Quantitative disease resistance

Quantitative disease resistance (QDR) is a type of polygenic plant immunity that has been observed in almost all studied plant pathosystem. It is controlled by multiple genomic regions known as quantitative trait loci (QTLs) that interact with each other. Their combined effects of QTLs provide a partial level of resistance that reduces disease severity, instead of completely preventing it. It is often more durable than qualitative resistance and exhibits race-nonspecific effects, which provides a broad-spectrum resistance to disease (Parlevliet, 2002; Poland *et al.*, 2009). Phenotypically, QDR is associated with the continuous distribution of resistance traits in a genetically segregating or variable population that does not fit Mendelian segregation ratios (St Clair, 2010).

In plant research, understanding QDR could contribute to the selection of cultivars with effective resistance or could be applied in breeding programs to develop more resilient crops. This would lead to an improvement of disease management and the control of epidemics (Roux *et al.*, 2014). To further study new sources of resistance or susceptibility that can improve the control and management of LLS affecting oilseed rape, it is necessary to study and characterise the genes involved in QDR through QTL mapping approaches. In previous studies (Table 1.1), major resistance loci have been identified and characterised in *B. napus* against *P. brassicae*, associated with black flecking and reduced asexual sporulation (Bradburne *et al.* 1999; Boys *et al.* 2012). Polygenic QDR has also been identified, with multiple quantitative trait loci (QTLs) being located in the hosts genome (Pilet *et al.*, 1998; Karandeni Dewage *et al.* 2022; Fell *et al.*, 2023).

#### 5.1.2. Bulk segregant analysis

Bulked segregant analysis (BSA) is a high-throughput mapping approach used for identifying QTLs associated with a specific trait, developed by Michelmore *et al.* (1991). The first step of BSA is to generate an F<sub>2</sub> population segregating for the trait of interest to obtain two phenotypically contrasting groups. This is achieved by crossing two extreme phenotypes. From the resulting segregating population, two bulks are created by selecting a small percentage of individuals with the most contrasting phenotypes. This technique relies on the principle that the alleles controlling the trait of interest would be enriched in either bulk, whereas those loci not affecting the trait would segregate randomly in both bulks (Zhang & Panthee, 2020). DNA from these two bulks is compared to find genetic markers that are significantly different between the two pools. These differences aid in identifying genomic regions that are likely to contain QTLs associated with a specific trait such as resistance or susceptibility to a pathogen (Majeed *et al.*, 2022).

BSA was significantly improved by the incorporation of Next-Generation Sequencing (NGS) for marker discovery and only requires the sequencing of the two extreme phenotype pools for comparison, speeding up QTL discovery. This hybrid method (BSA-seq) has been successfully used before in the *Brassica* genus to map QTLs to study clubroot resistance in *Brassica rapa* (Zhu

et al., 2019). It was specifically used in *B. napus* to study the angle at which the branches grow (Wang et al., 2016) and vernalisation requirements (Tudor et al., 2020).

The genetics of QDR in the *B. napus*-*P. brassicae* pathosystem may be studied using the BSA-seq method, for which it is necessary to generate an  $F_2$  population that segregates for a trait of resistance or susceptibility to *P. brassicae*, as outlined in Figure 5.1. The  $F_2$  population can be generated by crossing two extreme genotypes: a highly susceptible cultivar with a highly resistant one. The  $F_1$  generation directly obtained from this will be genotyped through a microsatellite marker assay to confirm they are hybrid offsprings of the parental cross. Microsatellite markers are short, repetitive nucleotide sequences that are highly variable between individuals, and can be used to track allele inheritance. After verification, the  $F_1$  generation will be selfed to subsequently generate the  $F_2$  population, which will be screened. The extreme individuals (most and least susceptible to the pathogen) will be selected, pooled and their DNA extracted and sequenced with NGS. The resulting data can be used to identify QTLs associated with QDR.

### 5.1.3. Aims and objectives

Expanding the existing knowledge on QDR of *B. napus* against *P. brassicae* will significantly contribute to the selection or development of oilseed rape cultivars with a more durable resistance to LLS, which will benefit both breeders and farmers. The aim of this chapter is to better the understanding of the genetics of QDR by identifying QTLs involved in resistance or susceptibility to LLS through a bulked segregant analysis.

It was hypothesized that a segregating  $F_2$  population will show a continuous distribution of resistance traits that do not fit Mendelian segregation ratios, associated with QDR rather than *R*-gene mediated resistance. Furthermore, a bulked segregant analysis will allow for the identification of host QTLs related to resistance to LLS.

The separate objectives contained in this chapter were:

1. To generate an *B. napus*  $F_2$  population that segregates for resistance against *P. brassicae*.
2. To determine whether susceptibility to the *P. brassicae* is a resistant or dominant trait through the study of the  $F_1$  generation.
3. To perform bulked segregant analysis of the *B. napus*  $F_2$  segregating population to identify QTLs associated with resistance or susceptibility to *P. brassicae*.

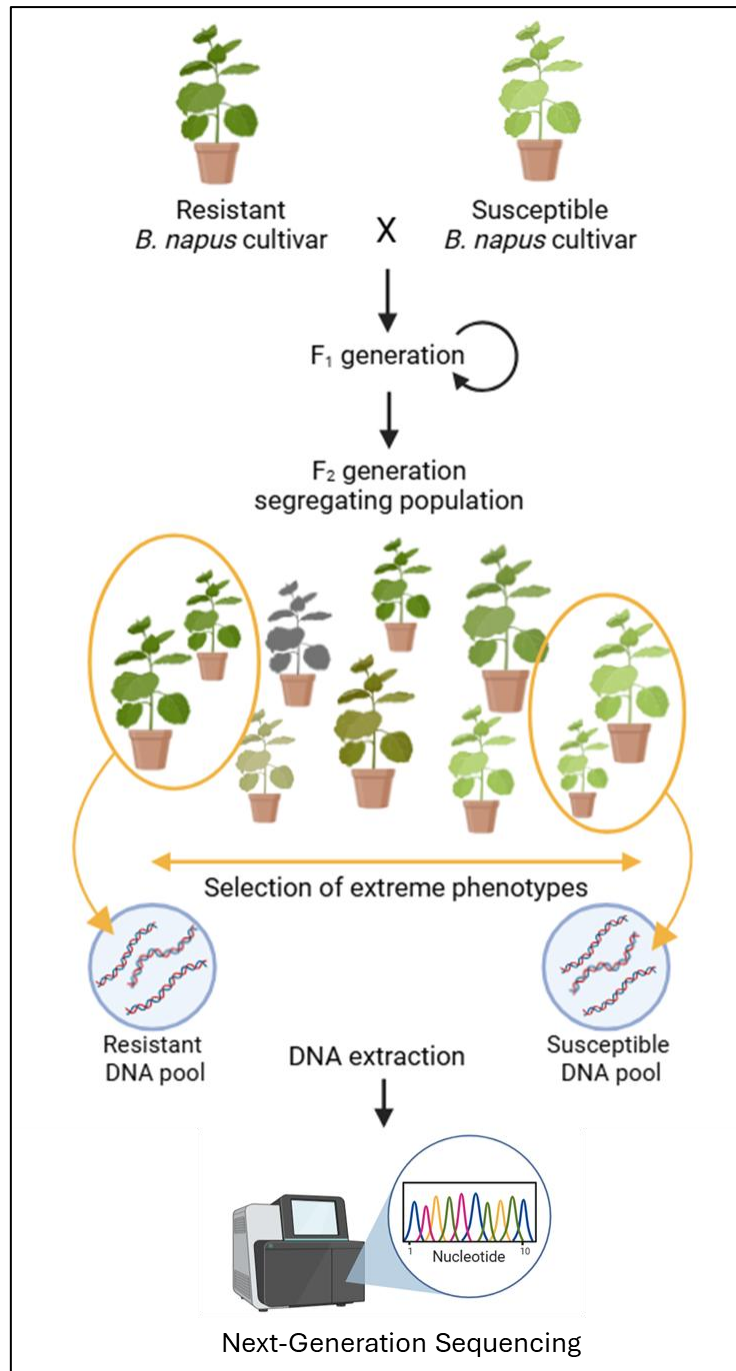


**Figure 5.1.** Representation of the BSA-seq approach for identification of QTLs associated with quantitative disease resistance of *Brassica napus* against *Pyrenopeziza brassicae*. It depicts the production of a segregating F<sub>2</sub> population, the selection of individuals with extreme phenotypes, the extraction of pooled DNA and its analysis through Next-Generation Sequencing.

## 5.2. Materials and methods

### 5.2.1. Generation of segregating F<sub>2</sub> population

To generate a segregating F<sub>2</sub> population, the first step was crossing two cultivars with extreme phenotypes. The *B. napus* cultivars chosen for the cross were cvs Abukuma and Lipid. These cultivars have been used in experimental research, and phenotyped using the visual assessment method, measured on an LLS 1-6 severity scale (Karandeni Dewage *et al.*, 2021). These cultivars were at opposite ends of the disease severity scale, fitting as the extreme parents needed for the cross. In addition, both cultivars had a consistent behaviour under different environmental conditions, which justified their selection.

The selected parental lines were grown in a glasshouse at the John Innes Centre (JIC), under speed-breeding conditions that accelerated their growth, shortened their breeding cycle and significantly accelerated the time of flowering (Ghosh *et al.*, 2018). The plants were in a LED-supplemented glasshouse, under an extended 22-hour photoperiod (22 h at 22°C and 2 h at 17°C), in contrast to the standard long day 16-h photoperiod. Photosynthetic photon flux density (PPFD), measured at plant canopy height, was 315  $\mu\text{mol m}^{-2} \text{s}^{-1}$  during the day and 220  $\mu\text{mol m}^{-2} \text{s}^{-1}$  during the night, on average.

Once both cvs. Lipid and Abukuma had flowered, they were crossed. For the crossing, unopen buds of female plants were carefully opened, and sepals, petals and anthers removed. The remaining stigma was pollinated with pollen taken from anthers of male plants of the opposite cultivar. Then, the pollinated stigmas were covered with transparent plastic bags to avoid cross-pollination. From the pollinated flowers, seeds pods containing F<sub>1</sub> seeds developed (Figure 5.2).

The seeds of the F<sub>1</sub> generation, directly obtained from the Lipid x Abukuma cross, were grown under the same glasshouse conditions. To verify the success of the cross and confirm the F<sub>1</sub> plants generated were indeed the hybrid offsprings of the parental cross, a microsatellite marker assay was performed. Biological duplicate samples were taken from the first true leaves of the F<sub>1</sub> plants, as well as from the parents cvs Lipid and Abukuma. An additional *B. napus* cultivar (cv. Leopard) was also included to rule out the possibility of it being one of the F<sub>1</sub> parents, since there were suspicions that a mistake had been made when crossing the cultivars. DNA was extracted as described in chapter 2.5 (DNA extraction). A PCR was prepared with using HotStarTaq<sup>R</sup> DNA Polymerase (QIAGEN, UK), following the manufacturer's instructions. The primers designed to target the microsatellite markers were BRMS-008-Forward (5'-AGGACACCAGGCACCATATA-3') and BRMS-008-Reverse (5'-CATTGTTGTCTTGGGAGAGC-3'), with an expected amplicon size of 145 bp (Suwabe *et al.*, 2002). The PCR was run following the cycling conditions recommended, with an annealing temperature of 60°C and a total of 30 cycles. An electrophoresis was run with the PCR products in a 2.5% agarose-TBE gel, prepared as described in Chapter 2.7 (Agarose gel electrophoresis); run at 80V for 2 hours, using a 100 bp ladder (Sigma Aldrich, UK). The F<sub>1</sub> plants, grown under the speed-breeding programme in the glasshouse, were self-pollinated to produce the F<sub>2</sub> segregating population.

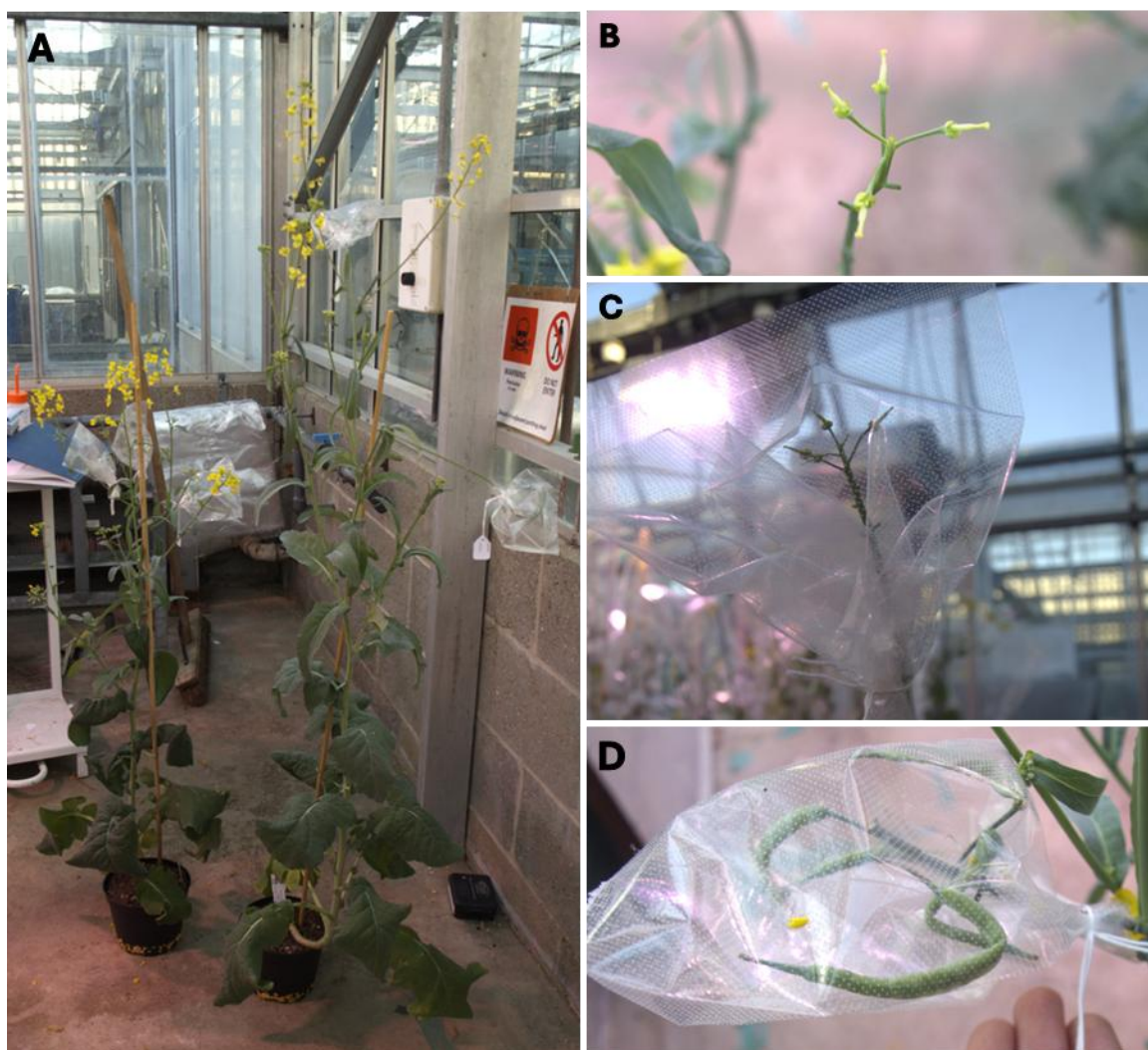


**Figure 5.2.** Pictures illustrating the process of crossing *Brassica napus* cultivars Abukuma and Lipid. **A)** Flowering *B. napus* plants ready for crossing. **B)** Stigma of female plants; petals, sepals and anthers removed. **C)** Pollinated stigma covered with transparent plastic bags. **D)** Seed pods developed from the pollinated flowers, containing  $F_1$  seeds.

### 5.2.2. Screening and phenotyping of the F<sub>1</sub> lines and F<sub>2</sub> population

In BSA, the bulks with the extreme phenotypes usually contain 20 to 50 individuals that constitute the 5-10% of the segregating population. To obtain bulks with at least 30 individuals, that would represent 5% of the segregating population, 600 plants from the F<sub>2</sub> population were needed to ensure enough extreme phenotypes were present. Glasshouses at the Bayfordbury campus, University of Hertfordshire, were used to grow approximately 600 F<sub>2</sub> plants under a 12-hour photoperiod, with baseline temperatures of 16°C during the light period and 14°C during the dark period. Actual temperatures recorded were outside this baseline range due to environmental fluctuations during the experiments (Appendix 8). Supplemental lighting was used for the day period using sodium high pressure lamps (Sylvania SHP-TS 400 W GroLux), which automatically switched on once natural daylight decreased to  $< 115 \mu\text{mol m}^{-2}\text{s}^{-1}$ . The intensity of supplemental lighting was set at  $250 \mu\text{mol m}^{-2}\text{s}^{-1}$ .

The 600 F<sub>2</sub> plants were grown in six batches of 100 individuals (experiments A to F), avoiding the more extreme conditions of summer or winter, favouring periods with moderate environmental conditions. Experiment A was done in September-October (2022), B in February-March (2023), C in March-April (2023), D in February-March (2024), E in March-April (2024) and F in April-May (2024). In each experiment, 5 individuals of each of the parental cultivars (cvs Abukuma and Lipid) were grown as controls. The first batch included 5 individuals of the F<sub>1</sub> generation. *B. napus* plants were grown as described in chapter 2.2 (Growing *Brassica napus* under controlled environment conditions). The plants were arranged in a randomised  $\alpha$ -design, laid out in blocks to distribute the parents and the F<sub>1</sub> individuals randomly amongst the F<sub>2</sub> individuals.

The plants were inoculated with a *P. brassicae* population from glasshouse experiments at Rothamsted Research (Muthayil-Ali, 2023), obtained as described in chapter 3.2.1 (*Pyrenopeziza brassicae* populations and isolates). The pathogen was sprayed as described in chapter 2.3 (*Pyrenopeziza brassicae* spray-inoculation method). The infected *B. napus* plants were screened for LLS using the visual assessment method (chapter 3.2.3) and spore counting (chapter 3.2.5). Leaf samples of each individual plant were stored in the freezer at -80°C.

### 5.2.3. Selection of extreme phenotypes

The results from screening the F<sub>2</sub> population and the controls with two different phenotyping methods (visual assessment and spore counting) were normalised so the data across experiments would be comparable, reducing variation between blocks that could affect the selection of extreme phenotypes. The data for spore counting was transformed from log spores/cm<sup>2</sup> into percentages by finding the highest value of each experiment and dividing the rest of the spore counting values by it.

The adjusted data was analysed using RStudio (R Core Team, 2023). Normal distribution was tested using Shapiro-Wilk's test and the data plotted in histograms. Non-parametric Kruskal-Wallis test was used, followed by *post-hoc* Dunn test, to check the significant variances between different genotypes, as well as between the separate glasshouse experiments. The data was represented using boxplots with jittered data points.

To select the extreme phenotypes, a linear mixed-effects model was used to compare the results from visual assessment and spore counting, improving the accuracy by considering both

phenotyping strategy methods. Spearman's test was used to check correlation between them, with a 95% confidence interval. Across all the experiments, 5% of the most extreme phenotypes were selected in both ends, the 30 individuals that showed the highest and 30 that showed the lowest susceptibility to *P. brassicae*.

#### 5.2.4. DNA extraction of extreme phenotypes

The harvested *B. napus* leaves were stored in the freezer at -80°C. Frozen leaf tissue from the parental cvs Lipid and Abukuma were placed in separate Falcon tubes and submerged in liquid nitrogen, then ground to a very fine powder using a sterile mortar and pestle. The same was done with the individuals from the F<sub>2</sub> population, selected for their extreme phenotypes. Their leaf tissue samples were pooled together and ground to a powder in sterile mortar and pestle, and thoroughly mixed, forming resistant and susceptible bulks.

For BSA-seq, high-quality DNA was needed from the extreme phenotype pools and from parental cvs Lipid and Abukuma. The extracted DNA was required to have a concentration higher than 20 ng/μL and a purity of OOD260/280=1.8-2.0, showing no degradation or contamination. Genomic DNA extraction kit, Nucleon PhytoPure (Cytiva, UK), was used to achieve this. The protocol provided by the manufacturer was followed, albeit with some additional steps and changes to improve the quality of the DNA.

The main difference with the manufacturers protocol was the addition of a sorbitol wash as a pre-extraction step to improve DNA purity. Sorbitol acts by drawing water out the cells before lysis, which helps separate and protect the desired DNA from other cellular components such as polysaccharides, which could potentially contaminate biological samples and are therefore removed. PVP-40 is another component of the sorbitol wash and acts as a binding agent to polyphenols, which renders them inert and protects the DNA against phenol oxidation. This step leads to a cleaner extraction that maintains the purity and integrity of the genetic material, preventing contaminants from interfering with downstream applications like sequencing (Jones & Schwessinger, 2020). For the preparation of the sorbitol wash, 31.88 g of D-sorbitol (0.35 M) and 5 g of PVP-40 (1% w/v) were diluted in a mix of 50 mL of Tris-HCl (1 M, pH 4.8), 5 mL of EDTA (0.5 M, pH 8.0) and 445 mL of sterile distilled water (SDW). The solution was mixed by manual agitation until it was transparent and stored at 4°C until required. Right before starting the DNA extraction protocol, aliquots were taken from this buffer and mixed with dithiothreitol (DTT), adding 0.15 mg per millilitre of sorbitol-wash and mixed thoroughly using a vortex. DTT is a reducing agent that prevents the dimerization of DNA in a solution. It is also used as an enzyme stabiliser, it breaks disulfide bonds in proteins and inactivate enzymes such as RNase and proteases that could degrade DNA (Fjelstrup *et al.*, 2017).

Approximately 100 mg of the leaf powder pools were transferred to a clean 2 mL screw-cap tube containing 500 μL of the sorbitol wash with DTT. The sample tubes were mixed by inversion and vortexed to ensure the powdered leaf material was completely covered by the sorbitol wash. The samples were centrifuged at 5000 x g for 5 minutes at room temperature. The supernatant was discarded, and the pellet was washed by repeating the previous step 5 times, resuspending it in excess sorbitol wash, centrifuging under the same conditions and discarding the supernatant. After these washes, the pellet is resuspended once more in the sorbitol wash and centrifuged at

4000 x g for 5 minutes at room temperature. The supernatant was carefully discarded to avoid disturbing the pellet, which was left to dry on blue roll upside down.

Once the pellet was dry, 600 µL of Reagent 1 were added to each sample tube and resuspended by pipetting. RNase was added to a final concentration of 20 µg/ml and incubated at 37°C in a thermal block for 30 minutes. Then, 200 µL of Reagent 2 were added to each sample tube and mixed by inversion. The samples were incubated at 65°C in a water bath for 10 minutes, mixing by inversion every few minutes, and then put in ice for 10 seconds. After this, 100 µL of potassium acetate (5M) were added and samples were incubated in ice for 20 minutes.

For the fundamental DNA extraction step, 500 µL of chloroform (kept at -20°C) were added and mixed by agitating manually so there were no separated phases. The tubes were gently inverted manually at room temperature for approximately 10 minutes, until the mix looked “milky”. The samples were centrifuged at 1300 x g for 10 minutes, which resulted on the samples separating into three phases. A maximum of 750 µL of the upper phase was transferred to a clean new 1.5 ml Eppendorf, careful to not disturb the middle or bottom layer.

Cold isopropanol was added in equal volumes to the previously upper phase transferred to a new tube, and gently inverted to precipitate the DNA, which might be seen in the tube in a viscous mucous form. The tubes were incubated at -20°C for an hour and the centrifuged at 4000 x g for 5 minutes to pellet the precipitated DNA. The supernatant containing the isopropanol was discarded and the pellet was washed by resuspending in excess cold 70% ethanol and centrifuging again in the same conditions. The supernatant containing the ethanol was discarded, and an additional quick spin was done to remove the remaining ethanol. The pellet was air dried for 10 minutes to fully remove the ethanol droplets. The pellet was finally resuspended in 25 µL of TE buffer and rehydrated in a water bath at 65°C for an hour, periodically mixing the suspension by gently tapping the tube. The DNA concentration was measured in ng/µL, quantified both with an Invitrogen™ Qubit™ 4 Fluorometer (Thermo Fisher Scientific, UK) using a broad range dsDNA kit (Thermo Fisher Scientific, UK), and with a Nanodrop ND-1000 spectrophotometer (Labtech International, UK), which also allowed to measure ratios of absorbance A260/280 and A260/230 to assess DNA purity. The quality control was crucial since high-quality DNA was required for sequencing.

#### **5.2.5. Next-Generation Sequencing**

The samples were sequenced with Next-Generation Sequencing (NGS) by Novogene. The samples went through a quality control to ensure the purity of the DNA and accurately quantify its concentration. Then, the genomic DNA was randomly sheared into 50-500bp fragments. These were end-repaired, A-tailed and further ligated with Illumina adapters. This was done using a PCR-free technique that simplified the process by combining DNA fragmentation and the attachment of adapters. The resulting fragments with adapters were size selected and purified. Libraries were then quantified using Qubit, pooled and sequenced. Sequencing was done with Illumina NovaSeq X Plus PE150, that uses sequencing by synthesis (SBS) to detect individual DNA bases as they are added to a growing strand. The parental samples Lipid and Abukuma were sequenced at a 30x coverage, whilst the F<sub>2</sub> DNA pools were sequenced at 80x coverage.

## 5.2.6. Bulk segregant analysis

### 5.2.6.1 Construction of sequence assemblies

The initial processing pipeline for this analysis was performed on the Galaxy EU platform (Galaxy Version 2.5.4+galaxy0). The quality of the raw sequencing data was checked using *Falco*, a high-speed and high-throughput FastQC alternative (de Sena Brandine & Smith, 2021) before the raw reads were processed with *Trimmomatic* (Bolger *et al.*, 2014), which removes low-quality bases and adapter sequences to improve downstream analysis. A 4-base wide sliding window was used and reads with <20 Phred quality scores were trimmed. The cleaned reads from both parents (cvs Lipid and Abukuma) and both susceptible and resistant bulks were aligned to the *B. napus* reference genome from cv. Darmor-bzh v10, obtained from Genoscope genomes (<http://www.genoscope.cns.fr/genomes>) using *Bowtie2* v2.5.4 (Langmead & Salzberg, 2012) to map the reads. The sequencing coverage of the alignments was calculated using *Samtools Flagstat* (Danecek *et al.*, 2021) to determine the number of mapped reads. *Freebayes* (Garrison, 2012) was used to detect genetic variations in the reads against the reference genome, also known as variant calling. Finally, *bcftools view* (Danecek *et al.*, 2021) was used to exclusively select the SNP type mutations.

### 5.2.6.2. Calculation of $\Delta$ SNP index

RStudio was used to filter SNPs, selecting only the ones that were homozygous in both parents, with a quality equal or higher than 20 and an allele frequency >20. Then, the read depth (DP) and alternate allele observations (AO) were examined. The DP value is the total number of reads aligned to a position and used in variant calling, and the AO value represents the reads that are different from the reference allele. For further and more thorough filtering, SNPs with their DP equal to AO were selected, as well as all the SNPs with an AO equal or higher than 10. This yielded high confidence SNPs in separate files for parents cvs Lipid and Abukuma, the resistant pool and the susceptible pool. Each of the extreme pools was contrasted with each of the parents. The allele frequencies of each pool were measured by calculating the SNP-index, which calculates the ratio of alternative reads to total reads for each SNP:

$$\text{SNP-index} = \frac{\text{AO}}{\text{DP}}$$

The files with the allele frequencies were processed using a step known as binning, typically used to efficiently manage an extensive amount of SNP data. It contributes to reducing noise and pinpointing SNPs that differentiate the two pools. It divides the genome into smaller, consecutive and non-overlapping segments (“bins”). This analysis used intervals of 5 kb for fine resolution and calculated the average SNP frequencies within each bin, also known as SNP density.

The  $\Delta$ SNP-index was calculated with a window size of 10 Mb. This measurement establishes the difference between the allele frequencies of the pools. When contrasting the pools to the susceptible parent (cv. Abukuma), the susceptible pool was subtracted from the resistant pool, and vice versa for the contrast of the pools against the resistant parent (cv. Lipid). The SNPs with  $\Delta$ SNP-index values higher than the thresholds, set at 95% and 99% confidence intervals ( $p < 0.05$  and  $p < 0.01$ , respectively), were considered to have a significant effect.

$$\Delta\text{SNP-index} = \text{SNP-index of resistant pool} - \text{SNP-index of susceptible pool}$$

Finally, the G-value method was used (Magwene *et al.*, 2011), a statistical approach that compares the SNP frequency between the two contrasting phenotypic pools that significantly reduces the noise, computing smoothed G' values within a 10 Mb sliding window along the chromosomes. This procedure also calculated *q*-values, adjusted *p*-values that consider the False Discovery Rate (FDR), that represents the expected proportion of false positives; therefore, *q*-values indicated highly significant SNPs. Where the corresponding G' value was higher than the established thresholds of *p*-value<0.01, the SNP was considered to have a significant effect.

## 5.3. Results

### 5.3.1. Confirmation of parentage

For the production of an  $F_2$  segregating population, *B. napus* cvs Lipid and Abukuma were chosen as parents. Lipid showed low disease scores both in glasshouse experiments (2.74) and field trials (2.67), associated with low susceptibility to *P. brassicae*. Abukuma, on the other hand, showed high disease scores associated with a high susceptibility to the pathogen, both in glasshouse experiments (4.77) and field trials (5.00).

It was crucial to confirm that the initial parental cross (Lipid x Abukuma) was successful and the  $F_1$  seeds obtained were a hybrid. A microsatellite assay was performed to track allele inheritance. These markers are short, repetitive DNA sequences that were amplified through PCR and run in a 2.5% agarose gel. The resulting gel showed a distinct distribution of bands for each of the samples examined (Figure 5.3). Highly susceptible parent cv. Abukuma generated a band of approximately 125 bp. Resistant parent cv. Lipid produced a band around 190 bp. The  $F_1$  sample yielded bands that corresponded with the parental ones: a band at 125 bp and a band at 190 bp. This confirmed the success of the parental cross, because the offspring produced both the characteristic bands of its parents. In contrast, the cultivar used as a negative control, cv. Leopard, generated two bands close to each other, at 160 bp and 190 bp. The smaller band is not present in the lanes of the  $F_1$  samples, confirming Leopard was not one of the parents. It was notable that the bands at the top of the Leopard and Lipid lanes corresponded to template DNA. The DNA samples used to run the PCR had much higher concentrations for these two than for Abukuma and  $F_1$ , causing these high molecular weight bands.

### 5.3.2. Screening and phenotyping of the $F_1$ lines and $F_2$ population

Approximately 600 individuals of the  $F_2$  population were screened for susceptibility to LLS in six separate glasshouse experiments (A to F), along with parental cvs Abukuma and Lipid as controls and  $F_1$  individuals. They were phenotyped using two different methods: visual assessment and spore counting. The values obtained from both strategies were normalised to make the data across blocks and replicates comparable.

The data of the  $F_2$  population, along with the parental lines (Abukuma and Lipid) and the  $F_1$  generation, was displayed as boxplots to show the difference between genotypes (Figure 5.4). A non-parametric Kruskal-Wallis test was used to confirm there was a significant difference between genotypes. An additional *post-hoc* Dunn test confirmed there was a significant difference between the parental lines. The parents were used as controls and, as such, their disease severity was at opposite ends of the distribution. *B. napus* cv. Lipid, which was selected as the parent with a lower susceptibility to *P. brassicae*, yielded scores that were concentrated between 1 and 2 for both the visual assessment and spore counting methods. Data ranged from 10 to 100 spores/cm<sup>2</sup> when counting spores. These values were consistent with the expected lower susceptibility to the pathogen. On the contrary, the disease scores for *B. napus* cv. Abukuma, the more susceptible parent, were significantly higher, concentrated between 5 and 6, with spore counts higher than 100,000 spores/cm<sup>2</sup>. These values were consistent with the expected higher susceptibility to the pathogen.

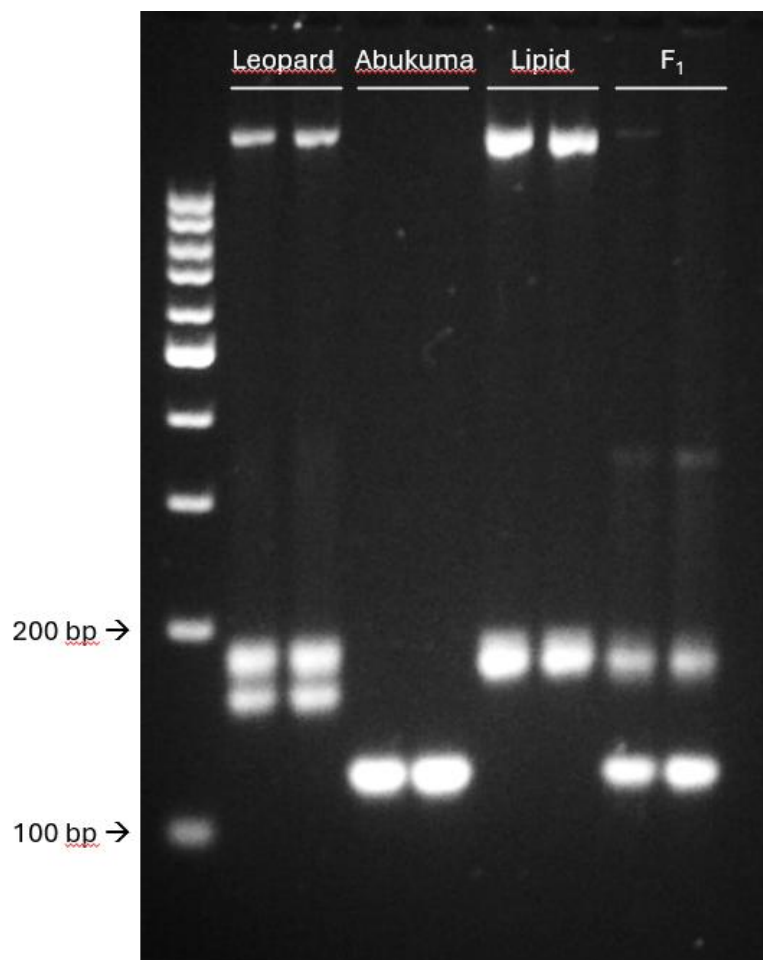


**Figure 5.3.** Microsatellite marker assay run with samples from the F<sub>1</sub> generation and *Brassica napus* cvs Leopard, Lipid and Abukuma, to confirm if the F<sub>1</sub> plants were descended from a parental cross Lipid x Abukuma. The PCR products were run in a 2.5% agarose-TBE gel with a 100 bp ladder.

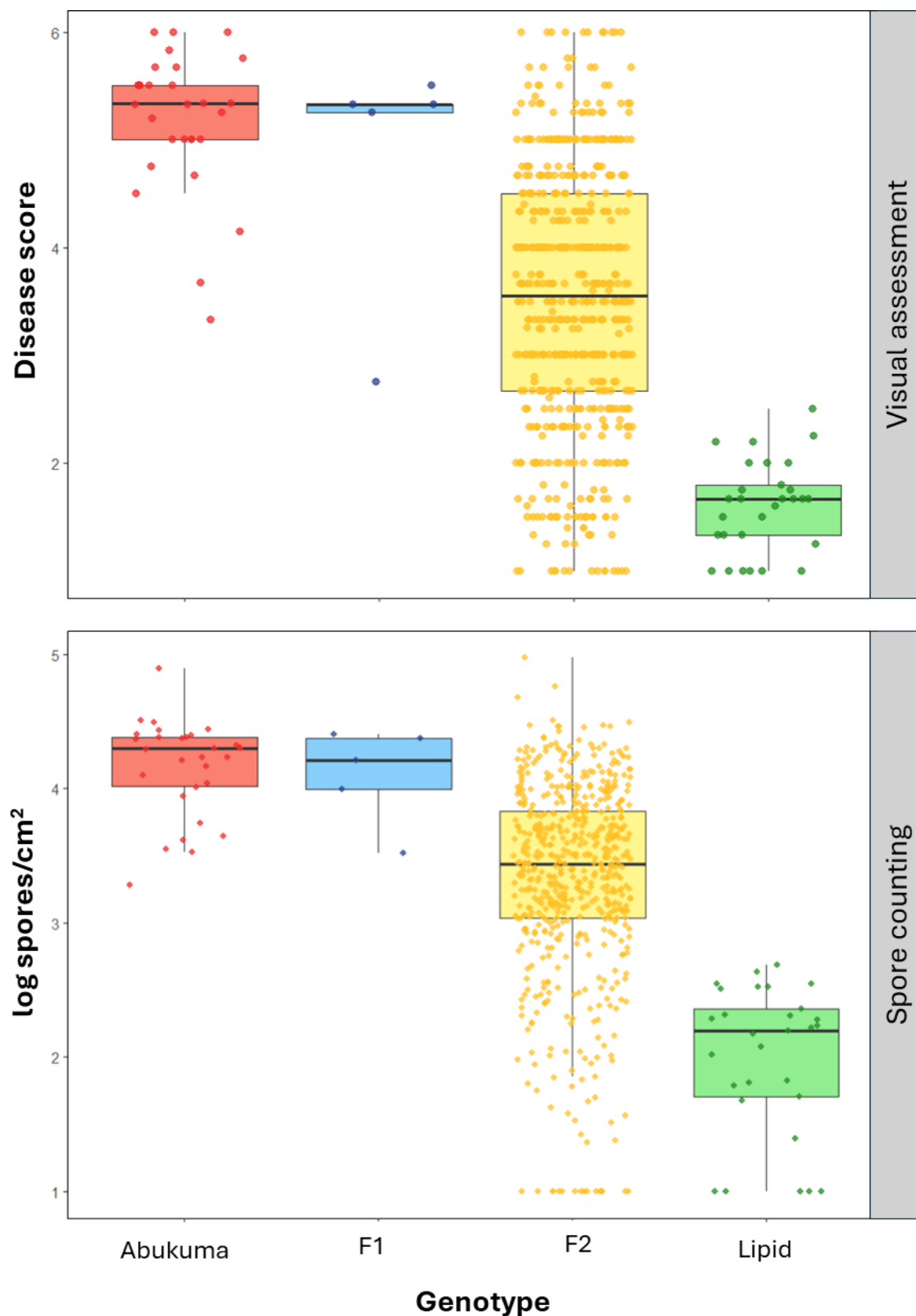


**Figure 5.4.** Screening of *Brassica napus* cultivars Abukuma and Lipid, hybrid F<sub>1</sub> population and segregating F<sub>2</sub> population, phenotyped for light leaf spot disease using visual assessment and spore counting.

Regarding the hybrid  $F_1$  lines, the *post-hoc* Dunn test confirmed there was a significant difference with Lipid, however  $F_1$  was not different from Abukuma.  $F_1$  individuals yielded disease scores between 5 and 6, and spore counts higher than 10,000 spores/cm<sup>2</sup>. These results predominantly aligned with the highly susceptible parent, cv. Abukuma, rather than with the resistant parent, suggesting that susceptibility to *P. brassicae* is inherited as a dominant trait.

The  $F_2$  population showed a wide range of disease severity when using both phenotyping method, continuously distributed between the extreme phenotypes associated with the parental cultivars, with a higher incidence in the intermediate values. A non-parametric *post-hoc* Dunn test confirmed there was a significant difference between the segregating population and the other genotypes. To further study its distribution, the adjusted values of the  $F_2$  population were plotted in as histograms to display the distribution of the severity of the disease across the segregating population when phenotyped with visual assessment and spore counting (Figure 5.5). Both graphs show a range of susceptibility to the disease, the lower disease scores or spore counts (log spores/cm<sup>2</sup>) representing low susceptibility to the pathogen, gradually increasing to higher values that correspond to a higher susceptibility. A Shapiro-Wilk test was used, and it was concluded that the distribution of the data was non-normal both for disease scores and spore counts ( $p$ -value = 9.485e-07 and  $p$ -value = 2.2e-16, respectively). The highest percentages in both graphs were between values 3-4, associated with an intermediate disease severity. The histograms exhibited a symmetrical unimodal distribution, with a slight skew to the right in the case of the spore counts. Despite there being a tendency to the right, associated with susceptibility, the density at the left end of the graphs, associated with the least susceptible phenotypes, is higher than that of the right end, linked to the highest susceptibility. There was a higher percentage of individuals that were totally resistant than individuals with an extremely high susceptibility to the pathogen. The overall distribution showed that a segregating  $F_2$  population did not fit Mendelian segregation ratios. This finding would suggest that susceptibility to LLS is associated with QDR rather than with *R*-gene mediated resistance, although there might be a contribution of some major resistance genes, indicated by the presence of individuals not supporting sporulation, explaining the disparity between the number of individuals with extreme phenotypes.

The data was also analysed according to their batch, six separate experiments (A to F), displayed as boxplots (Figure 5.6). This graph showed the controls at both end of each experimental distribution, resistant parent Lipid (in green) showing lower values than susceptible parent Abukuma (in red).  $F_1$  individuals could be seen in experiment A (in blue) with higher values similar to Abukuma's values. The values of  $F_2$  are continuously and evenly distributed in each experiment, as expected from QDR. The pattern is similar in each of the experiments, suggesting that the differences between them were not significant with the exception of experiment F when assessed with the spore counting method, that showed a slight difference with the rest, with lower spores/cm<sup>2</sup> values. This was confirmed through a non-parametric Kruskal-Wallis test and a *post-hoc* Dunn test. This was more evident in the spore counting graph, but still noticeable in the visual assessment one by the fact that the median is the lowest of all the experiments. This difference is possibly due to the time this experiment was done, since it finished in May, 2024. In late spring, the higher environmental temperatures and longer light periods could have affected the effectivity of *P. brassicae* infection and its production of spores. This indicates that the

differences between experiments was more evident when using spore counting method, which discriminates better between genotypes than visual assessment. Overall, however, the data across experiments was comparable.

### 5.3.3. Selection of extreme phenotypes

A linear mixed-effects model was used to compare the normalised results from the F<sub>2</sub> screening, using visual assessment and spore counting as phenotyping methods in separate experiments. Disease scores and spore counts were factors with a fixed effect, whilst the separate experiments were established as factors with a random effect. This comparison produced Figure 5.7, which showed the distribution of the different genotypes screened across the six separate experiments (A to F). The segregating F<sub>2</sub> population was continuously distributed for the severity of the disease measured as disease score and log spores/cm<sup>2</sup>. The parental cvs Lipid and Abukuma clustered at both ends of the distribution, Lipid presenting lower values as the parent with low susceptibility to *P. brassicae*, and Abukuma showing higher values as the highly susceptible parent. Regarding the individuals from the F<sub>1</sub> generation, their presence in experiment A showed, as was seen in previous figures, its concentration around cv. Abukuma, the highly susceptible parent, suggesting the dominant inheritance of the susceptibility trait.

All the separate experiments showed a positive correlation between both phenotyping strategies. For the bulked-segregant analysis, the 5% most extreme phenotypes out of the total 600 F<sub>2</sub> individuals screened were selected. For this selection, the results from both phenotyping methods were taken into consideration, however spore counting was prioritised since it was proven in Chapter 3 to be a more reliable technique. The F<sub>2</sub> individuals targeted for selection had phenotypical values similar to the parents or, when possible, more extreme. This can be seen in the graph, with selected individuals concentrated at both ends of the distribution, with the 30 most susceptible individuals selected from F<sub>2</sub> clustered around cv. Abukuma, and the 30 least susceptible individuals concentrated around cv. Lipid.

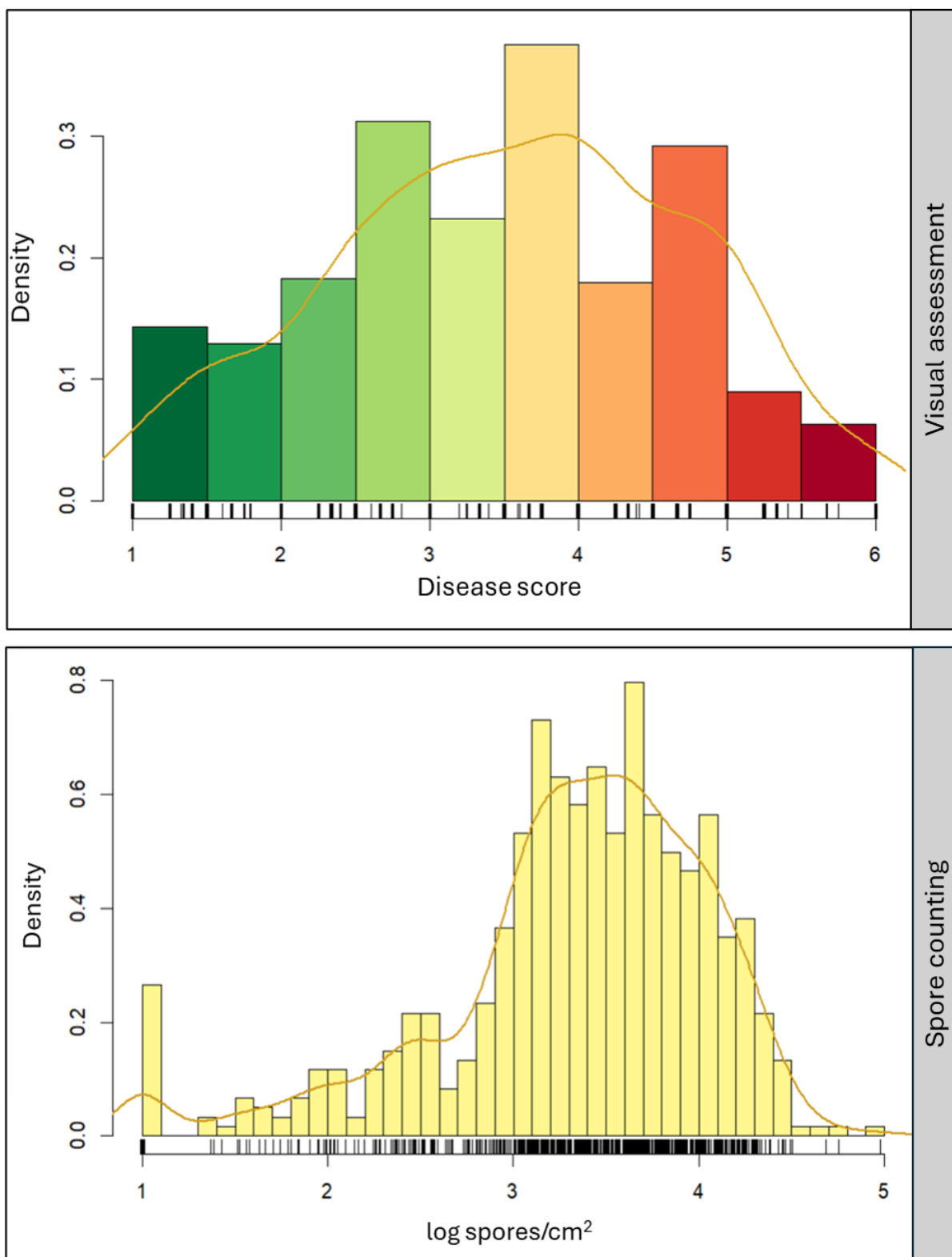


**Figure 5.5.** Histogram of the distribution of the severity of the light leaf spot disease of an  $F_2$  segregating population, measured using visual assessment or spore counting. The density represents the relative frequency of the data in each bin.

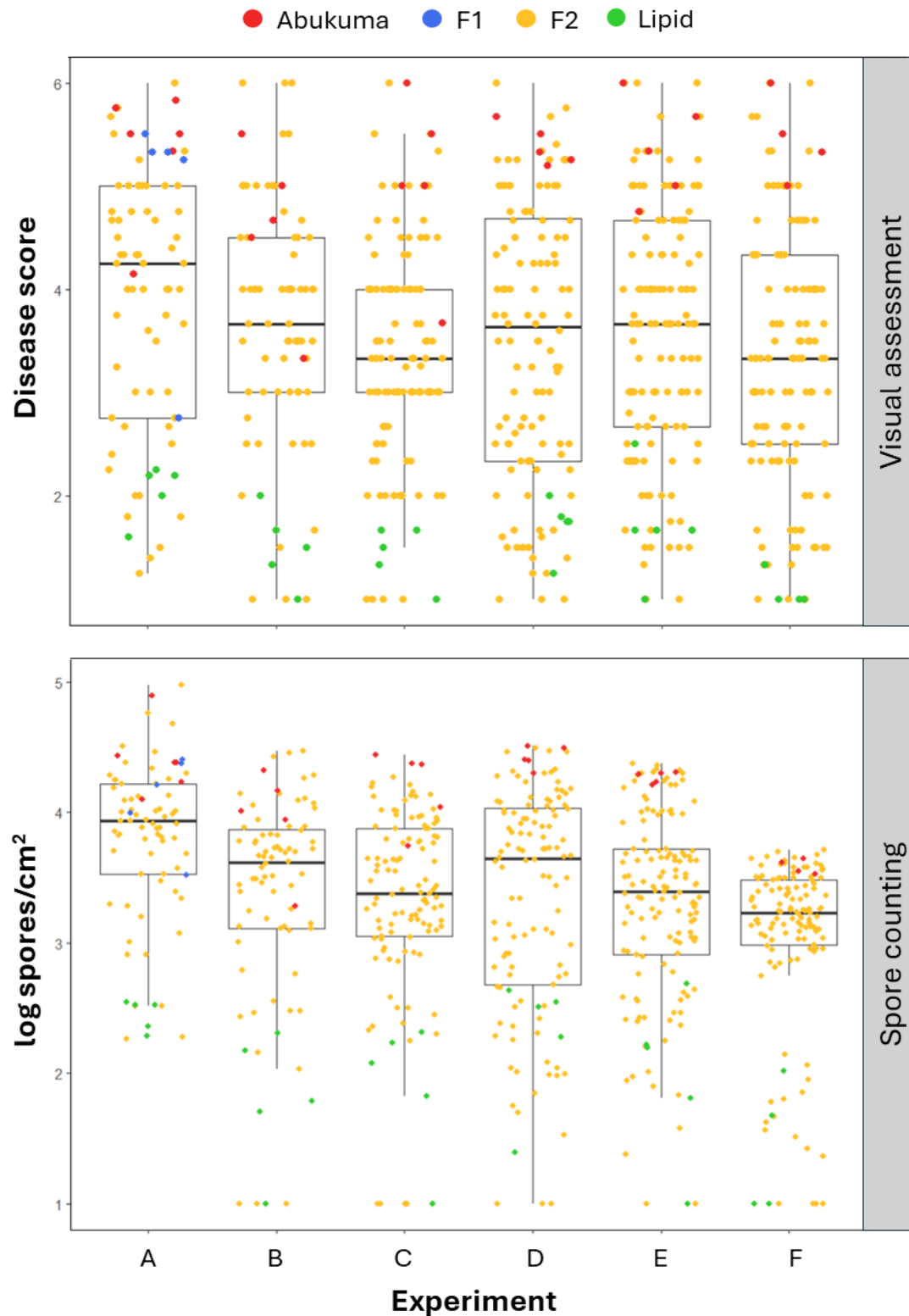


**Figure 5.6.** Distribution of the disease severity of *Brassica napus* cultivars Abukuma and Lipid, hybrid F<sub>1</sub> population and segregating F<sub>2</sub> population, in six separate experiments, phenotyped for light leaf spot disease using visual assessment and spore counting. Experiment A was done in September-October (2022), B was done in February-March (2023), C was done in March-April (2023), D was done in February-March (2024), E was done in March-April (2024) and F was done in April-May (2024).

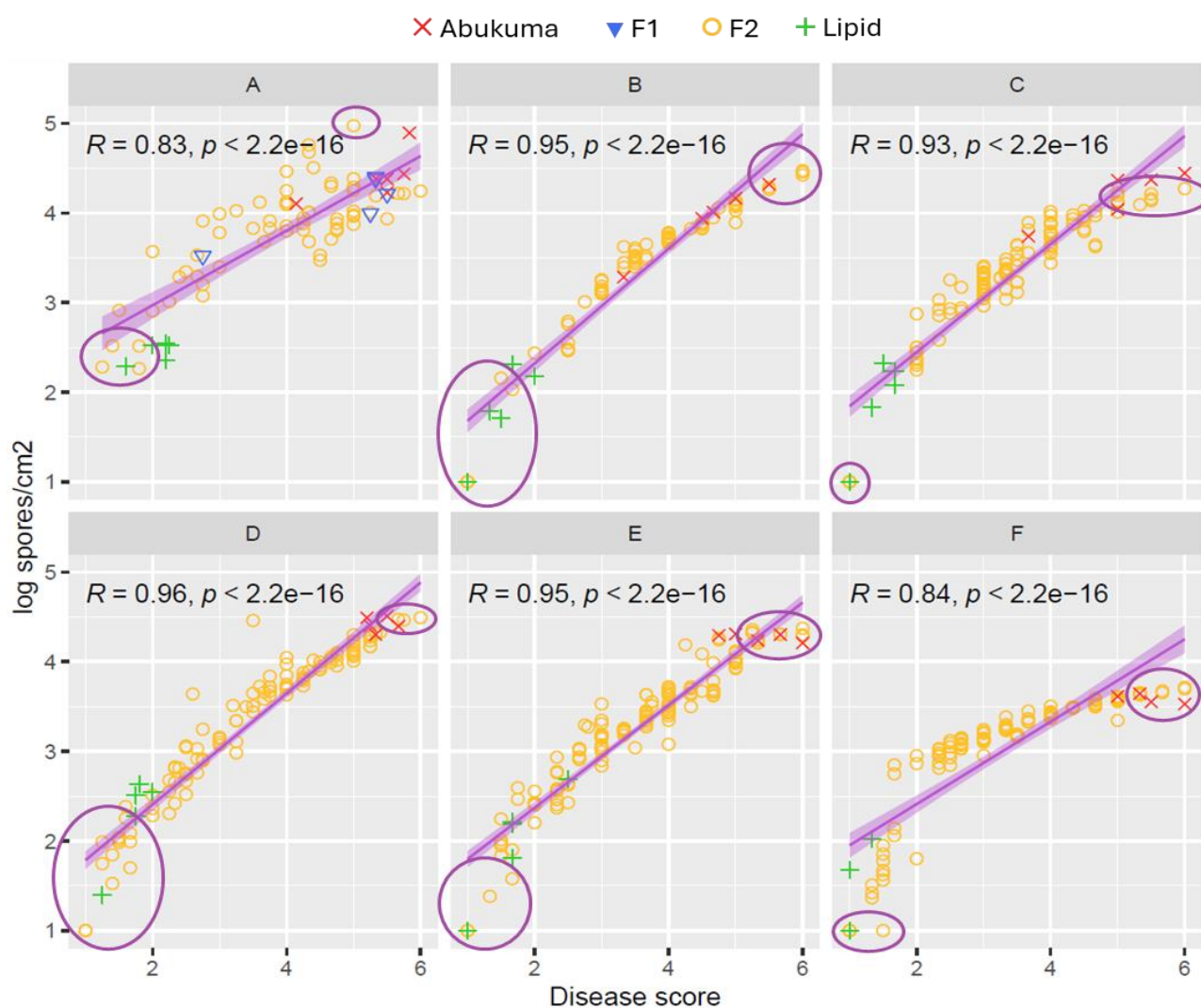


**Figure 5.7.** Linear mixed-effects model comparing visual assessment and spore counting for the selection of F<sub>2</sub> individuals with extreme phenotypes. Graphs A to F show separate experiments where different genotypes of *Brassica napus* were screened for susceptibility to light leaf spot (LLS) disease. The genotypes assessed were parental cultivars Abukuma and Lipid (with high and low susceptibility to LLS, respectively), hybrid F<sub>1</sub> and the segregating F<sub>2</sub> population. The selected F<sub>2</sub> individuals with extreme phenotypes were circled in purple. The lines represent linear regressions using Spearman's correlation test, with a 95% confidence interval (purple area).

#### 5.3.4. Identification of candidate loci

The DNA extracted from the parents cvs Lipid and Abukuma, and from susceptible and resistant pools, yielded high-quality DNA (Appendix 9), as required for NGS. The samples passed a quality control check, all of them had concentrations higher than 20 ng/μL with an absorbance (OD260/280) between 1.8 and 2.0, as required to proceed with the library preparation and sequencing through NGS. The total output of raw data from sequencing was 252.1 G. The total amount of reads and raw data for each sample can be seen in Table 5.1, along with the number of reads mapped and properly paired to the reference genome, and the amount of high confidence SNPs after filtering.

To identify genomic regions associated with plant defence between resistant and susceptible bulks,  $\Delta$ SNP-index was calculated and plotted for all the high confidence SNPs across subgenome A (Figure 5.8) and subgenome C (Figure 5.9). Thresholds set at 95% and 99% confidence intervals helped with finding loci associated with resistance or susceptibility to *P. brassicae*. Positive  $\Delta$ SNP-index values indicated that the specific SNP alleles were enriched in the resistant bulk and depleted in the susceptible bulk, and vice versa for the negative  $\Delta$ SNP-index values. This is because the  $\Delta$ SNP-index values were calculated by subtracting SNP-index values from the susceptible bulk from the SNP-index values from the resistant bulk.  $\Delta$ SNP-index=0 represented no deviation in allele segregation between bulks.

In subgenome A (Figure 5.8), four chromosomes appeared to have  $\Delta$ SNP-index values higher than those of the thresholds: A01, A03, A06 and A07. In subgenome C (Figure 5.9), no loci were identified as significantly relevant in any of the bulks, since none of the  $\Delta$ SNP-index values surpassed the thresholds. The findings from the relevant chromosomes were summarised in Table 5.2.

On chromosome A01, a 12 Mbp genomic QTL region had  $\Delta$ SNP-index values above the 95% confidence threshold. It contained 8,821 high confidence SNPs and 8,819 annotated sequences according to the Darmor-*bzh* reference genome, consisting of 1,389 genes, 6,082 coding sequences (CDS) and 1,348 untranslated regions (UTRs). Increasing the threshold to consider only variation at the 99% confidence value narrowed the region to 2.76 Mbp that contained 4,218 high confidence SNPs. This highly relevant sequence was associated with 1,905 annotated sequences within the reference genome, with 294 genes, 1,360 CDS and 251 UTRs. All the relevant regions identified in A01 chromosome had positive  $\Delta$ SNP-index values, meaning they were enriched in the resistant bulk, depleted in the susceptible one. This suggested the genetic contribution for resistance against *P. brassicae* within this region in A01 was determined by the resistant parent, Lipid, rather than by the susceptible Abukuma.

On chromosome A03, the negative  $\Delta$ SNP-index values for the first region of the chromosome seemed to surpass the 95% threshold within the plot; however, consideration of the *p*-values revealed no significant association in this region.

An 837 kbp peak in  $\Delta$ SNP-index values was detected on Chromosome A06, above the 95% threshold. It contained 45 SNPs of high confidence. Further analysis of this QTL revealed 3 genes, 7 CDS and 4 UTRs. The positive values of this region indicated that the SNPs in this QTL region were enriched in the resistant bulk, suggesting that the genes within this region could contribute to resistance against *P. brassicae*.

Finally, at a 95% confidence interval, chromosome A07 showed a 6 Mbp significant region that contained 26 high confidence SNPs and 715 annotated sequences, which included 86 genes, 552 CDS and 77 UTRs. The negative  $\Delta$ SNP-index values indicated that the SNPs in this QTL region were enriched in the susceptible bulk and depleted in the resistant one. The negative association of this region with resistance to the pathogen could indicate that it may contain genes linked to susceptibility to *P. brassicae*.

To further investigate these relevant regions, the  $G'$  values and  $q$ -values were calculated with a 99% threshold. These statistical approach removes noise, enabling the identification of regions that are significantly associated with the traits of interest. The  $G'$  results validated the results calculated using the  $\Delta$ SNP-index, they both coincided in finding regions of interest in chromosomes A01 and A07 (Figure 5.10A). Additionally, the  $G'$  results further confirmed that there were no loci of interest in subgenome C (Figure 5.10B).

**Table 5.1.** Total amount of raw, mapped and paired reads for each of the samples sequenced through NGS by Novogene, as well as the resulting high confidence SNPs.

| Genotype                          | Raw reads | Raw data | Mapped reads          | Properly paired reads | High confidence SNPs |
|-----------------------------------|-----------|----------|-----------------------|-----------------------|----------------------|
| Abukuma                           | 225257840 | 33.8     | 224840736<br>(96.64%) | 218054944<br>(93.72%) | 1054966              |
| Lipid                             | 233824026 | 35.1     | 216335594<br>(96.50%) | 210861748<br>(94.05%) | 1069132              |
| F <sub>2</sub> resistant pool 1   | 43007830  | 91.4     | 589729263<br>(97.38%) | 566487808<br>(93.54%) | 1411655              |
| F <sub>2</sub> resistant pool 2   | 566399006 |          |                       |                       |                      |
| F <sub>2</sub> susceptible pool 1 | 112484466 | 91.9     | 578393714<br>(95.14%) | 427633944<br>(70.34%) | 1404815              |
| F <sub>2</sub> susceptible pool 2 | 34058918  |          |                       |                       |                      |
| F <sub>2</sub> susceptible pool 3 | 465930646 |          |                       |                       |                      |

**Table 5.2.** Significant regions in the *Brassica napus* genome, associated with resistance or susceptibility to *Pyrenopeziza brassicae*, identified using the  $\Delta$ SNP-index. Thresholds set at 95% and 99% confidence intervals (CI). The analysis included the position of the identified loci and its length, the number of high confidence SNPs included in the region and the results from an annotation analysis. Annotation elements included the number of coding sequences (CDS), messenger RNA (mRNA) and untranslated regions (UTR).

| Chromosome | CI  | Position (bp)           | Region length (bp) | Number of SNPs | Annotations |       |       |
|------------|-----|-------------------------|--------------------|----------------|-------------|-------|-------|
|            |     |                         |                    |                | Genes       | CDS   | UTR   |
| A01        | 95% | 16,491,492 - 28,518,227 | 12,026,736         | 8,821          | 1,389       | 6,082 | 1,348 |
| A01        | 99% | 19,596,249 - 22,364,188 | 2,764,940          | 4,218          | 294         | 1,360 | 251   |
| A06        | 95% | 21,581,673 - 22,418,748 | 837,076            | 45             | 3           | 7     | 4     |
| A07        | 95% | 131,579 - 743,151       | 611,573            | 26             | 86          | 552   | 77    |

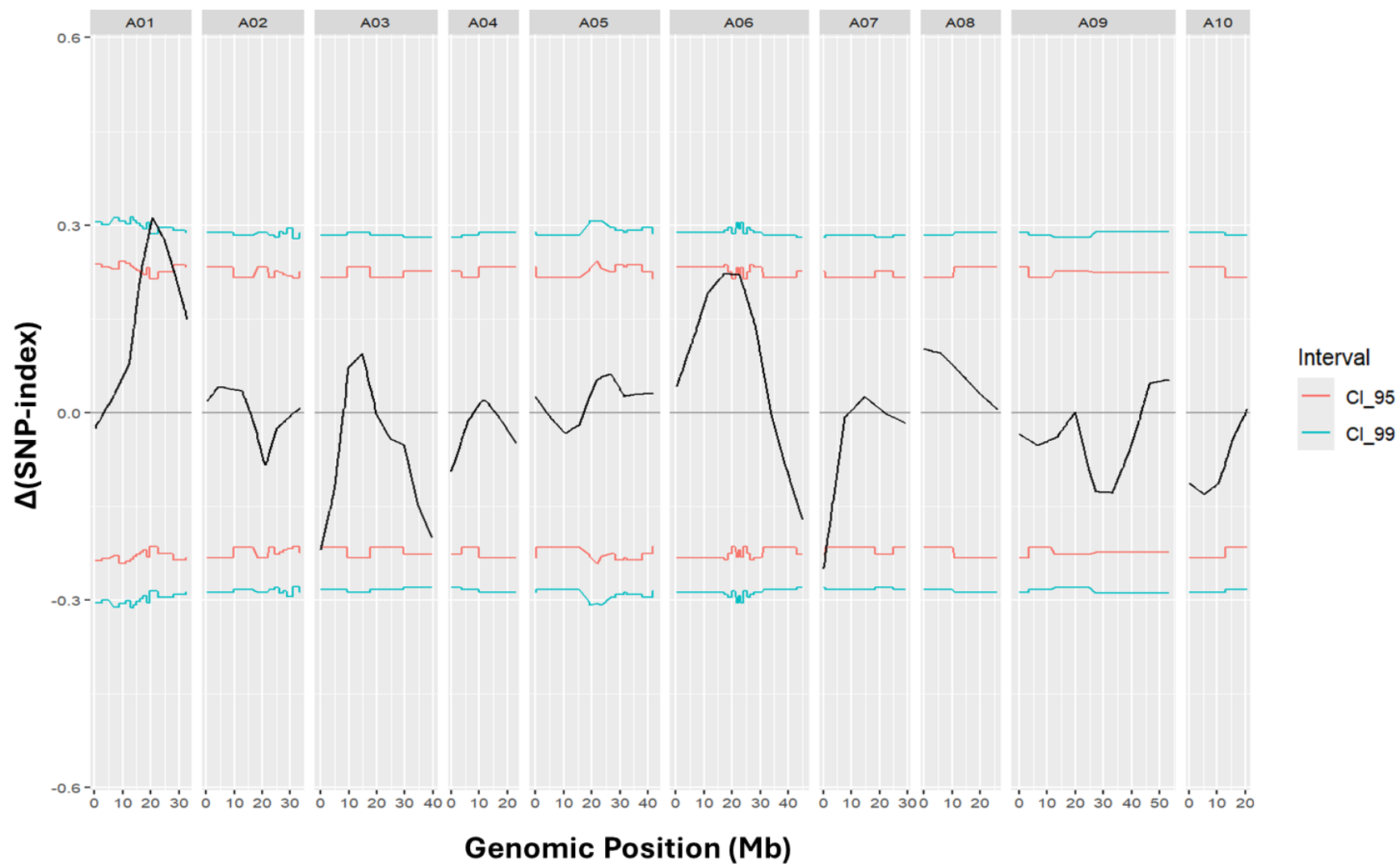


**Figure 5.8.** Results of a bulk segregant analysis showing  $\Delta$ SNP-index for resistance and susceptibility of *Brassica napus* to *Pyrenopeziza brassicae*, against the chromosomal position according to Darmor-bzh in *B. napus* subgenome A.  $\Delta$ SNP-index=0 represented no deviation in allele segregation between bulks (horizontal line). Thresholds to determine significance were set at confidence interval 95% and 99%.

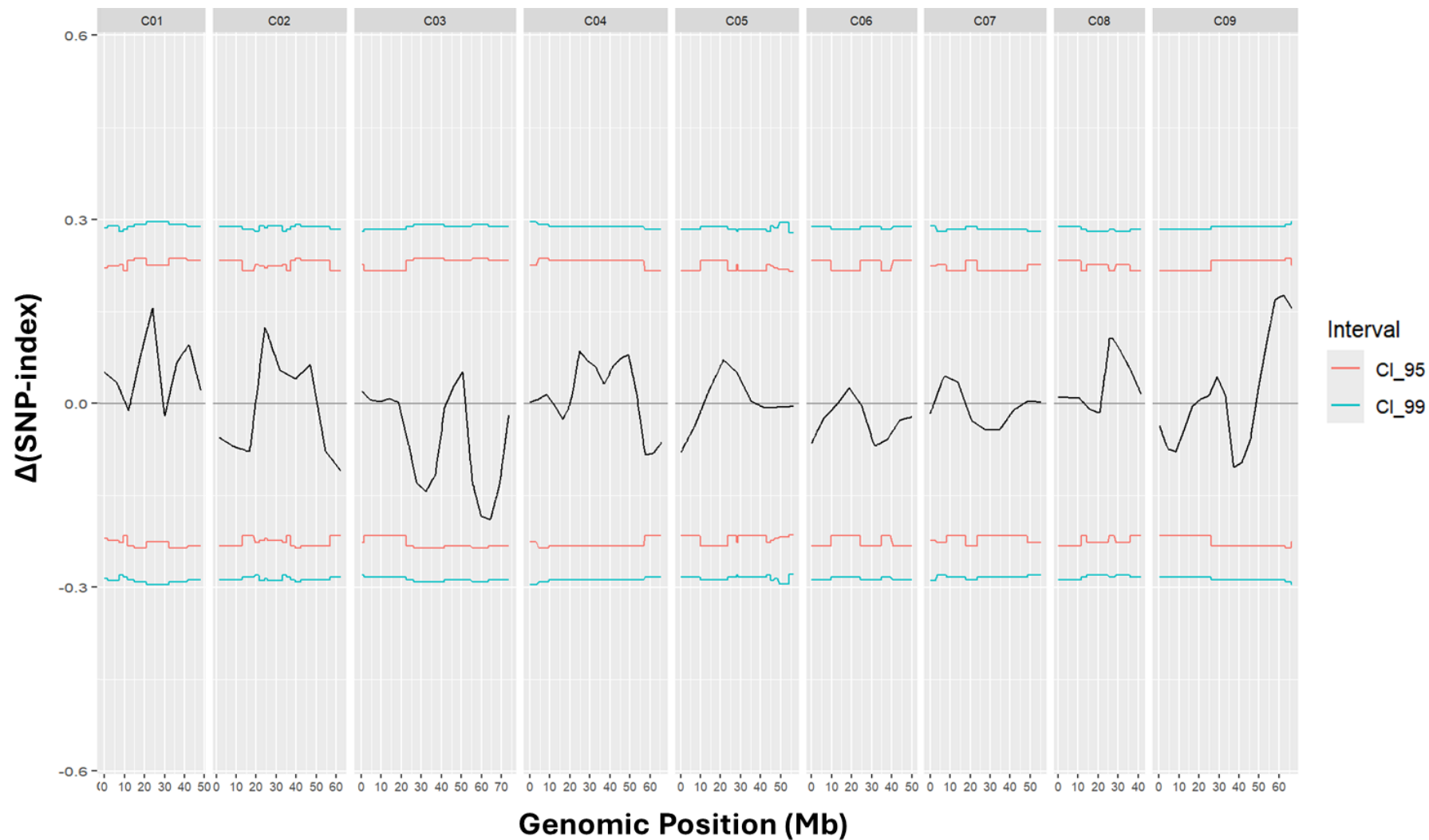


**Figure 5.9.** Results of a bulk segregant analysis showing  $\Delta$ SNP-index for resistance and susceptibility of *Brassica napus* to *Pyrenopeziza brassicae*, against the chromosomal position according to Darmor-bzh in *B. napus* subgenome C.  $\Delta$ SNP-index=0 represented no deviation in allele segregation between bulks (horizontal line). Thresholds to determine significance were set at confidence interval 95% and 99%.

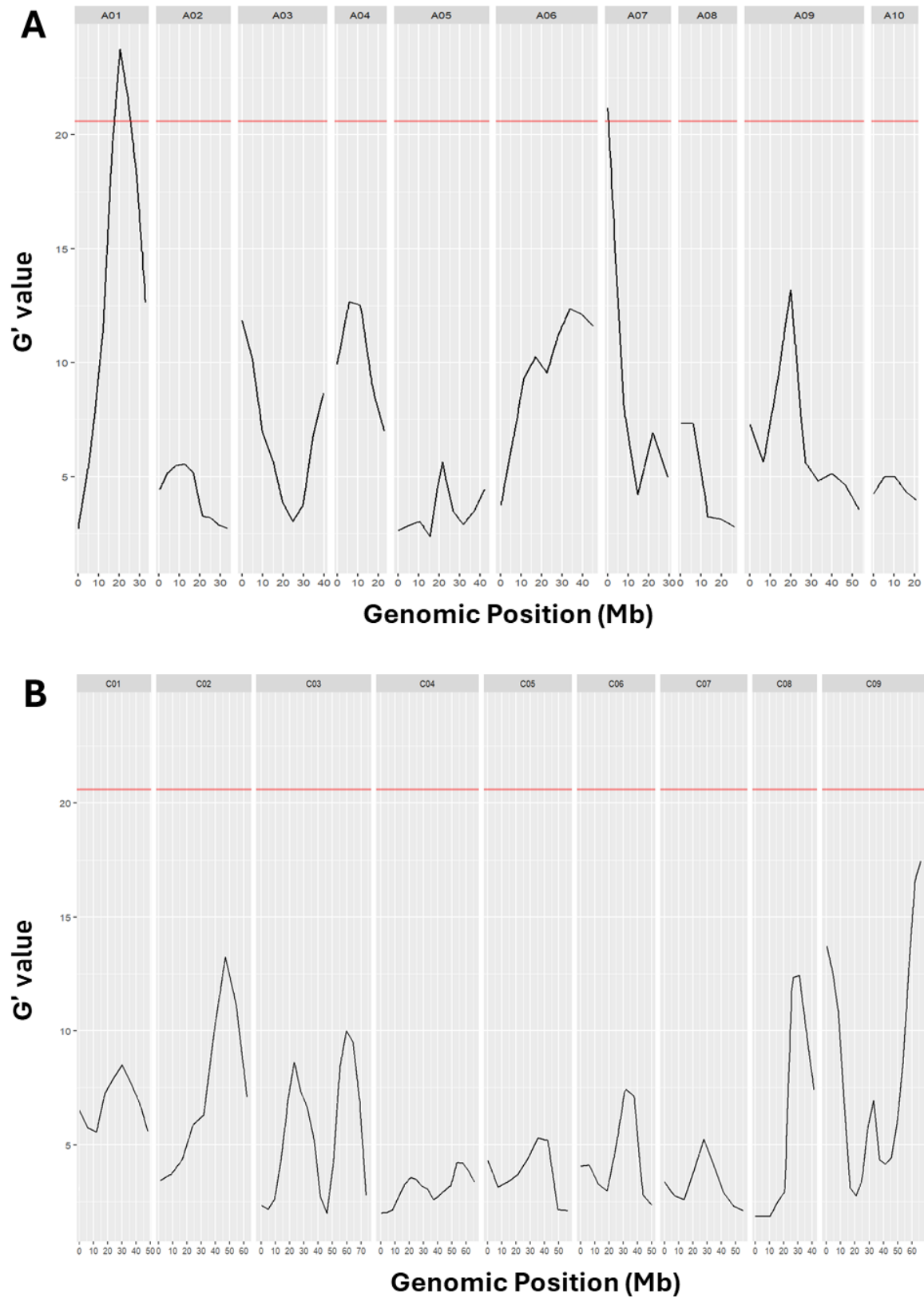


**Figure 5.10.** Results of a bulk segregant analysis showing the G' values for resistance and susceptibility of *Brassica napus* to *Pyrenopeziza brassicae*, against the chromosomal position according to Darmor-*bzh* in *B. napus* **(A)** subgenome A and **(B)** Subgenome C. Threshold to was set at a confidence interval of 99% to determine significance.

## 5.4. Discussion

Bulked segregant analysis mapping approach was used to identify QTL regions in the *B. napus* genome that were significantly associated with resistance against *P. brassicae* in an effort to expand the knowledge on QDR genetics. The cross of a highly susceptible cultivar (Abukuma) and a resistant one (Lipid) generated F<sub>1</sub> and F<sub>2</sub> populations that were inoculated with *P. brassicae* and phenotyped. The hybrid F<sub>1</sub> showed results that significantly aligned with the susceptible parent, cv. Abukuma, which suggested that susceptibility is inherited as a dominant trait. The F<sub>2</sub> population segregated for resistance against the pathogen, showing a continuous distribution of resistance levels, widely ranging between the extreme phenotypes. As was hypothesised, this distribution of the F<sub>2</sub> population did not fit Mendelian segregation ratios that are typically associated with *R*-gene mediated resistance. Thus, it could be inferred that host resistance is associated with QDR.

The BSA-seq analysis resulted in the identification of QTLs in the *B. napus* genome that were related to resistance or susceptibility to *P. brassicae*. A 12 Mbp genomic region was identified on chromosome A01 that appeared to contribute to resistance against the fungal pathogen. Previous studies have identified associations between regions on this chromosome and resistance to *P. brassicae*. A major resistance locus (*PBR1*) was identified by Bradburne *et al.* (1999) on chromosome A01, although it was not associated with any specific phenotype at the time. However, this gene was later studied by Boys *et al.* (2012), who reported that this locus was more likely to be associated with QDR than with *R*-gene mediated resistance because its phenotype was associated with black flecking after inoculation, no asexual sporulation of the fungus and a decrease in hyphal growth. This would significantly deter the spread of the disease and reduce its effect on the host, which is typically associated with QDR, rather than lead to pathogen death, commonly linked to the effect of *R* genes.

A QTL region of interest was also found on chromosome A06, with an estimated sequence length of 837 kbp positively associated with resistance to the pathogen. This coincided with previous research that reported a significant QTL associated with LLS resistance. Pilet *et al.* (1998) found a region in a linkage group on chromosome A06 that was considered to be a “multiple disease resistance” QTL. It was potentially involved in common resistance mechanisms against different fungal pathogens: *P. brassicae* and *Leptosphaeria maculans* (phoma stem canker). This was further confirmed by Sapelli (2025), who discovered that this QTL on chromosome A06 was colocalised with a QTL for resistance against *L. maculans*.

A 6 Mbp region of interest was found on chromosome A07, although it contributed to susceptibility to *P. brassicae* instead of resistance against it. Pilet *et al.* (1998) reported a significant linkage group on this chromosome, but it was associated with host resistance. There have been no reports on relevant QTL regions on chromosome A07 associated with susceptibility to *P. brassicae*; thus, it could be novel genomic QTL loci.

From these results, it could be inferred that QTL regions on the *B. napus* chromosomes A01 and A06 were associated with resistance against *P. brassicae*, and a QTL region on chromosome A07 linked to susceptibility to the pathogen. This coincided with the initial hypothesis for this experimental chapter that proposed that a bulked segregant analysis would reveal loci associated with QDR. It is likely that the different loci in A01 and A06 might have a pyramiding

effect that significantly contributes to host resistance. Further investigation would strengthen this research by mapping the specific genes contained in the QTL regions identified and characterising them in relation to host resistance and susceptibility.

## **5.5. Conclusion**

A bulked segregant analysis done on an  $F_2$  population confirmed that *B. napus* host resistance was caused by quantitative disease resistance (QDR) rather than by *R*-gene mediated resistance because the phenotype of the  $F_2$  population presented a continuous distribution of resistance against *P. brassicae* that did not fit Mendelian segregation ratios that are typically linked to the effect of *R*-genes. Instead, the phenotypical distribution supported the hypothesis of QDR being responsible for host resistance. Furthermore, the resulting phenotype of hybrid  $F_1$ , similar to the susceptible parent cv. Abukuma, suggested that susceptibility is inherited as a dominant trait. Finally, the analysis of the BSA-seq identified two significant QTL regions associated with resistance against *P. brassicae* in the *B. napus* genome, specifically on chromosomes A01 and A06. It also identified a QTL region on chromosome A07 that was associated with susceptibility to the pathogen, which has never been reported before and could be a novel source of susceptibility to *P. brassicae*.

## Chapter 6: General discussion

The first aim of this study was to improve phenotyping strategies that determine disease severity by developing, testing and comparing quantitative techniques that yield more reliable data than qualitative visual assessment. Additionally, this research aimed to expand the existing knowledge of the *Brassica napus*-*Pyrenopeziza brassicae* pathosystem by studying the genetic basis of host resistance, focusing on quantitative disease resistance (QDR). This included the characterisation of the *BnHAT1* gene, potentially involved in susceptibility to *P. brassicae*, the study of gene expression in cultivars with different phenotypes after pathogen inoculation, and the identification of QTLs involved in resistance or susceptibility to LLS through a bulked segregant analysis.

### **6.1 Assessment of quantitative phenotyping methods**

The four quantitative phenotyping strategies studied (acervuli quantification, spore counting, WAC assay and qPCR) provided with a non-subjective portrayal of the interaction between *B. napus* and *P. brassicae*. They were used for identification of the pathogen, early detection *in planta* and determination of disease severity, which is fundamental for the study of host resistance. They were able to distinguish between genotypes and phenotypes efficiently, offering more reliable and accurate data than the traditional visual assessment, prone to human error.

In order of efficiency and ability to differentiate between the cultivars tested for resistance to the pathogen, the qPCR technique was the most efficient phenotyping method for *P. brassicae* detection by quantifying pathogen DNA. The next most accurate method was the WAC assay, which was first used in this report for the measurement of relative fungal biomass in the *B. napus*-*P. brassicae* pathosystem. Spore counting could efficiently distinguish phenotypes and performed better than acervuli quantification that, even though it had the lowest ability to differentiate between the cultivars, still yielded more reliable results than visual assessment.

Acervuli quantification could be optimised by using machine learning and artificial intelligence to develop a statistical algorithm that would be able to count the number of acervuli on a picture of an inoculated leaf. Attempts at this during this project resulted in multiple challenges. The main issue that arose was the presence of acervuli on the leaf veins because both are white and the program was unable to efficiently differentiate between them, which resulted in large amounts of acervuli structures being dismissed in the quantification. Additionally, the physical difference between leaves in terms of colour and relief complicated the training of the program, which was efficient at detecting the acervuli in contrast to darker shades of flat leaf surface but not significant enough when the colour was lighter or the leaf was more distorted. These issues could be solved by increasing the number of picture samples in the database, training the algorithm for a larger variety of possibilities. Other types of imaging methods have been reported to detect the presence of the LLS pathogen *in planta* before the most significant symptoms appeared, using multispectral imaging (Veys *et al.*, 2019).

**Chapter 3: Improvement of quantitative phenotyping strategies to reliably determine disease severity**

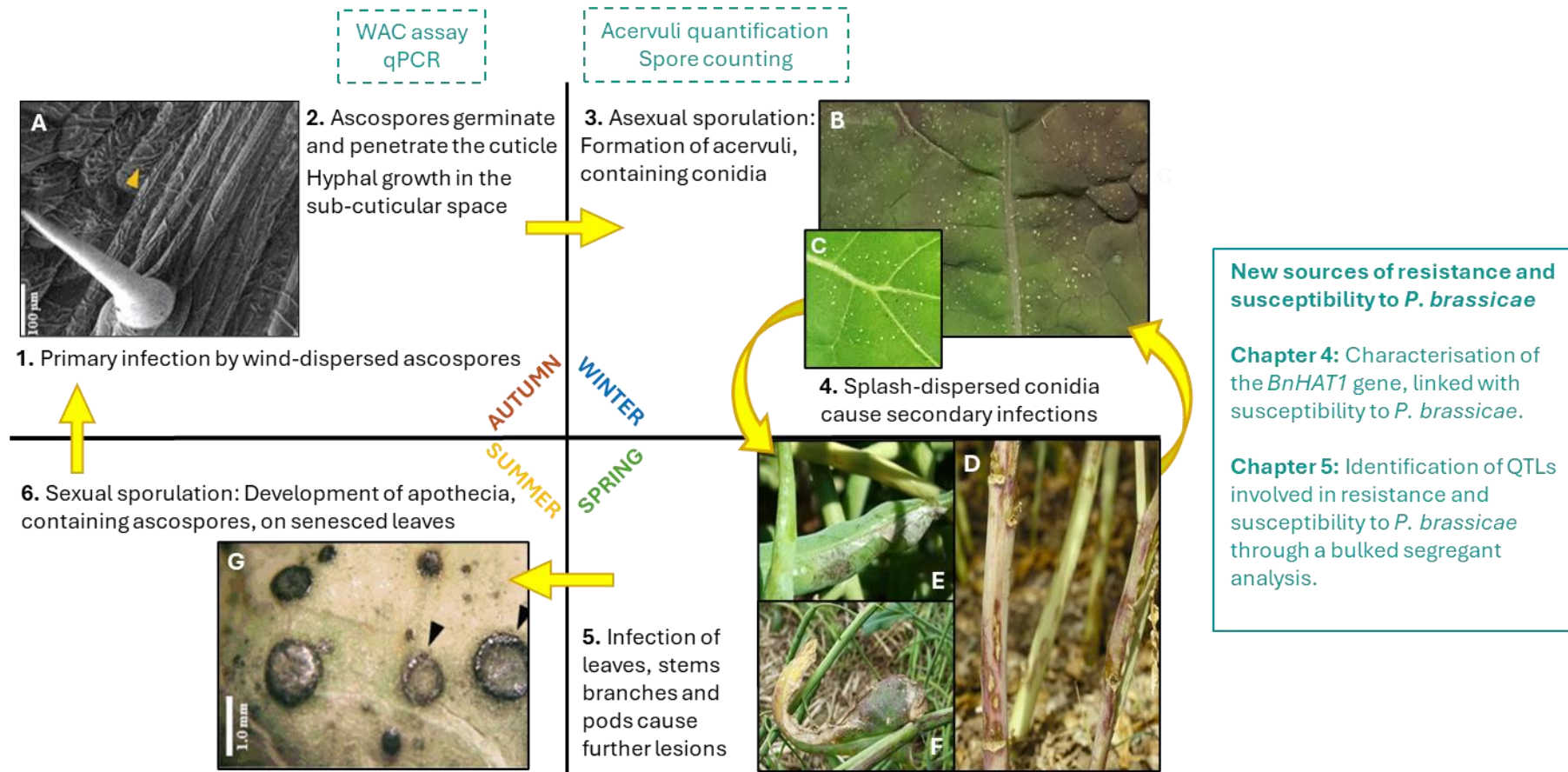


**Figure 6.1.** Visual abstract summarising the findings and outcomes of this research on the quantitative assessment and genetics of the interaction between the light leaf spot pathogen *Pyrenopeziza brassicae* and its oilseed rape host *Brassica napus*, integrated on the life cycle of the fungal pathogen.

The development and study of these quantitative phenotyping methods is fundamental for the study of host resistance in *B. napus*. It is crucial to establish disease severity levels and higher accuracy would increase the reliability of any experimental procedure down the line. Throughout this project, the screening of all the *B. napus* individuals inoculated with *P. brassicae* was done using both the qualitative visual assessment and the quantitative spore counting. On every occasion, the spore counting yielded more precise and accurate results. This was observed in when the TILLING mutant lines were observed with the wild type individuals in Chapter 4, or when studying the segregating TILLING mutant line 3B, where it was important to determine phenotypical differences within the genotypes. However, it was the most vital during the bulked segregant analysis, where a crucial step was carefully selecting the most extreme phenotypes of the F<sub>2</sub> population and spore counting offered stricter and significantly more reliable results.

The development of improved monitoring strategies would aid in the selection of the most suitable method to control the spread of light leaf spot epidemics. Accurate pathogen identification, early detection *in planta* and disease severity determination would allow for targeted and localised treatment of the disease, reducing the need for blanket spraying of fungicides. This would be significantly beneficial for farmers and breeders because it would reduce crops yield loss and fungicide applications, with the consequent reduction in expenditure in disease management procedures. A lower reliance on this chemical treatment would also benefit the ecosystem by minimising the spread of these chemicals in water and soil, as well as reducing the development of fungicide resistance in the pathogen population. This could be aided by forecasting tools that predict the effects of the disease. Up until 2023, the AHDB (Agriculture and Horticulture Development Board) provided with an online forecast system able to predict 25% of plants affected by light leaf spot disease in the spring. This served as a guide for efficient fungicide applications. However, in lieu of that system not being available any longer, it is crucial to turn to reliable monitoring strategies such as the ones examined throughout this study.

## **6.2 BnHAT1 gene involved in susceptibility to *P. brassicae***

The function of the *BnHAT1* gene (*BnaA04g20860D*) was characterised using TILLING mutants that had SNP mutations in the gene of interest. The results indicated that this gene is significantly associated with susceptibility to *P. brassicae*. This was supported by the results from an RNA-seq analysis of a gene expression experiment, which revealed that the expression of the *BnHAT1* gene was statistically higher in susceptible cultivars than in resistant ones. The first step of a future work programme would be to complete the experiment in Chapter 4.3.6, that attempted to study the gene expression of the *BnHAT1* gene in TILLING mutant 2A. Since the RNA was extracted from each of the samples, the remaining part of the experimental process would be to complete the RT-qPCR as described in Chapter 4.4.5.

Another potential experiment that could be of much interest would be a metabolomic analysis that would compare the concentration of multiple metabolites in wild type *B. napus* individuals with TILLING mutant lines, after *P. brassicae* inoculation. This would elucidate what metabolites the *BnHAT1* could be associated with, and if it has an influence on any of them. This study could potentially lead to the discovery of the substrates and products of this acyltransferase enzyme.

An experiment like this was started on 2025 by Dr Henrik Stotz and Fred Beaudoin, but no results have been obtained yet.

Furthermore, the *BnHAT1* gene could be further studied by studying protein expression in heterologous systems such as *Escherichia coli* and *Arabidopsis thaliana* using recombinant technology. This experiment would examine if expressing the BnHAT1 protein in *A. thaliana*, that is not susceptible to *P. brassicae*, would affect its resistance to the disease when inoculated with the pathogen. A preliminary plan to do this experiment using Gateway Cloning was designed and pOPINK vectors for the transformation, but this experiment went beyond the extension of this project and, thus, it was not accomplished.

The molecular structure of the BnHAT1 protein was studied using 3D models, which confirmed the importance of the highly conserved HXXXD motif typically found in BAHD-acyltransferases. The aspartic acid residue from this motif appeared to play a structural role by establishing a salt bridge that seemed to contribute to the integrity of the protein. A TILLING mutant line whose SNP mutation resulted in the absence of this salt bridge showed an increase in resistance against the pathogen. This reinforced the hypothesis that the BnHAT1 protein plays a role as a susceptibility factor in *B. napus* against fungal pathogen *P. brassicae*. This protein could be studied through crystallography to confirm the 3D atomic structure of the molecule, determine interaction with substrates and products, and establish its mechanism of action, how does it affect susceptibility to *P. brassicae*, contributing to functional characterisation.

### **6.3 Discovery of genes associated with resistance against *P. brassicae***

The RNA-seq analysis of a gene expression experiment discovered three upregulated genes (*BnaA01g03020D*, *BnaA09g13870D* and *BnaA08g16100D*) and a downregulated gene (*PDF1.2*) that, by looking at the annotation of *A. thaliana*, they were significantly associated with host resistance. The study of their *A. thaliana* orthologues provided with information about their potential biological functions and mechanisms of action. As potential future work, it would be interesting to examine these genes closer to determine the role they play in *B. napus* in relation with fungal pathogen *P. brassicae*. It would also be of interest to study how these genes are expressed under different treatments since the RNA-seq analysis revealed conflicting results as to what signalling pathways induced their gene expression. The RNA-seq analysis could be extended to examine different interactions within the transcriptome and further annotation of discovered genes.

### **6.4 QTL regions associated with host resistance**

The analysis of the F<sub>2</sub> population that derived from a cross between resistant Lipid and susceptible Abukuma confirmed that quantitative disease resistance (QDR) is responsible for host resistance, rather than *R*-gene mediated resistance. This was inferred from the continuous distribution of disease scores throughout the entire F<sub>2</sub> population, which did not fit Mendelian ratios. The bulked segregant analysis (BSA) revealed three significant QTL regions associated with host resistance in chromosomes A01, A06 and A07. The regions of interest in A01 and A06 were positively associated with resistance to the pathogen, whereas in A07 it was associated

with susceptibility to *P. brassicae*. This latter one has not been reported before, therefore it could constitute a novel source of susceptibility worth examining further.

As part of the future work related to these discoveries, it would be of great interest to look into the genes inside the QTL regions, specifically map them and study annotations using gene ontology, as well as examining their orthologue genes for some insight into their biological and molecular functions. It would be of high interest to further examine if the genes within the QTL regions found in Chapter 5 were differentially expressed in the RNA-seq analysis from Chapter 4. Potentially, some of the genes contained in these regions could have a higher gene expression in resistant cultivars or vice versa, which provide invaluable insight into new sources of resistance to *P. brassicae*.

Furthermore, some of the significant genes identified in Chapter 4 (Figure 4.13; Tables 4.5 & 4.6) corresponded to the chromosomes highlighted in the BSA analysis A01 and A07. There were three upregulated genes on chromosome A01: the *BnaA01g03020D*, *BnaA01g28440D* and *BnaA01g28930D*. The first one had an ortholog in *A. thaliana* (*UGT73B3*) with an established function associated with host resistance. The *BnaA07g26670D* gene, present on the A07 chromosome, was downregulated in the RNA-seq analysis. It would be beneficial to check if these genes are within the identified QTL region of the chromosome and, if such, how they might contribute to host resistance.

The identified quantitative sources of resistance and susceptibility could be used down the line in breeding and bioengineering programs to develop resistant *B. napus* cultivars able to withstand light leaf spot epidemics. Potentially, further investigation and research could lead to the generation of oilseed rape varieties in which the genes associated with resistance are enhanced, or the susceptibility genes have been silenced or bred out. As previously mentioned, the use of resistant cultivars would be favourable for breeders and farmers from an economic perspective. It would also be substantially beneficial for the environment.

## **6.5 Conclusion**

Quantitative phenotyping methods proved to be significantly more reliable than the traditional method visual assessment, far more accurate and precise in establishing the levels of disease resistance. This is a fundamental step in the study of the *B. napus*-*P. brassicae* pathosystem. More specifically, qPCR yielded the most significant results, with the highest ability to distinguish not only between resistance phenotypes, but also between genotypes. The WAC assay was used for detection of fungal biomass of *P. brassicae* in *B. napus* for the first time in this report, with significant results. Spore counting was highly significant and used often as a substantially improved method in comparison to qualitative visual assessment. Acervuli quantification, although it should be optimised for efficient use, also performed better than visual assessment.

The *BnHAT1* gene that had been identified as a potential susceptibility gene against *P. brassicae* was successfully characterised using TILLING mutant lines and RNA-seq analysis of a gene expression experiment. It was confirmed it plays a role in *B. napus* as a susceptibility factor against the pathogen. Additionally, through the RNA-seq analysis, new genes were discovered that were differentially expressed across genotypes and pathogen inoculation time points. This indicated that they might be related to host resistance and are worth further examination.

Finally, the continuous distribution of the resistance phenotype of an F<sub>2</sub> population confirmed that QDR was responsible for host resistance in the *B. napus*-*P. brassicae* pathosystem, rather than *R*-gene mediated resistance. The bulked segregant analysis of the F<sub>2</sub> population discovered relevant QTL regions on chromosomes A01 and A06, associated with resistance to the pathogen, and a novel region on chromosome A07, associated with susceptibility, never reported before. The genes within this QTLs could be new sources of resistance or susceptibility to *P. brassicae*, worth further investigation.

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## Appendices

**Appendix 1:** Key stages of oilseed rape growth (Sylvester-Bradley *et al.*, 1984; Sylvester-Bradley, 1985).

| Developmental stages in plant | Growth stage (in decimals) | Description                                  |
|-------------------------------|----------------------------|----------------------------------------------|
| Germination and emergence     | 0.0                        | Dry seed                                     |
| Leaf production               | 1.0                        | Both cotyledons unfolded and green           |
|                               | 1.1                        | First true leaf emerged                      |
|                               | 1.2                        | Second true leaf emerged                     |
|                               | 1.3 etc.                   | Third true leaf emerged                      |
| Stem extension                | 2.0                        | No internodes (rosette)                      |
|                               | 2.5                        | About five internodes                        |
| Flower bud development        | 3.0                        | Only leaf buds present                       |
|                               | 3.1                        | Flower buds present but enclosed by leaves   |
|                               | 3.3                        | Flower buds visible from above ('green bud') |
|                               | 3.5                        | Flower buds raised above leaves              |
|                               | 3.6                        | First flower stalks extending                |
|                               | 3.7                        | First flower buds yellow ('yellow bud')      |
| Flowering                     | 4.0                        | First flower opened                          |
|                               | 4.1                        | 10% all buds opened                          |
|                               | 4.3                        | 30% all buds opened                          |
|                               | 4.5                        | 50% all buds opened                          |
| Pod development               | 5.3                        | 30% potential pods                           |
|                               | 5.5                        | 50% potential pods                           |
|                               | 5.7                        | 70% potential pods                           |
|                               | 5.9                        | All potential pods                           |
| Seed development              | 6.1                        | Seeds expanding                              |
|                               | 6.2                        | Most seeds translucent but full size         |
|                               | 6.3                        | Most seed green.                             |
|                               | 6.4                        | Most seed green-brown mottled                |
|                               | 6.5                        | Most seeds brown                             |
|                               | 6.6                        | Most seed dark brown                         |
|                               | 6.7                        | Most seed black but soft                     |
|                               | 6.8                        | Most seed black and hard                     |
|                               | 6.9                        | All seeds black and hard                     |
| Leaf senescence               | 7.0                        | Leaf senescence                              |
| Stem senescence               | 8.1                        | Most stem green                              |
|                               | 8.5                        | Half stem green                              |
|                               | 8.9                        | Little stem green                            |
| Pod senescence                | 9.1                        | Most pods green                              |
|                               | 9.5                        | Half pods green                              |
|                               | 9.9                        | Few pods green                               |

**Appendix 2:** Phenotyping of the light leaf spot disease, using visual assessment to determine the severity of *Pyrenopeziza brassicae* symptoms on *Brassica napus* field plots (AHDB). Colour formatted with green, yellow and red ranges, from lower to higher resistance to *P. brassicae*.

| Score | Description                                                                         |
|-------|-------------------------------------------------------------------------------------|
| 1     | All plants stunted or dead                                                          |
| 2     | >50% plants stunted or dead                                                         |
| 3     | <50% plants stunted or deformed<br>>50% plants with <i>P. brassicae</i> sporulation |
| 4     | 35-50% plants with <i>P. brassicae</i> sporulation                                  |
| 5     | 20-35% plants with <i>P. brassicae</i> sporulation                                  |
| 6     | 5-20% plants with <i>P. brassicae</i> sporulation                                   |
| 7     | 1-5% plants with <i>P. brassicae</i> sporulation                                    |
| 8     | Traces of <i>P. brassicae</i> sporulation visible on close inspection               |
| 9     | No disease observable                                                               |

**Appendix 3:** Quantification of the percentage of *P. brassicae* DNA present in different *B. napus* samples. Total DNA extracted was extracted from *B. napus* samples and quantified using Qubit™. The Through qPCR, the *P. brassicae* DNA was calculated. Knowing that the final DNA extracted was diluted in 40 µL of TE buffer, the amount of *P. brassicae* was adjusted. The total amount of DNA was compared to the specific *P. brassicae* DNA to obtain the final percentages.

| Cultivar             | Total DNA (ng) | <i>P. brassicae</i> DNA (ng)* | Adjusted <i>P. brassicae</i> DNA (ng)* | % <i>P. brassicae</i> per DNA per <i>B. napus</i> sample |
|----------------------|----------------|-------------------------------|----------------------------------------|----------------------------------------------------------|
| Cabriolet            | 8640           | 0.048                         | 1.9                                    | 0.022 %                                                  |
| Ambassador           | 2056           | 24.668                        | 986.7                                  | 47.99 %                                                  |
| RV31                 | 1904           | 7.378                         | 295.1                                  | 15.50 %                                                  |
| Nikita               | 1952           | 0.179                         | 7.2                                    | 0.37 %                                                   |
| Lipid                | 1752           | 0.689                         | 27.5                                   | 1.57 %                                                   |
| *Multi-copy gene ITS |                |                               |                                        |                                                          |

**Appendix 4:** TILLING mutant lines with their corresponding SNPs, resulting amino acid changes and type of mutation they produce. Highlighted in orange are the lines that were sent from the JIC.

| <b>TILLING mutants</b> | <b>SNP</b> | <b>Amino acid change</b>          | <b>Type of mutation</b> |
|------------------------|------------|-----------------------------------|-------------------------|
| 1                      | 499 G → A  | 167 Aspartic acid → Asparagine    | Missense                |
| 2*                     | 499 G → A  | 167 Aspartic acid → Asparagine    | Missense                |
| 3*                     | 516 G → A  | 172 Tryptophan → Stop codon       | Stop-codon              |
| 4*                     | 534 G → A  | 178 Tryptophan → Stop codon       | Stop-codon              |
| 5                      | 154 G → A  | 52 Aspartic acid → Asparagine     | Missense                |
| 6*                     | 191 C → T  | 64 Serine → Phenylalanine         | Missense                |
| 7                      | 227 C → T  | 176 Alanine → Valine              | Missense                |
| 8                      | 253 G → A  | 85 Glutamic acid → Lysine         | Missense                |
| 9                      | 256 G → A  | 86 Aspartic acid → Asparagine     | Missense                |
| 10                     | 259 G → A  | 87 Aspartic acid → Asparagine     | Missense                |
| 11                     | 269 C → T  | 90 Alanine → Valine               | Missense                |
| 12                     | 448 G → A  | 150 Glutamic acid → Lysine        | Missense                |
| 13                     | 503 G → A  | 168 Glycine → Glutamic acid       | Missense                |
| 14                     | 511 G → A  | 171 Valine → Isoleucine           | Missense                |
| 15                     | 511 G → A  | 171 Valine → Isoleucine           | Missense                |
| 16                     | 551 C → T  | 184 Threonine → Isoleucine        | Missense                |
| 17                     | 553 G → A  | 185 Glycine → Serine              | Missense                |
| 18                     | 553 G → A  | 185 Glycine → Serine              | Missense                |
| 19                     | 557 G → A  | 186 Arginine → Glutamine          | Missense                |
| 20                     | 571 C → T  | 191 Proline → Serine              | Missense                |
| 21                     | 587 G → A  | 196 Glycine → Glutamic acid       | Missense                |
| 22                     | 613 C → T  | 205 Proline → Serine              | Missense                |
| 23                     | 613 C → T  | 205 Proline → Serine              | Missense                |
| 24                     | 626 C → T  | 209 Proline → Leucine             | Missense                |
| 25                     | 725 C → T  | 242 Serine → Phenylalanine        | Missense                |
| 26                     | 844 G → A  | 282 Glutamic acid → Lysine        | Missense                |
| 27                     | 459 C → T  | 153 Aspartic acid → Aspartic acid | Silent                  |
| 28                     | 240 C → T  | 80 Valine → Valine                | Silent                  |

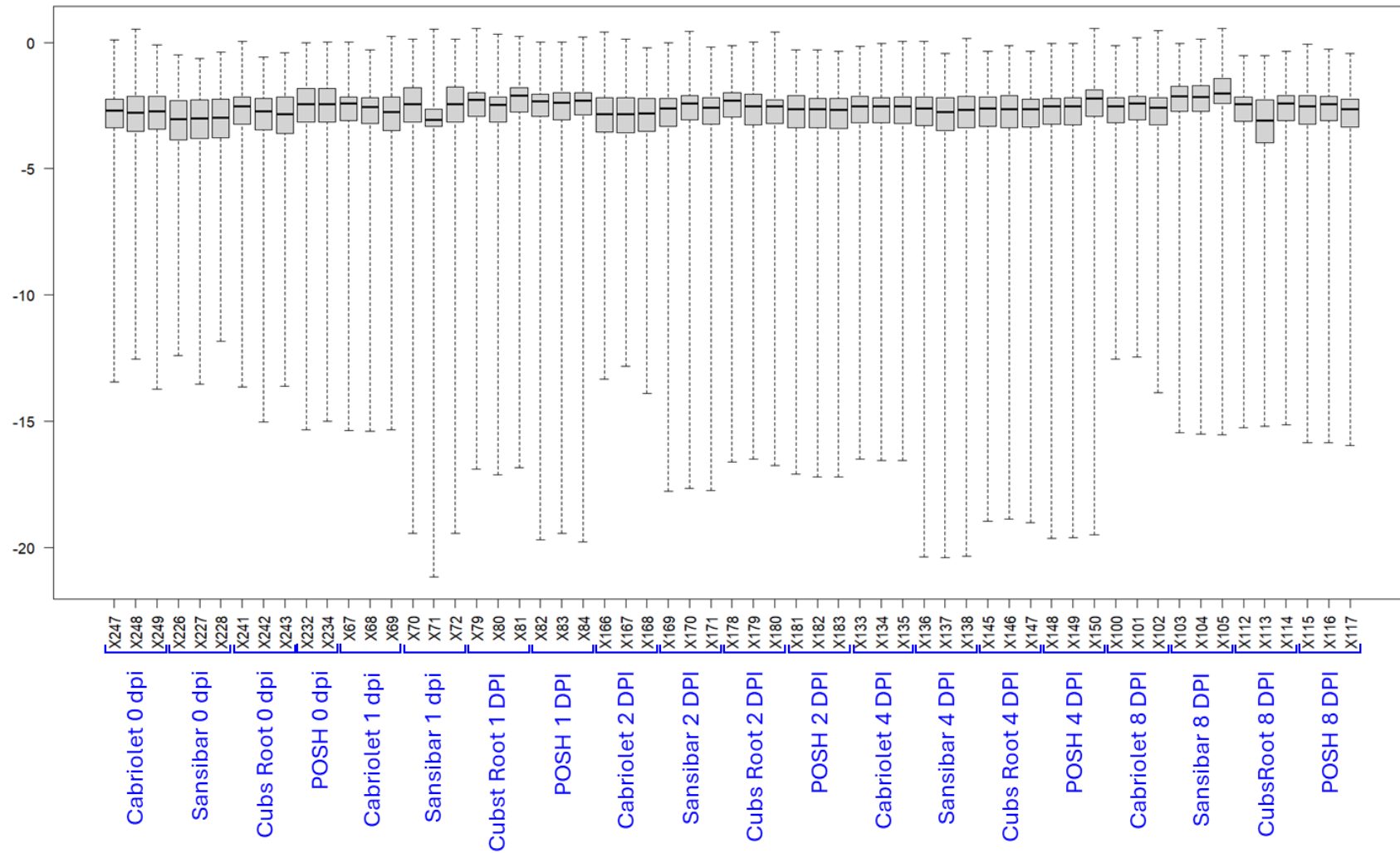
\*TILLING mutant lines selected for further study.

**Appendix 5:** Coding sequence (CDS) of the *BnHAT1* gene (*BnaA04g20860D*). The three exons are highlighted in pale orange. SNPs in the TILLING mutants are featured in bold and underlined, and the affected codons highlighted. TILLING mutant line 2 is highlighted in orange, line 3 in red, line 4 in blue and line 6 in green. The CDS that correspond to the conserved HXXXD and DFGWG domains are framed by a dark orange rectangle.

**DNA sequence of the *BnaA04g20860D* gene**

TATTTAAATAATTAACATATGGAGATATATTATTACAACATTAAATTATATATATCTAAT  
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TTGTGTTTTGTAAAGAAAAAATGATGAAATTAGATTAATATAAATAGTTCGCAGATCCC  
TCGCAGTCTATCATACTACTCCCATCCATTGGTGTAATAAAAAAGAAAAAGAAGATA  
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GTCTCTGAGCTTAAAGCTAAAGCCAACGGCGAGGTTGGTGGTTCGGATAATGTCTCGTCG  
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CGATTGTTGTTGGTTCTTTGCAACCGTTGCGATGTCCCATCGACACGTGGCGATCCGCGAT  
TGATGCACCTTTTAACTTTTTTTTTTATTAGACAAAAAAGATAAAAAAATTTAAGAA  
ACCA

**Appendix 6:** Visualisation of the distribution of the raw reads for each of the samples from different *Brassica napus* cultivars inoculated with a *Pyrenopeziza brassicae* population from Rothamsted Research (Muthayil-Ali, 2023) at different inoculation time points.



**Appendix 7:** Selected *Brassica napus* genes and their descriptions.

| <b>Selected <i>B. napus</i> genes</b> | <b>Gene description</b>                                        |
|---------------------------------------|----------------------------------------------------------------|
| <i>BnaA04g20860D</i>                  | BAHD acyltransferase ( <i>BnHAT1</i> )                         |
| <i>BnaC03g45470D</i>                  | Pathogenesis related protein ( <i>PR1</i> )                    |
| <i>BnaC04g14330D</i>                  | Cinnamate-4-hydroxylase ( <i>C4H</i> )                         |
| <i>BnaA02g07530D</i>                  | Phosphatidylinositol-specific phospholipase C4 ( <i>PLC4</i> ) |
| <i>BnaC07g27610D</i>                  | 40s Ribosomal subunit protein S24e                             |
| <i>BnaC07g38240D</i>                  | β-adaptin                                                      |
| <i>BnaA10g05830D</i>                  | Polyadenylate-binding protein 1 ( <i>PBP1</i> )                |
| <i>BnaC08g24910D</i>                  | Universal stress protein ( <i>USP</i> )                        |
| <i>BnaA05g34100D</i>                  | <i>SNF1</i> kinase homolog 10 ( <i>KIN10</i> )                 |
| <i>BnaC02g23620D</i>                  | Plant defensin 1.2 ( <i>PDF1.2</i> )                           |

**Appendix 8:** Environmental temperature conditions during glasshouse experiments in the Bayfordbury campus, University of Hertfordshire, studying susceptibility of *Brassica napus* to *Pyrenopeziza brassicae*. Temperature values were obtained from United Kingdom Weather History in Luton, U.K. (51.88 °N, 0.38 °W).

| Experiment | Sowing and transplanting <sup>1</sup> |        |        | Inoculation and sampling <sup>1</sup> |        |        |
|------------|---------------------------------------|--------|--------|---------------------------------------|--------|--------|
| A          | September 2022                        |        |        | October 2022                          |        |        |
|            | Max °C                                | Avg °C | Min °C | Max °C                                | Avg °C | Min °C |
|            | 18.60                                 | 14.44  | 10.90  | 16.39                                 | 12.88  | 9.29   |
| B          | February 2023                         |        |        | March 2023                            |        |        |
|            | Max °C                                | Avg °C | Min °C | Max °C                                | Avg °C | Min °C |
|            | 9.36                                  | 5.93   | 2.46   | 9.52                                  | 6.66   | 4.00   |
| C          | March 2023                            |        |        | April 2023                            |        |        |
|            | Max °C                                | Avg °C | Min °C | Max °C                                | Avg °C | Min °C |
|            | 9.52                                  | 6.66   | 4.00   | 12.97                                 | 8.17   | 4.03   |
| D          | February 2024                         |        |        | March 2024                            |        |        |
|            | Max °C                                | Avg °C | Min °C | Max °C                                | Avg °C | Min °C |
|            | 9.83                                  | 6.96   | 4.21   | 11.00                                 | 7.63   | 4.71   |
| E          | March 2024                            |        |        | April 2024                            |        |        |
|            | Max °C                                | Avg °C | Min °C | Max °C                                | Avg °C | Min °C |
|            | 11.00                                 | 7.63   | 4.71   | 13.13                                 | 9.22   | 5.80   |
| F          | April 2024                            |        |        | May 2024                              |        |        |
|            | Max °C                                | Avg °C | Min °C | Max °C                                | Avg °C | Min °C |
|            | 13.13                                 | 9.22   | 5.80   | 17.84                                 | 13.53  | 9.55   |

<sup>1</sup>Sowing, transplanting, inoculation and sampling refer to the stages of *B. napus* seedlings.

**Appendix 9:** Concentration and quality of DNA pools for Next-Generation sequencing.

| <b>Genotype</b>                 | <b>Concentration<br/>Qubit (ng/ul)</b> | <b>OD 260/280</b> |
|---------------------------------|----------------------------------------|-------------------|
| Abukuma                         | 77.30                                  | 1.85              |
| Lipid                           | 63.50                                  | 1.89              |
| F <sub>2</sub> susceptible pool | 67.75                                  | 1.80              |
| F <sub>2</sub> resistant pool   | 51.55                                  | 1.91              |

# Detecting light leaf spot in oilseed rape: study of quantitative phenotyping strategies

Gimenez Molina, Laura; Fell, Heather; Stotz, Henrik

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University of Hertfordshire **UH**



## ABSTRACT

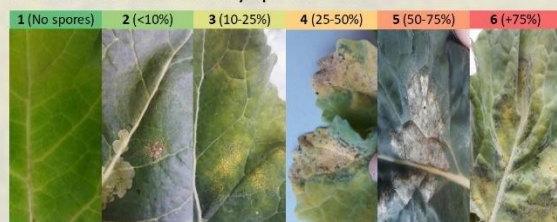
*Pyrenopeziza brassicae* is responsible for **light leaf spot**, the most economically damaging disease affecting oilseed rape (*Brassica napus*) across Europe. Cultural and chemical control practices are insufficient to control its spread, therefore the study of genetic resistance has become of increasing importance. To study **host resistance**, it is fundamental to establish a reliable phenotyping method, however the strategies commonly used are based on visual assessment<sup>2</sup> and prone to human error. A quantitative method (**spore count**<sup>3</sup>) was studied as a more reliable alternative. It was determined that spore concentration is a reliable strategy since it can be statistically correlated with the disease severity.



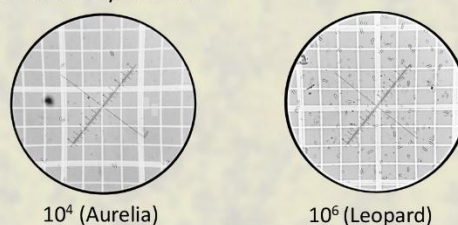
## MATERIALS & METHODS

Field trial conducted in Thriplow (managed by KWS). 21 *B. napus* accessions (2 repetitions) sown in September, 2021. Samples collected in March, May and June (2022) by picking 10 leaves at random per cultivar, wrapped in paper towels and placed in plastic bags that were sprayed with water to create humidity. The sealed bags were kept at 4°C for 7 days to induce sporulation, after which samples were assessed using the following methods:

**Visual assessment:** Leaves are assessed on a 1-6 scale based on area covered by sporulation.

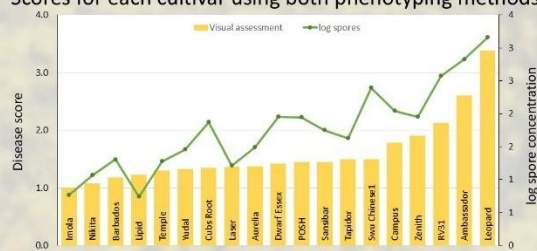


**Spore count:** Spores are washed from leaves and counted with a haemocytometer.

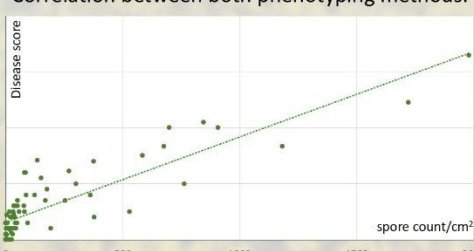


## RESULTS

Scores for each cultivar using both phenotyping methods.



Correlation between both phenotyping methods.



## CONCLUSIONS

Spore concentration is statistically correlated to disease severity, therefore it is a reliable quantitative phenotyping method.

**What is there left to do?**

- ❖ Glasshouse trial
- ❖ Study other quantitative phenotyping strategies (qPCR, ImageJ, WAC assay)

## REFERENCES

- <sup>1</sup> Karandeni-Dewage, C.S. et al. (2017) Host-pathogen interactions in relation to management of light leaf spot disease (caused by *P. brassicae*) on *Brassica* species. *Crop Pasture Sci*, 69(1), 9-19
- <sup>2</sup> Karandeni Dewage, C.S. et al. (2021) Interactions in the *B. napus* - *P. brassicae* pathosystem and sources of resistance to *P. brassicae* (light leaf spot). *Plant Pathol J*, 70, 2104-2114
- <sup>3</sup> Guimarães, R.L. et al. (2004). Resistance to *Botrytis cinerea* in *Solanum lycopersicoides* is dominant in hybrids with tomato, and involves induced hyphal death. *Eur. J. Plant Pathol*, 110, 13-23

## Appendix 10.2:

# Detection of light leaf spot in oilseed rape: Study of quantitative phenotyping strategies

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In collaboration with breeding company KWS

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University of  
Hertfordshire **UH**

**Perry**  
FOUNDATION

**KWS**

### ABSTRACT

*Pyrenopeziza brassicae* is a phytopathogenic ascomycete responsible for **light leaf spot (LLS) disease**, the most economically damaging disease that affects winter oilseed rape (*Brassica napus*) crops across the UK and Europe. Current cultural and chemical control practices are insufficient to manage epidemics of the disease, therefore the study of the **genetic host resistance** against *P. brassicae* has become of increasing importance as an effective strategy to control LLS. To study the interaction of the *B. napus* - *P. brassicae* pathosystem<sup>1</sup>, it is fundamental to establish a reliable method to detect and quantify the phenotypical changes related to colonisation and to determine the severity of the disease. Currently, the strategies used to assess this are based on the presence of acervuli on the surface of the leaves, visually measuring the percentage of area covered by sporulating structures and classifying the disease severity on a scale of 1 to 6. Visual assessment<sup>2</sup>, however, can lead to error; therefore, **spore count**<sup>3</sup> was used as a quantitative phenotyping method. Spores washed from leaf samples were counted using a haemocytometer. The data obtained was compared to data generated from a glasshouse experiment, to determine the reliability of the method. Spore counting was a more reliable phenotyping method than visual assessment. There was a significant positive correlation between spore concentrations and the visual disease assessments.



February, 2022



May, 2022

### MATERIALS & METHODS

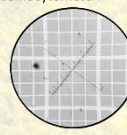
A field trial was done in Thriplow (managed by KWS). Twenty one *B. napus* accessions (2 repetitions) were sown in September, 2021. Samples were collected in March, May and June (2022) by removing 10 leaves at random per cultivar, wrapping in paper towels, spraying with sterilised water to create high humidity and placing in plastic bags.. The sealed bags were incubated at 4°C for 7 days to induce sporulation, after which samples were assessed using the following methods:

**Visual assessment:** Leaves were assessed on a 1-6 scale based on area covered by sporulation.



**Figure 1.** Areas covered by *P. brassicae* sporulation on the surface of different leaves, corresponding to the different values of the 1-6 scale.

**Spore count:** Spores were washed from leaves with sterile distilled water. The spore suspension was filtered through Miracloth and counted with a haemocytometer.



10<sup>6</sup> (Aurelia)



10<sup>6</sup> (Leopard)

**Figure 2.** Spore suspensions in the haemocytometer as seen through the microscope. More spores can be observed in the susceptible cultivar Aurelia than in more resistant Aurelia.

### RESULTS

Cultivars were assessed using two phenotyping strategies: visual assessment and spore counts. Both assessments were plotted in a single graph for comparison (Fig. 3). Furthermore, the correlations between a glasshouse experiment (Rothamsted Research) and the field trial (Thriplow) were determined using both phenotyping methods, i.e. less biased spore counting and visual estimates of disease severity, the latter of which might be prone to error. A Pearson correlation test (R) was done and a regression between glasshouse and field experiments for both assessment methods. A 95% confidence interval was included (Fig. 4).

Both methods have a significant correlation between the different environmental conditions, with *p*-values lower than 5%. Spore count, however, generated a higher correlation coefficient (0.75) and lower *p*-value (0.00076) than visual assessment (*R*=0.48 and *p*=0.04), therefore it can be considered that spore count is a more reliable method.

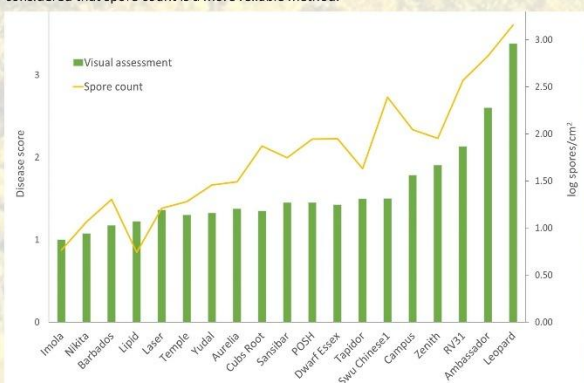


**Figure 3.** Comparison of visual assessment and spore count as phenotyping methods to determine the severity of the disease. The correlation coefficient between disease score and spore count is *R*=0.6.

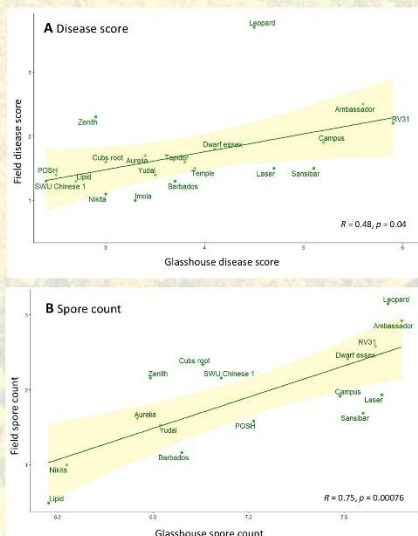


**Figure 4.** Scatter plots of the different methods used to phenotype the severity of the disease: visual assessment (A) and spore count (B). The graphs show the positive correlation between the data obtained in the glasshouse experiment and the field trial. Circles represent the cultivars that were tested. The black line represents regression with associated Pearson's correlation (*R*) and *p* value (*p*). The gray area represents the confidence interval.

### DISCUSSION

The development of a reliable quantitative method is fundamental to identify and differentiate resistant and susceptible phenotypes, in order to efficiently study host-resistance and develop durable disease-management strategies. This will also contribute to early detection of the disease in oilseed rape crops. The results obtained showed that spore count can be considered as an alternative to visual assessment, with a higher correlation coefficient. Moreover, there is a significant positive correlation between both phenotyping strategies (*R*<sup>2</sup> = 0.6).

#### What is there left to do?

- ❖ Evaluate other quantitative phenotyping strategies (qPCR, Wheat Germ Agglutinin Chitin (WAC) assay)
- ❖ Use these quantitative methods to phenotype an F2 segregating population to better understand host-resistance.

### REFERENCES

- Karandeni Dewage, C.S., et al. (2017). Host-pathogen interactions in relation to management of light leaf spot disease (caused by *P. brassicae*) on Brassica species. *Crop Pasture Sci.* 69(1), 9-19
- Karandeni Dewage, C.S. et al. (2021). Interactions in the *B. napus* - *P. brassicae* pathosystem and sources of resistance to *P. brassicae* (light leaf spot). *Plant Pathol. J.* 70, 2104-2114
- Guimarães, R.L., et al. (2004). Resistance to *Botrytis cinerea* in *Solanum lycopersicon* is dominant in hybrids with tomato, and involves induced hyphal death. *Eur. J. Plant Pathol.* 110, 13-23

## Appendix 10.3:

# Resistance of oilseed rape against the light leaf spot disease

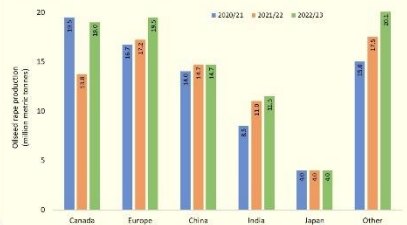
Laura Gimenez-Molina<sup>1\*</sup>; Ajisa Mutayil-Ali<sup>1</sup>; Heather Fell<sup>1</sup>; Catherine Chinoy<sup>2</sup>; Rachel Wells<sup>2</sup>; Henrik Stotz<sup>1</sup>

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## WHY OILSEED RAPE?

Around 80 million metric tons of **oilseed rape** (*Brassica napus*) are produced every year worldwide, predominantly cultivated in areas with colder climates. The UK contributes with 10% to the total of the European production.

Currently, 60% of oilseed rape is used in the food industry and 3% for animal feed. The remaining 37% is used for industrial purposes and biofuel.



## LIGHT LEAF SPOT DISEASE

Oilseed rape crops are largely affected by a variety of pests and diseases. Currently, the **light leaf spot (LLS) disease** is the most important disease across the UK and Europe, more than 90% of the cultivated crops have been affected by this epidemic, causing significant yield losses and economic losses that have surpassed £100M per year. It is caused by the fungus *Pyrenopeziza brassicae*, a pathogen with a polycyclic life cycle with a long symptomless phase that makes its detection and treatment challenging.

## Control of LLS disease

- Cultural control
- Chemical control
- Cultivar resistance:** Using oilseed rape varieties that are naturally more resistant. The study of **genetic host resistance** against the pathogen has become of increasing importance as an effective control strategy. The study of new sources of quantitative resistant is fundamental, since it is more durable than R-gene mediated resistance.

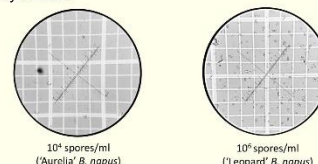
## MATERIALS & METHODS

In a previous study, 195 varieties of *B. napus* were screened against *P. brassicae*, which revealed a specific gene associated with **susceptibility** to the pathogen (BnaA04g20860)<sup>1</sup>. Mutation of this gene was induced (TILLING mutation), and TILLING mutant 2A was confirmed as homozygous for the mutation. Ten individuals of this mutant were grown in a controlled environment along with ten wild-type 'Cabriolet' *B. napus* individuals, where they were spray-inoculated with the pathogen. The resistance of the plants was assessed using two different methods of pathogen detection: visual assessment and spore counting.

**Visual assessment:** Leaves are assessed on a 1-6 scale based on the area covered by sporulation<sup>2</sup>.

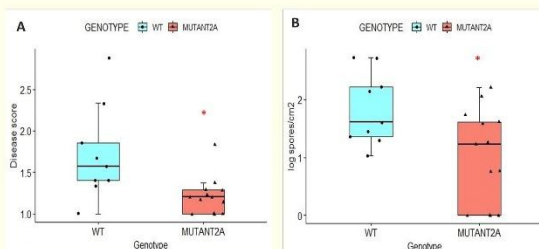


**Spore count:** Spores are washed from leaves and counted with a haemocytometer.



## RESULTS

The assessment of host resistance of TILLING mutant 2A showed a statistical difference with the host resistance of wild-type 'Cabriolet' both using visual assessment ( $P$ -value = 0.0283) and the spore count ( $P$ -value = 0.0190).



## DISCUSSION

The mutation of the candidate gene of interest (BnaA04g20860) resulted in a significant increase of host resistance against the pathogen, supporting the hypothesis that the mutated gene of interest acts as a **susceptibility factor** against *P. brassicae* in the plants that present it in their genome, making them significantly more susceptible to the light leaf spot disease.

Other TILLING mutants will be tested to check how other mutations affect host resistance.

## REFERENCES

- Fell, H. et al. (2023). Novel gene loci associated with susceptibility or cryptic quantitative resistance to *Pyrenopeziza brassicae* in *Brassica napus*. *Theoretical and Applied Genetics*, 136(71).
- Karandeni Dewage, C.S. et al. (2021) Interactions in the *B. napus* – *P. brassicae* pathosystem and sources of resistance to *P. brassicae* (light leaf spot). *Plant Pathol J*, 70, 2104–2114

## Assessment of quantitative resistance against *Pyrenopeziza brassicae* in *Brassica napus* TILLING mutants

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### ABSTRACT

*Pyrenopeziza brassicae* is a pathogenic fungus responsible for the **light leaf spot (LLS) disease**, one of the most important diseases affecting winter oilseed rape (*Brassica napus*) crops across the UK and Northern Europe, currently considered the most economically damaging disease affecting this UK crop. Current cultural and chemical control practices are insufficient to manage epidemics of the disease, therefore the study of the **genetic host resistance** against *P. brassicae* has become of increasing importance as an effective control strategy. In a previous study, 195 lines of *B. napus* were screened against *P. brassicae* and analysed using association transcriptomics and a genome-wide association (GWA) mapping. This led to identification of eight gene expression markers associated with infection. One of them, an **HXXXD-type acyl transferase gene**, was negatively correlated with quantitative disease resistance, suggesting its function as a **potential susceptibility gene**. The expression of this gene was induced during infection in susceptible, but not in resistant lines, of *B. napus*. The aim of this study is to characterise the function of this candidate gene. Screening of a **TILLING** mutant with a D167N substitution in the HXXXD-motif was done in a controlled environment with spray inoculation using conidia of *P. brassicae*. The resistance phenotype was evaluated using visual assessment (1-6 scale) and the counting of spores released from acervuli. The D167N mutant was significantly more resistant than background cv Cabriolet according to the 1-6 scale visual assessment ( $P = 0.0283$ ) and spore counting ( $P = 0.0190$ ) methods. These results show that the **D167N mutation** that altered the conserved HXXXD motif in the candidate gene had a significant effect by increasing the resistance of the host against the pathogen. It supports the hypothesis that the HXXXD-type acyl transferase acts as a susceptibility factor against *P. brassicae*.

### MATERIALS & METHODS

To characterise the putative susceptibility gene, **TILLING** technique was used to randomly mutate the genome of *B. napus* Cabriolet WT. TILLING mutants 2, 3, 4 and 6 were grown in the glasshouse (Rothamsted Research), leaves were collected, and their DNA extracted to do a **KASP marker analysis** that determined if the SNPs of interest were homozygous or heterozygous.

**KASP marker analysis** (Kompetitive Allele Specific PCR) is a fluorescence-based variant of PCR that allows the differentiation of SNPs due to its allele-specific primers and fluorescent labels (FAM for WT nucleotide; HEX for SNP nucleotide) (Figure 1).

TILLING mutant 2A (**D167N**) was selected to further the characterisation process: 10 TILLING biological replicates and 10 WT Cabriolet *B. napus* were grown in CE cabinets (John Innes Centre). After a month of growth, they were infected with a *P. brassicae* population obtained from KWS. Samples were harvested 21 days post inoculation, kept for a week at 4°C to induce sporulation on the leaves, after which they were evaluated using **visual assessment** and the **spore counting** methods.

### RESULTS

Results from the KASP marker analysis revealed that only biological replicates 2A and 6A are homozygous for the mutant allele (Table 1).

The assessment of TILLING mutant 2A, compared to the control WT Cabriolet, showed a statistical difference between the host resistance of WT Cabriolet and TILLING mutant 2A according both to visual assessment ( $P$ -value = 0.0283) and the spore count ( $P$ -value = 0.0190) (Figure 2).

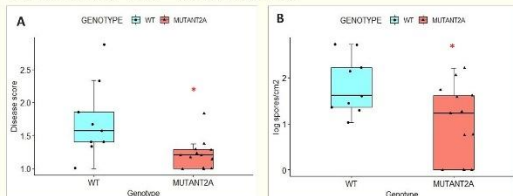


**Figure 2.** Significant differences in host resistance for each of the genotypes (TILLING mutant 2A and WT Cabriolet) evaluated through **A**) visual assessment ( $P$ -value = 0.0283), and **B**) spore counting ( $P$ -value = 0.0190).

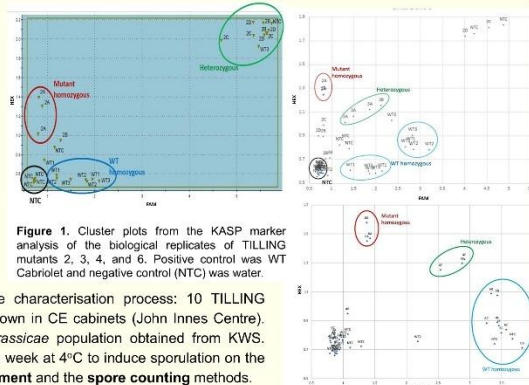


**Figure 1.** Cluster plots from the KASP marker analysis of the biological replicates of TILLING mutants 2, 3, 4, and 6. Positive control was WT Cabriolet and negative control (NTC) was water.

**Table 1.** TILLING mutants with their corresponding SNP. Heterozygosity/Homozygosity was determined through KASP marker analysis.

| TILLING mutant | Biological replicate | Mutation Change | Het/Hom    | Missense                                 | Truncations                         |
|----------------|----------------------|-----------------|------------|------------------------------------------|-------------------------------------|
| 2              | A                    | G>A             | Mutant Hom | 499G>A:167D>N (Aspartate to Asparagine)  |                                     |
| 2              | B                    | G>A             | Het        | 499G>A:167D>N (Aspartate to Asparagine)  |                                     |
| 2              | C                    | G>A             | Het        | 499G>A:167D>N (Aspartate to Asparagine)  |                                     |
| 2              | D                    | G>A             | Het        | 499G>A:167D>N (Aspartate to Asparagine)  |                                     |
| 3              | A                    | G>GA            | Het        |                                          | 516G>GA:172W>X (Tryptophan to Stop) |
| 3              | B                    | G>GA            | Het        |                                          | 516G>GA:172W>X (Tryptophan to Stop) |
| 4              | E                    | G>GA            | WT Hom     |                                          | 534G>GA:178W>X (Tryptophan to Stop) |
| 4              | F                    | G>GA            | Het        |                                          | 534G>GA:178W>X (Tryptophan to Stop) |
| 6              | A                    | C>CT            | Mutant Hom | 191C>CT:84S>SF (Serine to Phenylalanine) |                                     |

### DISCUSSION

The D167N mutation resulted in a significant increase of host resistance against the pathogen, supporting the hypothesis proposed that the mutated gene of interest acts as a **susceptibility factor** against *P. brassicae*.

## Appendix 10.5:

# Assessment of quantitative resistance against *Pyrenopeziza brassicae* in *Brassica napus* TILLING mutants

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## INTRODUCTION

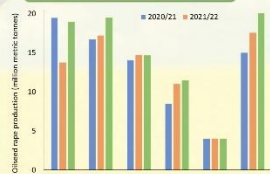


Figure 1. Leading producing countries of oilseed rape in million metric tonnes (USDA, 2023).

Around 80 million metric tons of oilseed rape (*Brassica napus*) are produced yearly worldwide (Figure 1). However, this crop is severely affected by *Pyrenopeziza brassicae*, a pathogenic leotiomycetes responsible for the light leaf spot (LLS) disease, currently considered the most economically damaging disease affecting this crop across the UK and Northern Europe, with economic losses that surpass £100M/year (Figure 2). Current cultural and chemical control practices are insufficient to manage LLS, hence the study of the genetic host resistance against *P. brassicae* has become of increasing importance as an effective control strategy.

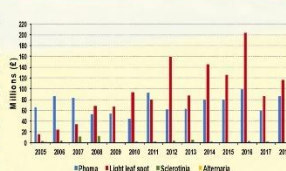


Figure 2. Winter oilseed rape yield losses caused by major diseases in England and Wales. ([www.cropmonitor.co.uk/](http://www.cropmonitor.co.uk/))

## GENE EXPRESSION

In a previous study, 195 lines of *B. napus* were screened against *P. brassicae* and analysed using association transcriptomics and a genome-wide association (GWA) mapping. This led to identification of an HXXXD-type acyl transferase gene (BnaA04g20860) that was negatively correlated with quantitative disease resistance, suggesting its function as a potential susceptibility gene<sup>1</sup>. The expression of this gene was induced during infection in susceptible, but not in resistant lines, of *B. napus* (Figure 3)<sup>2</sup>.

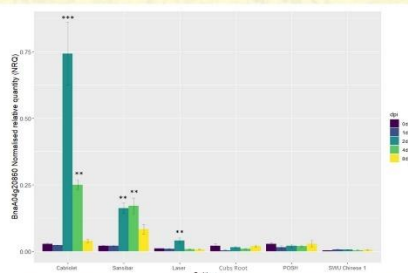


Figure 3. Normalised relative quantity of BnaA04g20860 (HXXXD-type acyl transferase). Susceptible (Cabriolet, Sansibar, Laser) and resistant (Cubs Root, POSH, SWU Chinese 1) cvs/lines inoculated with 10<sup>6</sup> conidia/ml of *P. brassicae*. (Ajisa Mutayil-Ali, 2023)

## PHENOTYPIC CHARACTERISATION

TILLING mutant 2A with a homozygous D167N substitution in the HXXXD-motif was grown in controlled environment cabinets, spray-inoculated with conidia of *P. brassicae* and incubated for 21 days. The resistance phenotype was evaluated using visual assessment (1-6 scale) and counting of spores released from acervuli<sup>3</sup>.

The D167N mutant was significantly more resistant than background cv. Cabriolet according to the 1-6 scale visual assessment and spore counting methods (Figure 4).

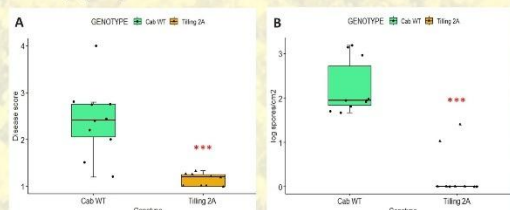


Figure 4. Significant differences in host resistance for each of the genotypes (TILLING mutant 2A and WT Cabriolet) evaluated through A) visual assessment ( $P$ -value = 0.0008676), and B) spore counting ( $P$ -value = 0.001945).

## ACKNOWLEDGEMENTS

University of Hertfordshire

John Innes Centre  
Unleashing Nature's Diversity

iPerry  
FOUNDATION

KWS

Please visit poster P3.1-012 to learn more about LLS

## TILLING SCREENING

To characterise the function of this putative susceptibility gene, TILLING technique was used to randomly mutate the genome of *B. napus* Cabriolet WT. Selected TILLING mutants were grown in the glasshouse (Rothamsted Research), leaves were collected, and their DNA extracted to do a KASP marker analyses that determined if SNPs of interest were either homozygous or heterozygous (Table 1).

Table 1. TILLING mutants with their corresponding SNPs. KASP marker analysis was used to determine if the mutants were heterozygous (highlighted in orange) or homozygous (highlighted in green).

| TILLING mutant | Biological replicate | Mutation Change | Het/Hom    | Missense                                  | Truncations                          |
|----------------|----------------------|-----------------|------------|-------------------------------------------|--------------------------------------|
| 2              | A                    | G>A             | Mutant Hom | 499G>A:167D>N (Aspartate to Asparagine)   |                                      |
| 2              | B                    | G>A             | Het        | 499G>A:167D>N (Aspartate to Asparagine)   |                                      |
| 2              | C                    | G>A             | Het        | 499G>A:167D>N (Aspartate to Asparagine)   |                                      |
| 2              | D                    | G>A             | Het        | 499G>A:167D>N (Aspartate to Asparagine)   |                                      |
| 3              | A                    | G>GA            | Het        |                                           | 516G>GA:172W>X (Tryptophane to Stop) |
| 3              | B                    | G>GA            | Het        |                                           | 516G>GA:172W>X (Tryptophane to Stop) |
| 4              | E                    | G>GA            | WT Hom     |                                           | 534G>GA:178W>X (Tryptophane to Stop) |
| 4              | F                    | G>GA            | Het        |                                           | 534G>GA:178W>X (Tryptophane to Stop) |
| 6              | A                    | C>CT            | Mutant Hom | 191C>CT:64S>S/F (Serine to Phenylalanine) |                                      |

## DISCUSSION

A significant difference in BnaA04g20860 (HXXXD-type acyl transferase gene) induction was observed between susceptible and resistant genotypes. The expression of the gene of interest was greater in susceptible cultivars/lines, specifically at 2 and 4 days post-inoculation.

Furthermore, the assessment of TILLING mutants show that the D167N mutation that altered the conserved HXXXD motif in the candidate gene had a significant effect by increasing the resistance of the host against the pathogen. This supports the hypothesis proposed that the gene of interest acts as a susceptibility factor against *P. brassicae*.

## Future work:

1. Generation of a segregating population from heterozygous mutants 2C and 2D.
2. Assessment of the segregating population
  - Genotyping: KASP marker analysis
  - Phenotyping: assessment of host resistance against *P. brassicae*

## REFERENCES

1. Fell, H. et al. (2023). Novel gene loci associated with susceptibility or cryptic quantitative resistance to *Pyrenopeziza brassicae* in *Brassica napus*. *Theoretical and Applied Genetics*, 136.
2. Mutayil-Ali, A. (2023). Molecular genetics and biochemistry of quantitative resistance against *Pyrenopeziza brassicae* in *Brassica napus*. PhD thesis, University of Hertfordshire.
3. Karandeni-Dewage, C.S. et al. (2021) Interactions in the *B. napus* - *P. brassicae* pathosystem and sources of resistance to *P. brassicae* (light leaf spot). *Plant Pathology Journal*, 70, 2104 - 2114.

