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Project leader:	Dr Charlotte Nellist (NIAB EMR), Prof Jim Dunwell (University of Reading)
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Key staff:	Matteo Luberti
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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

Matteo Luberti			
PhD Student			
NIAB EMR			
Signature	Matteo Luberti	Date	
Dr Charlotte Nel	llist		
Group Leader			
NIAB EMR			
Signature	Charlotte Nellist	Date	
Report authoris	sed by:		
Report authoris [Name]	sed by:		
	sed by:		
[Name]	sed by:		
[Name] [Position] [Organisation]	sed by:		Date
[Name] [Position] [Organisation]			Date
[Name] [Position] [Organisation]			Date
[Name] [Position] [Organisation] Signature			Date
[Name] [Position] [Organisation] Signature [Name]			Date

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GROWER SUMMARY

Headline

• This project aims to identify sources of durable resistance to *Phytophthora cactorum* in strawberry and apple in order to integrate them in the UK breeding programmes.

Background

The genus *Phytophthora* is comprised of a number of pathogenic oomycete (water-mould) species responsible for substantial damages to crops worldwide. *Phytophthora cactorum* can cause disease in over 250 plant hosts, including economically important crops such as cultivated strawberry (*Fragaria* x *ananassa*) and apple (*Malus* x *domestica*). *P. cactorum* is homothallic and it can produce both sexual and asexual spores (Erwin and Ribeiro, 1996). The sexual oospores are able to persist in the soil for several decades, while asexual zoospores are bi-flagellate, motile zoospores released in wet conditions which are able to swim towards a suitable host to initiate infection (Khew and Zentmyer, 1973).

P. cactorum is able to cause severe disease (crown and leather rot) in strawberry (Erwin and Ribeiro, 1996) Crown rot infection causes wilting of the plant, usually beginning from the youngest leaves, and red-brown lesions within the crown while leather rot causes the fruit to acquire a "leathery" texture, unpleasant smell and an altered taste resulting in high economic losses at all stages of production. Up to 40% of total strawberry crops were lost in Norway to crown rot (Stensvand *et al.*, 1999) and 20-30% in commercial farms in Ohio due to leather rot (Ellis and Grove, 1983).

Due to the high costs associated with orchard establishment, *P. cactorum* infection can be particularly damaging in perennial plants such as apple and pear (Harris, 1991). Thus, the production of resistant varieties is an important objective in breeding programs. In apple, *P. cactorum* can cause crown, collar and root rot (Erwin and Ribeiro, 1996). It can enter the tree through wounds and it reportedly often initiates infection at the graft union site, producing a moist rot, while below ground the bark tends to turn black and is decomposed by soil microorganisms (Harris, 1991).

Previous work on resistance to *P. cactorum* in strawberry at NIAB EMR has identified three major effect <u>Q</u>uantitative <u>T</u>rait <u>L</u>oci (QTL) in a bi-parental cross and additional QTL from a preliminary genome-wide association study (Nellist *et al.*, 2019). In comparison to strawberry where resistance is known to be quantitative, there is very little know about resistance to *P. cactorum* in apple. Although reports exist that suggest the presence of a major resistance gene in the 'Northern Spy' cultivar (Knight and Alston, 1969), suggesting qualitative

resistance. Thus, one of the key aims of this project is to identify markers associated with resistance and to elucidate the underlying molecular plant-pathogen interactions.

Initially, we employed an excised shoot assay test to screen 29 apple genotypes of interest to NIAB EMR's breeding programme for resistance/susceptibility to two UK *P. cactorum* isolates. This allowed us to identify an existing bi-parental cross of parents M.27 and M.116 called M432 segregating for resistance to *P. cactorum*. In 2019, using the same artificial inoculation technique to allow for larger number of replicates we phenotyped the individuals in this population and identified a QTL present on linkage group 6 highly associated with resistance to *P. cactorum*. This year we repeated the phenotyping of the same mapping population including a greater number of individuals and confirming the presence of a resistance-associated QTL on linkage group 6. We have also screened 99 apple rootstock and scion accessions. Using the phenotyping data we performed a preliminary genome-wide association study (GWAS) identifying two resistance associated QTL loci, on linkage group 5 and 6 (at the same locus identified in the M432 population). These results suggest resistance to *P. cactorum* in apple is controlled by a major effect locus and other smaller effect loci, and indicate a potential break-down of previously reported sources of resistance.

Summary

In the second year of this PhD programme, we have employed an excised shoot assay to screen the wider UK apple germplasm preserved within NIAB EMR's Genebank and the National Fruit Collection for resistance to *P. cactorum*. We screened 99 apple rootstock and scion varieties relevant to the apple breeding programs at NIAB EMR. We identified several levels of resistance and susceptibility within the UK germplasm, identifying a number of resistant varieties of both apple scions and apple rootstocks. Using genotypic data previously generated at NIAB EMR in the framework of several projects, including AHDB funded projects we were subsequently able to perform a GWAS. This allowed us to identify two resistance associated loci on linkage groups 5 and 6. We also performed a second round of phenotyping on previously assessed apple population. Our analysis confirmed the presence of a major effect resistance QTL responsible for 58.2% of the phenotypic variation observed in the population. Future work will focus on the identification of robust markers associated with resistance, as well as aiding in the identification of robust markers associated with resistance to be employed in the rootstock breeding programme for the deployment of resistance in the breeding lines. In summary:

• UK apple germplasm genotypes pertinent to the apple breeding programs were screened using an excised shoot assay in order to identify sources of resistance to *P. cactorum.*

- A major effect resistance QTL previously identified using a segregating population phenotyped was validated with a second round of phenotyping and mapping.
- A preliminary genome-wide association study was performed allowing us to identify two resistance-associated loci on linkage groups 5 and 6. Notably the locus on linkage group 6 is the same as the one previously identified in the course of this PhD project.
- Future work will focus on identifying and characterising the genes responsible for resistance. As well as aiding in the identification of robust markers associated with resistance to be employed in the rootstock breeding programme.

Financial Benefits

This report summarises the work that has been carried out in the first and second year of this PhD project. As this is a four-year programme, there are no direct financial benefits to be reported as of yet. However, the methods developed during the past two years and the data presented in this annual report will aid in the selection of genotypes to be employed in apple breeding programmes.

Action Points

• There are no action points at this early stage of the project.

SCIENCE SECTION

Introduction

The *Rosaceae* family contains a large number of economically important edible fruit and ornamental plant species. Amongst the most important genera contained within this family are the *Meloideae* (apple and pear) and the *Rosoideae* (brambles and strawberry). It is estimated that the combined economic value of these species amounts to around 180 billion dollars (Hummer and Janick, 2009). In 2017, world apple production was estimated at around 83 million tonnes (FAOSTAT) with a UK market value of ~140 million pounds (GOV.UK). in the same year strawberry production was estimated at 9.2 million tonnes (FAOSTAT) with a market value in the UK of over 280 million pounds (GOV.UK).

The modern cultivated apple varieties employed for commercial production are derived from the *Malus* x *domestica* species. The genome of the domesticated apple has a size of approximately 740Mb with a chromosome number of 17 (Velasco *et al.*, 2010). Phylogenetic analysis has identified *Malus sieversii*, a Central Asian wild-apple species, as the primary progenitor of the domesticated apple (Robinson, Harris and Juniper, 2001). They also identified substantial contributions from other *Malus* species, in particular *Malus sylvestris* (Cornille *et al.*, 2012). Despite the existence of 25 *Malus* species and over 7,000 domesticated apple varieties, modern breeding programs utilise very few of them as founding clones. Interspecific crosses between *Malus* x *domestica* and other *Malus* species have also reportedly been utilised to introduce traits such as red flesh and disease resistance in commercial apple production, root and crown rots caused by *Phytophthora* species can severely impact apple production (Harris, 1991; MacHardy, 1996; MacKenzie and Iskra, 2005).

Unlike animals, plants do not possess an adaptive immune system and have to rely on an innate array of defence mechanisms composed of both physical barriers and biochemical mechanisms to prevent and overcome pathogen infection (Jones and Dangl, 2006). Fungi and oomycetes can enter plant cells directly employing invasive structures called hyphae. They also produce haustoria, structures that allow them to interface with the plant cell's plasma membrane to exchange nutrients and secrete effector molecules (Jones and Dangl, 2006).

The plant's immune system is commonly described as employing two interplaying branches for the identification and containment of pathogenic organisms. One employs pattern recognition receptors (PRRs), transmembrane proteins able to recognise pathogen associated molecular patterns (PAMPs) and microbe associated molecular patterns (MAMPS) outside the plant cell initiating the PAMP triggered immunity (PTI) response (Zipfel, 2014). The other allows the host to recognise pathogen-secreted effector molecules within the cell resulting in effector triggered immunity (ETI) which often results in cell death as a mean of containing infection. The evolutionary arms race between hosts and pathogens has been a major driving factor resulting in a great variety of both host receptors and pathogen-secreted effectors (Boller and He, 2009). Plants have developed a large array of defence-related protein structures devoted to the identification of pathogens and their effectors and the initiation of the immune response (Zipfel, 2008). One of the best understood protein domains associated with disease resistance are the nucleotide-binding domain (NB) and the leucine-rich repeat (LRR) domain. *FLS2* is one of the best characterised LRR receptors and is able to recognise *flg22* a 22-amino acid sequence present in bacterial flagellin (Dodds and Rathjen, 2010). Another example of resistance related structure is given by the U-box protein CMPG1. An E3 ligase associated with the immune response and targeted by the *P. infestans* effector *AVR3a* (Bos *et al.*, 2010).

The nature of plant disease resistance can be divided into qualitative and quantitative disease resistance. Qualitative disease resistance is described as a monogenic type of resistance that confers complete or high level of resistance in the host plant and it can be mapped down to the presence or absence of a particular allele of a gene. Quantitative resistance on the other hand is described as a continuous-scale phenotypic variation in the level of resistance to a disease in the plant host (St.Clair, 2010). In the past few decades marker assisted breeding has gained increasing traction in the breeding industry as a means to introduce sources of resistance in commercial cultivars. The ever decreasing costs of sequencing as well as the availability of a growing number of single nucleotide polymorphism (SNP) markers has resulted in a more widespread use of this technique (Baumgartner *et al.*, 2016).

Many economically important traits such as yield and resistance to certain pathogens are multigenic and often the phenotype only becomes apparent after the plant has reached maturity. The term quantitative trait locus (QTL) was introduced by Geldermann in 1975 to indicate a genomic locus that segregates with a quantitative trait (Geldermann, 1975). Recently, high-density genetic markers, cheap high-throughput sequencing technologies as well as powerful statistical methods to find correlations between markers and phenotypes have been developed (Collard *et al.*, 2005). This has allowed for the discovery of several QTLs associated with economically important traits, as well as their introgression into commercial cultivars via marker assisted selection (MAS), which is the selection of individual progenies based on the presence/absence of molecular markers (Collard *et al.*, 2005).

While several methods have been developed for the discovery of novel QTLs have been developed, they all share several core elements. The use of polymorphic DNA markers, the

phenotyping of a population for the trait of interest, the genotyping of the population and the creation of a linkage map, and finally the use of a statistical model to associate marker polymorphism to phenotypic variation (Sehgal, Singh and Rajpal, 2016). Interval mapping is the most common method employed in QTL detection. A number of statistical approaches to interval mapping have been developed such as interval mapping by maximum likelihood, by regression and composite interval mapping (Sehgal *et al.*, 2016).

Here we report screening the UK apple germplasm relevant for the apple breeding programmes using an excised shoot inoculation technique (see 'Materials and methods'). This allowed us to identify two putative resistance-associated loci. We also repeated the phenotyping of a previously reported segregating population to validate the presence of a major effect QTL. Subsequently, analysis of the apple genome allowed us to identify a panel of genes potentially responsible for the observed resistance.

Materials and methods

Plant material

Dormant first year growth apple shoots were collected (1 to 5 shoots per genotype) from each one of the 99 apple accessions in January 2020 from NIAB EMR's Genebank and the National Fruit Collection (Table 1; Appendices) to be employed in the germplasm screen. Actively growing shoots collected in July 2020 were employed in the second round of phenotyping of the M432 segregating population (one to four shoots per genotype) were employed in this assessment. All were cut to a length of 22 cm and surface-sterilised by immersing them in a 10% bleach solution for 15 minutes and then rinsed three times with sterile distilled water. One centimeter was cut off from each end and then both ends were sealed by being dipped in molten paraffin wax to avoid drying.

Mycelium production

P. cactorum isolate R36/14, isolated at the NIAB EMR site (UK) in June 2014, was employed in the assessment of M432 and the apple accessions. The *P. cactorum* isolate was revived from long term storage 14 days prior to inoculation. The isolate was grown on V8 agar at 20°C

in the dark, as described in Nellist *et al.* (2019). The isolate was re-subbed after seven days to ensure enough inoculum was produced.

Set-up and inoculation

A cork borer (4 mm diameter) was used to produce a wound in the middle section of each shoot and the outer bark was removed with a scalpel. Agar plugs of the same diameter containing the growing edge of *P. cactorum* mycelium were placed mycelium-side down onto the wound to inoculate the shoots. Mock inoculation of one shoot per accession was also performed in the germplasm screen. Queen Cox was used as a susceptible control and mock for the assessment and was inoculated using sterile V8 agar plugs, this was due to the restricted availability of plant material. Damp paper towels were placed at the bottom of each box and the shoots were arranged on raised racks made of rolled aluminium foil (Figure 1a). The boxes were sealed in clear plastic bags to maintain humidity and were placed in a controlled environment room, with a constant temperature of 22 °C (\pm 2 °C) and a 16/8 h, light/dark cycle. Dormant shoots were assessed 28 days post inoculation and actively growing shoots were assessed 21 days post inoculation.

Disease assessment

The bark surrounding the wound was carefully removed using a scalpel to reveal the full extent of the lesion (Figure 1b). The maximum lesion length was measured using a digital caliper as a measure for resistance/susceptibility. The length of the original wound (4mm) was subtracted to the final measurement.



Figure 1. Example of experimental set-up and disease assessment (a) Layout of inoculated shoots in box. (b) Disease assessment of shoots inoculated with *Phytophthora cactorum* isolate R36/14 (Luberti *et al.*, submitted).

Interval mapping

All individuals in the population as well as the parental genotypes were genotyped employing the International RosBREED SNP Consortium (IRSC) 8K SNP array (Chagné *et al.*, 2012). A linkage map was produced using JoinMap4® following user manual specification (Van Ooijen, 2011). The interval mapping was performed employing MapQTL5® software following user manual specifications (Van Ooijen, 2009). The best linear unbiased estimator (BLUE) value (Goldberger, 1962) of infection length for each genotype was used in the calculation. The same software was employed to perform a permutation test to determine a threshold of significance.

Preliminary genome-wide association study

All the individual apple accessions screened had been previously genotyped using a 20k SNP array (Bianco *et al.*, 2014). The linkage map employed in this study was developed by Di Pierro *et al.* (2016). The GWAS analysis was performed using the GWASpoly software in the

R environment (R version 4.0.3 "Bunny-Wunnies Freak Out"). Again, the best linear unbiased estimator (BLUE) value of infection length for each accession was used in the calculation.

Results

Germplasm screen and preliminary genome-wide association study (GWAS)

The apple accessions assessed in this study showed varying levels of susceptibility to *P. cactorum* isolate R36/14. The most susceptible genotypes were the scion varieties "Delicious" and "Duchess-of-Oldenburg" and the rootstock varieties "Mac 24" and "M14" (average lesion length > 150mm). While the most resistant apple varieties assessed in this study were the rootstock varieties "A469-4", "Budagovsky 9", "CG-11", "M24" and "M8" as well as the scion variety "Grimes-Golden" (no infection lesion detected).

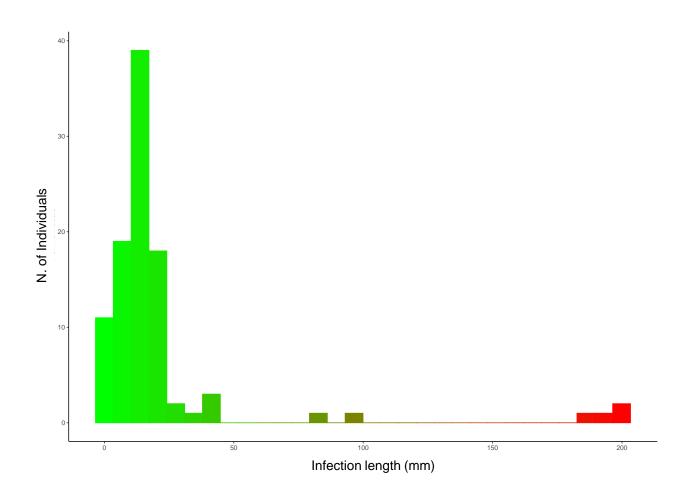


Figure 2. Distribution of susceptibility scores for the individual apple accessions assessed during the course of this study.

The preliminary GWAS identified two putative resistance loci on linkage groups 5 and 6. Notably the QTL on linkage group 6 is located within the same QTL region previously identified in the M432 mapping population. They have been estimated to explain 27.7% and 28.34% of the variation observed during the course of this study.

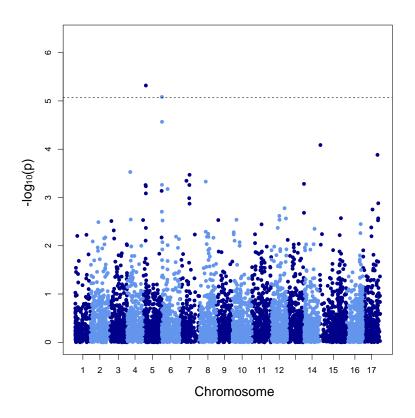


Figure 3. Preliminary GWAS of 99 accessions screened. The dots indicate the scores for the association of each of the single nucleotide polymorphism markers present on each of the 17 linkage groups of the apple genome with resistance to *Phytophthora cactorum*. The dotted line indicates the significance threshold ($-\log_{10}(p) = 5.05$).

Second round of interval mapping confirms the presence of a QTL on chromosome six

The distribution of the phenotypic scores for the second round of phenotyping was plotted to reveal a binomial distribution similar to that previously observed, indicating a major effect locus (Figure 4). Interval mapping was performed using MapQTL5® and revealed a highly associated region on chromosomes six. A permutation test was performed to determine a significance threshold of LOD= 11.6(Figure 4). This putative resistance QTL explains 58.2% of observed variation.

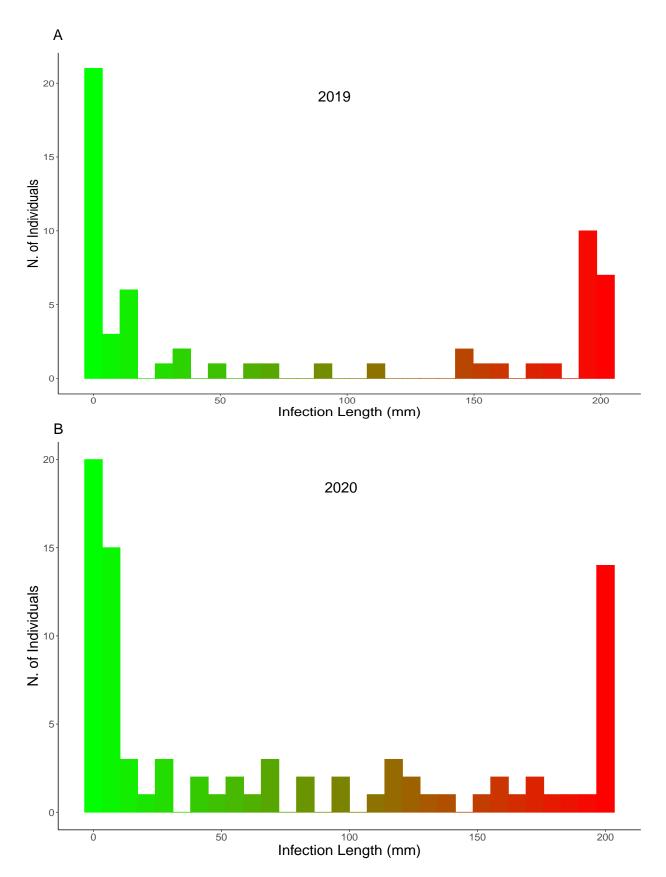


Figure 4. Distribution of the values for the average length of infection of each individual for the phenotyping experiments performed in 2019 (A) and 2020. (B). The binomial distribution observed in the assessment suggests the presence of a major effect quantitative trait locus responsible for most of the observed variation.

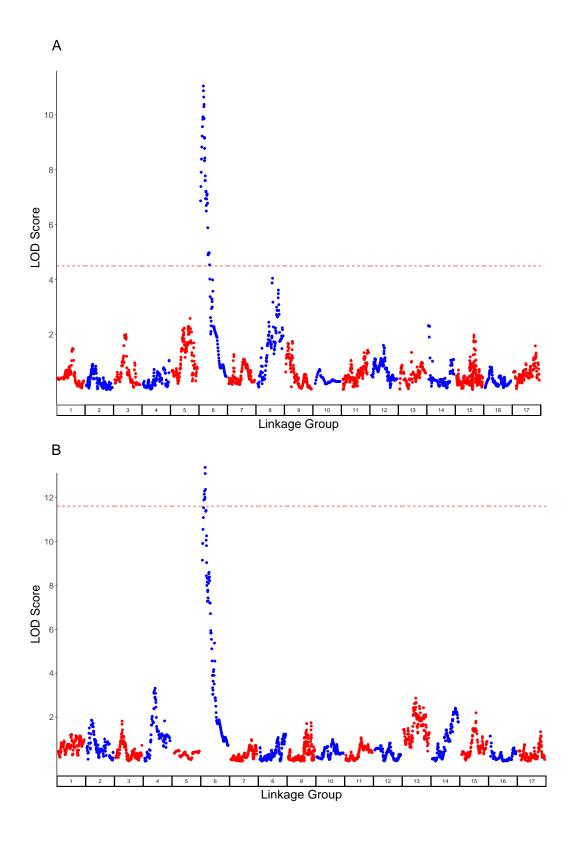


Figure 5. Interval mapping of the M432 population for the years 2019 and 2020. The coloured dots represent the scores for the association of each of the single nucleotide polymorphism markers present on each of the 17 linkage groups of the apple genome with resistance to *Phytophthora cactorum.* The red dotted line indicates the significance threshold 4.5 for 2019 (A) and 11.6 for 2020 (B) (p=0.05) determined using a permutation test. The presence of a highly significant QTL on linkage group 6 was confirmed.

Presence of known classes of resistance genes within QTL region

Using the annotated version of the 'Golden delicious' genome (GDDH13 Version 1.1) we analysed the QTL region on linkage group six. We identified 15 genes containing resistance associated motifs. Of these, 3 contained nucleotide binding (NB-ARC) domains and 2 contained leucine rich repeat (LRR) domains and represents good potential candidates. We also identified a number of other resistance associated motifs-containing genes, including mildew locus O (MLO) family genes, Really Interesting New Gene/U-box (RING/U-box) superfamily genes, Enhances Disease Resistance (EDR) genes and brassinosteroid (BR) signaling kinase genes. Analysis of the QTL region on linkage group five is still ongoing.

Discussion

During the course of this study we confirmed the presence of a highly significant QTL on chromosome six of the *Malus x domestica* genome. This putative resistance QTL was found to explain 58.2% of the phenotypic variation observed. We also identified several genes containing resistance associated motifs within the QTL region. Interestingly, one of the LRR genes was found to be a paralog of the *HcrVf2* gene, a known apple scab (*Venturia inaequalis*) resistance gene (Broggini *et al.*, 2009). A brassinosteroid (BR) signalling kinase was also identified within the QTL region. This class of signalling kinases is strongly involved in with plant resistance mechanisms, increased levels of BR signalling has been linked to increased resistance to *Pseudomonas syringae* (Yu *et al.*, 2018). A homolog of a cassava basic leucine zipper transcription factor (bZIP) was found within the QTL region. This was recently found to confer resistance to bacterial blight (*Xanthomonas campestris*; Li *et al.*, 2017).

Conclusions

The work reported here is the product of the second year of this PhD, therefore many of the results presented will need to be further explored. Nevertheless, the main conclusions that can be taken from this work are:

- The UK germplasm contains several sources of resistance that can be integrated in the current apple breeding programs.
- A previously identified major effect QTL explaining 58.2% of observed phenotypic variation in resistance in the biparental cross 'M.27' x 'M.116' was validated.

• A preliminary genome-wide association study has provided further validation for the previously identified QTL as well as allowing the identification of a further putative resistance locus.

Knowledge and Technology Transfer

The student attended and presented at:

• CTP-Day November 2020 (presentation)

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