Final Report

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Title: Precision diagnostic technologies in horticulture - exploring the genomics of fungal diseases in UK apple, strawberry, and raspberry production

Genomics of fungal diseases in apple, strawberry & raspberry

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1. Industry Summary

The overarching objective behind this study was to progress towards in-field diagnosis-by-sequencing of UK horticultural pathogens. A need for improved surveillance, detection, and diagnostic tools in agriculture has been identified by the UK Department for Environment, Food and Rural Affairs. Rapid and precise diagnosis of pathogens is necessary for tailored, efficient, and sustainable disease management. Diagnosis-by-sequencing methodologies require a basic knowledge of pathogen genetics. To achieve this objective, this study investigated the genetics of three of the most serious pathogens in UK horticulture: *Venturia inaequalis*, which causes apple scab disease; *Podosphaera leucotricha*, which causes apple powdery mildew disease; and *Podosphaera aphanis*, which is the cause of powdery mildew disease of raspberry and strawberry.

Quantitative Trait Loci mapping was used to identify genetic regions associated with reduced susceptibility to tebuconazole in apple scab. This resulted in the identification of multiple distinct regions in the genome. One region was found close to the gene which encodes the target of azole fungicides, implying that altered expression of of this target contributes to resistance. A second region was found on a separate part of the genome.This second region could indicate that a second resistance mechanism, not related to the azole target gene, has developed in apple scab.

Genome assemblies were constructed for apple, strawberry and raspberry powdery mildew. These include the first genome assemblies for strawberry and raspberry powdery mildew and the first assembly for a European population of apple powdery mildew. Collection of field samples avoided difficulties maintaining powdery mildew cultures, whilst still yielding high-quality genome assemblies following stringent controls for purity. This method has the potential to be applied to other challenging plant pathogens which cannot be cultured easily. The genomic resources developed in this study will accelerate research into apple and strawberry powdery mildew. Additionally, unique effector genes were identified that determine which plants each pathogen can infect, these could support diagnosis-by-sequencing to the level of a particular race or host-adapted form within each species.

This research lays the groundwork for the development of sequencing-based molecular diagnostics for these species, allowing detection of the pathogens and fungicide resistance. Early detection enables growers to identify and respond to disease outbreaks before they become severe, thereby preventing crop damage and reducing the need for extensive spraying. Furthermore, enhanced diagnostic precision can help growers to determine the exact pathogen strain present in the crop, enabling the selection of effective agrochemicals for control and reducing the risk of resistance to these chemicals over time.

2. Introduction

Fruit production is an important component of the UK's economy, with the value of fruits and berries increasing by nearly 150% between 2010 and 2020, compared to only a ~20% increase in the value of grains and vegetables produced in the same period (FAOSTAT, 2020). Apple and strawberry are by far the most important fruit crops, representing ~70% of production value. However, pests and diseases are a persistent threat to agricultural productivity, and it's been estimated that one-third of the world's crop yield is lost due to disease each year, costing over $220 billion (Agrios, 2005; Silva et al., 2021). Apple scab, apple powdery mildew, and strawberry powdery mildew are particularly destructive diseases for UK apple and strawberry growers. Therefore, a study was conducted to investigate the genetics of the fungal pathogens responsible for these diseases: *V. inaequalis, P. leucotricha,* and *P. aphanis*, in order to improve molecular techniques for pathogen identification and develop better strategies for disease management.

2.1. Fungicide Resistance in Apple Scab

The mechanisms underlying the quantitative DMI resistance observed in *V. inaequalis* have not yet been fully elucidated. There is evidence that target-site mutations, overexpression of the gene *CYP51*, and overexpression of drug efflux transporters may each contribute to resistance (Vijaya Palani and Lalithakumari, 1999; Schnabel and Jones, 2001; Köller et al., 2004; Villani et al., 2016; Yaegashi et al., 2020; Hoffmeister et al., 2021). It is also possible that different resistance mechanisms are prevalent in different scab populations (Xu et al., 2010; Villani et al., 2016; Yaegashi et al., 2020). A fuller understanding of the genetics underlying resistance would enable better predictions of how DMI resistance will develop in the future.

V. inaequalis' ability to rapidly evolve and overcome host resistance genes and fungicides may be aided by its life cycle. Asexual reproduction in the growing season generates enormous numbers of progeny and facilitates the rapid spread of adaptive mutational events, whilst sexual reproduction over winter facilitates novel combinations of mutations and can accelerate evolution (Goddard et al., 2005). This life cycle also allows for crossing experiments to investigate the genetics behind traits in *V. inaequalis*. Important genomic regions, known as Quantitative Trait Loci (QTLs), which are associated with fungicide resistance, can be identified by combining phenotypic and genotypic information from a crossing population (Sierotzki and Gessler, 1998). The traits (level of fungicide susceptibility) and associated genomic regions from each parent are expected to segregate among the progeny of a cross. DMI resistance QTLs, outside of the *CYP51* gene or immediate upstream region, have not been previously reported in apple scab (Schnabel and Jones, 2001; Villani et al., 2016, Yaegashi et al., 2020; Hoffmeister et al., 2021). Fortunately, good quality

reference genome assemblies of the pathogen have become available in the last few years, which can assist with the accurate mapping of traits.

The following objectives were addressed:

- Modelling of dose-response curves of isolates from a mapping population, varying in resistance to tebuconazole
- Generation of sequencing data for the mapping population
- Calling Single Nucleotide Polymorphisms (SNPs) from experimental isolates against the published *V. inaequalis* assembly
- Identification of polymorphic SNPs in the mapping population
- Generation of a *V. inaequalis* linkage map
- Mapping QTLs for the tebuconazole resistance trait

The mapping of multiple tebuconazole resistance QTLs would confirm the hypothesis that resistance is polygenic. Conversely, if only one QTL is identified, this would support the null hypothesis that resistance results from a single variable allele.

2.2. Generation of New Genomic Resources for *Podosphaera leucotricha* and *Podosphaera aphanis*

The availability of genomic resources can accelerate research on a given pathogen (Johnston et al., 2019; Müller et al., 2021; Zaccaron et al., 2021). A multi-gene analysis utilising 72 genomes has recently led to a rearrangement of the Leotiomycetes clade, placing the powdery mildews within the order Helotiales *(*Johnston et al., 2019). In *Erysiphe necator*, a powdery mildew pathogen affecting grapevine plants, repetitive genomic regions with frequent structural variations have been associated with adaptive responses to fungicide stress (Zaccaron et al., 2021). Meanwhile Müller et al. (2021) have utilised high-quality assemblies of *Blumeria graminis* in order to describe how hybridisation between *forma speciales* has allowed adaptation onto a new host. Genomic sequences could be used to address questions about the evolution and population structure of *Podosphaera* spp., including what makes powdery mildew fungi specific to a given host (Spanu and Panstruga, 2012).

The generation of high-quality genome assemblies for *Podosphaera* spp. is also a necessary first step towards the development of molecular diagnostics for these species.

Such assemblies would facilitate comparative genomics studies to identify informative genetic markers beyond those *Podosphaera* Internal Transcribed Spacer (ITS) sequences currently available. Developments in sequencing technology and bioinformatics make the sequencing of powdery mildews from the field plausible.

Genomic assemblies were generated for powdery mildew species affecting UK horticultural crops, specifically; apple, strawberry, and raspberry. These resources will facilitate comparative genomic analyses. This entailed the following:

- Collection of powdery mildew samples from multiple independent disease outbreaks
- Extraction of DNA from samples of sufficient quality and quantity for genome assembly
- Extraction RNA from samples, sufficient to guide genome annotation
- Development of a bioinformatic pipeline to remove non-mildew contaminants and generate single-species genome assemblies
- Assessment of assembly completeness

2.3. Comparison of Powdery Mildew Effector Compliments

Attempts to study gene group gain, loss, or distribution in powdery mildew affecting horticultural crops have, until now, been hindered by the lack of available genomic resources for *Podosphaera* spp.. This also applies to the identification of *Podosphaera* effector complements. Powdery mildew genomes typically encode hundreds of small secreted proteins which reside in highly dynamic gene clusters, often displaying lineage-specific expansion or copy number variation (Menardo et al., 2017; Frantzeskakis et al., 2018; Müller et al., 2018). These proteins match the broad criteria for candidate effectors.

The identification of effectors is an ongoing challenge in plant pathology. Effector paralogues are thought to rapidly lose sequence similarity through diversification (Jiang et al., 2008; de Guillen et al., 2015). Effector sequences are, therefore, highly diverse, and their identification relies upon very broad criteria (Sperschneider et al., 2015a; Sperschneider et al., 2015b). They are typically small, lack known functional domains, and rarely have homologues in unrelated species (Ellis et al., 2009; Kim et al., 2016). This is true of powdery mildew effectors. Most powdery mildew genes have homologues in other ascomycetes (92% in *E. necator*), and are under purifying selection (Jones et al., 2014). In contrast, Müller et al. (2018) found that of 235 effector families identified in *Blumeria* spp., only 3.7-5.1% had leotiomycete homologues outside of powdery mildew species; indeed, Liang et al. (2018) found that only one orthologous group of CSEPs was shared between *Blumeria* spp., *Erysiphe quercicola* and *E. necator.*

Effector targets were identified that were unique to strawberry, raspberry, and apple powdery mildew. This entailed the following:

- Annotating genes in the genomes of *P. aphanis* and *P. leucotricha*
- Identifying species unique orthogroups for *P. aphanis* and *P. leucotricha*
- Identifying candidate effector proteins from gene predictions

Identification of unique genes or genomic regions that determine host range could support diagnosis-by-sequencing to the level of a particular race or host-adapted form within a species.

3. Materials and methods

3.1. Fungicide Resistance in Apple Scab

3.1.1. Generation of a Mapping Population

The generation of a cross population and fungicide tests were conducted as part of a previous PhD project (Cordero-Limon, 2018). Briefly, a susceptible isolate 'AF28' (ED_{50} = 0.12 mg L^{-1} (ED_{50} = the effective dose required to cause a 50% reduction in mycelial growth)) was sampled from a non-commercial orchard, namely Ash Farm, Worcestershire, that has never been treated with fungicides and was crossed with a resistant isolate 'Spartan 1' (ED_{50} = 3.36 mg L^{-1}), sampled from a Kent orchard where fungicide resistance had been reported. Crossing of the two strains was performed by sub-culturing mycelial plugs of each parent onto the same leaf decoction plate, chilled to 4 °C to facilitate sexual reproduction.

The two parents and 81 progeny were assessed for their level of resistance to tebuconazole. The commercial fungicide Folicur 25 EW (250 g L^{-1} tebuconazole) from Bayer Crop Science was used to amend PDA media (39 g L^{-1}) onto which 4 mm diameter circular plugs of each isolate were sub-cultured. Fungal growth was recorded at four fungicide concentrations (4 mg L^{-1}, 1 mg L^{-1}, 0.1 mg L^{-1} and 0.01 mg L^{-1}) as well as a control with no fungicide. Two perpendicular diameters were measured for each colony three weeks after sub-culturing. For each combination of isolate and concentration, there was one plate with three to four mycelial plugs, and the entire experiment was repeated once.

3.1.2. Calculation of Effective Dosages

Effective doses were recalculated from the raw data gathered by Cordero-Limon (2018). In this study, the data were transformed to allow fixing of the upper and lower asymptotes of the dose-response curve, as opposed to fixing the slope of the curve, as was done in Cordero-Limon et al. (2021).

Using two perpendicular diameter measurements taken after three weeks of growth, the growth area of a given colony was estimated, assuming an oval shape and subtracting the initial plug area. Colony growth areas for each isolate, at each concentration, were averaged each time the experiment was repeated. Results from different repetitions were considered as independent replicates. Following this, the data for each isolate were transformed to a percentage of maximum growth, with the average growth on fungicide-free control plates taken as 100%.

The R package ‘drc’ was used to model the dose-response curve for each isolate (Ritz et al., 2015). Following initial exploratory plots for each isolate a two-parameter log logistic model, with upper asymptote fixed at 100 and the lower asymptote fixed at zero, was selected as fitting the data best across all isolates. Isolates with fewer than two doses in the declining phase of the response curve were discarded, as it was not possible to model the shape of the curve in this instance. Additionally, one datapoint, growth of isolate ‘RS11’ at zero fungicide concentration, was discarded as anomalous; growth measured for RS11 in this case was far below both the other replicates at zero fungicide concentration and at higher fungicide doses. A log logistic model was then used to estimate ED_{10}, ED_{50}, and ED_{90} values for each isolate (effective dose required for a 10, 50, and 90% reduction in growth respectively).

3.1.3. DNA Extraction

In this study, DNA was extracted from frozen mycelia, stored at -20 °C since the prior work of Cordero-Limon (2018). Premade buffers were combined to form lysis buffer (Buffer A: 0.35 M sorbitol; 0.1 M TrisHCl; 5 mM EDTA, pH 8 ● Buffer B: 0.2 M Tris-HCl; 50 mM EDTA, pH 8; 2 M NaCl; 2% CTAB ● Buffer C: 5% Sarkosyl N-lauroylsarcosine sodium salt ● Buffer D: PVP40 10% ● Buffer E: PVP10 10%) in the ratios 5:5:2:1:1, 10 µL (10 kU) RNase A was added was added per 14 ml of lysis buffer. Fungal samples were ground in a geno/grinder® with 2 ball bearings inserted into 2 ml Eppendorf tubes at 1500 rpm in 20 s bursts for a total of 2 min, chilling with liquid nitrogen between bursts. Samples were then incubated at room temperature for 30 min in 1.5 ml lysis buffer whilst being inverted, ball bearings were left in the tubes to increase mixing, 20 µL of proteinase K was then added and incubation continued for another 30 min. Following this, samples were cooled on ice for 5 min before 250 µL of 5 M potassium acetate was added, cooling was then continued for an additional 5 min. Samples were then centrifuged for 12 min at 5000 × g, and the supernatants were transferred to safe lock tubes. Washing was then carried out by the addition 1:1 of Phenol:Chloroform:Isoamyalcohol 100 mM Tris-EDTA pH 8 (P:C:I), samples were mixed by inversion for 2 min and centrifuged for 10 min at 4000 × g before transfer of the supernatant to a fresh tube. This wash was repeated three times per sample, followed by a fourth wash using Chloroform:Isoamyl alcohol (C:I) in place of P:C:I. DNA precipitation was performed with 200 µL of sodium acetate (3 M, pH 5.2) and 800 µL isopropanol, added to ~1 ml of supernatant and mixed by inversion at room temperature for 10 min. DNA was pelleted by centrifugation at 8,000 × g for 30 min. The supernatants were then removed, and the pellet was washed three times by resuspension in 1.5 ml of 70% ethanol, centrifugation at 13,000 × g for 5 min, and removal of the supernatant. Following the final wash step, remaining ethanol was allowed to evaporate for 30 min before the DNA pellet was dissolved in 100 µL of 10 mM Tris pH 8.5 at room temperature for 30 min. The quality of extracted DNA was assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific), if

these results were in the target range (OD260/230 = 1.8-2.0, OD260/280 = 1.8-2.0) samples were submitted for sequencing.

3.1.4. DNA Sequencing

DNA samples were submitted to the commercial sequencing company Novogene for total RNA depletion, purification, fragmentation, cDNA library construction, enrichment, and sequencing. Sequencing was performed on an Illumina NovaSeq platform using paired-end chemistry (PE150).

3.1.5. Calling Single Nucleotide Polymorphism Markers

Raw sequence data were subjected to a quality control check using FastQC before and after sequences were trimmed and adapters removed using Trimmomatic v0.39 (Bolger et al., 2014). Sequencing depth for each isolate was assessed using the K-mer Analysis Toolkit v2.4.2 function 'kat plot spectra-cn' (Mapleson et al., 2017).

The *V. inaequalis* genome generated by Passey et al. 2018 was used as a reference to call SNPs. Trimmed reads were aligned to this reference genome using Bowtie 2 v2.4.1 (Langmead and Salzberg, 2012). SAMtools was used to prepare Bowtie 2 mappings for SNP calling by removing multimapping reads, discordant reads, and duplicates, and adding read group and sample names to each mapped read.

SNP calling and genotyping were performed using GATK4 (McKenna et al., 2010). Picard tools v2.26.11 was used to create a sequence dictionary for the reference scab sequence, following this SNPs were detected with the GATK 'HaplotypeCaller' function, 'combineGVCFs' was used to combine samples allowing joint genotyping via the 'GenotypeGVCFs' function.

As no pre-existing SNP or indel data were available for *V. inaequalis*, variant calling was performed without applying base quality score recalibration. The vcflib toolkit v0.1.16 and GATK4 were used to perform hard filtering and removal of erroneous or low-quality SNPs. Using vcflib, variants were discarded that had not been successfully genotyped in 95% of individuals, were below a minimum quality score of 30 (base call accuracy of 99.9%), below a minimum mean read depth of 5, above a maximum allele frequency of 60%, below a minimum allele frequency of 40%, or below a minor allele count of 3. Following this, the call data set was further filtered using GATK 'VariantFiltration', retaining only passing variants: QUAL < 30.0 (variant confidence is the phred-scaled variant quality score); QD < 2.0 (QualByDepth is the qual score normalised by allele depth for a variant); SOR > 3.0 (StrandOddsRatio is allele specific strand bias estimated by symmetric odds ratio test); FS > 60.0 (FisherStrand is phred-scaled probability that there is bias at a site); MQ < 40.0

(RootMeanSquaredMappingQuality of reads across all samples); MQRankSum < -12.5 (MappingQualityRankSumTest is the u-based z-approximation from the Rank Sum Test for mapping qualities); ReadPosRankSum < -8.0 (ReadPosRankSum is the u-based z-approximation from the Rank Sum Test for site position within reads). The resulting SNPs were plotted using RStudio (RStudio Team, 2020).

3.1.6. Linkage Map Generation

Filtered SNPs were converted from variant call format to HapMap format using Tassel v5.2.81, and subsequently from HapMap to raw mapmaker format via a custom Python script (https://github.com/harrisonlab/nano_diagnositcs/blob/master/V_inaequalis/hapmap2mapmaker.py). SNPs with missing data, where both parents had the same variant, or where one variant was found in none of the progeny isolates, were excluded.

Joinmap 5 was used to calculate a genetic linkage map for *V. inaequalis* using SNP data; linkage phases were determined using pairs with an independence LOD larger than one. The 'exclude similar loci' function was used to exclude second locus pairs with similarity equal to one, thereby removing identical SNPs to leave 861 informative SNP markers. Ten linkage groups were calculated at LOD = 3.4. Two groups, 9a and 9b, consisted of markers on the same single reference assembly contig and were therefore considered to be on the same chromosome. The order of SNPs within resulting linkage groups was curated using knowledge of the physical position of SNPs on contigs within the Passey et al. (2018) genome assembly. A total of 16 contigs were covered only by SNPs with segregation identical to SNPs on other contigs, these contigs were therefore excluded from the map when using only informative SNP markers and subsequently added back to the map.

3.1.7. Mapping of Quantitative Trait Loci

Effective dose estimates (ED_{10}, ED_{50} & ED_{90}) were transformed by taking the square root in order to normalise the distribution of the data. One-way ANalysis Of VAriance (ANOVA) was performed for each of the 861 informative SNP markers versus effective dose. Following false discovery rate correction, markers found to be associated with increased effective dose estimates $p \geq 0.05$ were considered to represent significant QTLs. Effect size of a given QTL was calculated as the difference in mean effective dose for isolates carrying the resistant parent genotype and those carrying the susceptible parent genotype, divided by the standard deviation in isolates carrying the susceptible genotype. Proportion of Variance Explained (PVE) was calculated for significant QTLs. ANOVA was applied to a linear regression model with peak SNP marker as the independent variable and the square root effective dose as the dependent variable. The sum of squares for the SNP marker was

then divided by the total sum of squares, giving the proportion of the total variation in effective dose that explained by a given SNP marker.

3.2. Generation of New Genomic Resources for *Podosphaera leucotricha* and *Podosphaera aphanis*

Powdery mildew was sampled directly from naturally occurring outbreaks observed at NIAB EMR, Kent, UK, 51.2859° N, 0.4533° E, across three growing seasons. These infections matched the expected field symptoms of powdery mildew, and when viewed under a microscope, conidia of the expected morphology were visible (oval, unicellular (25-38 × 15-23 µm) produced in chains). In order to generate sufficient material for sequencing, spores from different plants in the same field or polytunnel were combined to form one bio-sample.

P. aphanis was collected from infected leaf material of *Fragaria* × *ananassa* 'Malling Centenary' plants growing under polytunnel conditions in May 2020 (ambient environmental conditions: 13.5 ± 14.5 °C, 70.1 ± 11.3% RH) and from infected fruit and stolons of *F. ananassa* 'Malling Ace' plants growing in polytunnel conditions in July 2021 (ambient environmental conditions: 17.9 ± 12.6 °C, 79.9 ± 10.4% RH). An additional *P. aphanis* bio-sample was collected from leaves of a *Rubus* × *idaeus* population growing in outdoor field conditions in July 2020 (ambient environmental conditions: 17.2 ± 18.1 °C, 76.0 ± 21.2% RH). *P. leucotricha* was collected: from leaves of a population of one year old *Malus* × *domestica* seedlings growing in glasshouse conditions in April 2020 (ambient environmental conditions 10.5 ± 13.6 °C, 75.3 ± 21.9% RH) as well as from leaves of established *M. domestica* trees growing in exposed orchard conditions in April 2019 (ambient environmental conditions: 9.2 ± 13.9 °C, 79.2 ± 16.3% RH) (varieties: 'M1 I'; 'Idared'; 'Loopspy'; 'M18'; 'MM106'; 'Gala'; 'Idared'; 'Jonathan', binned together) and June 2021 (ambient environmental conditions, 16.9 ± 12.0 °C, 78.3 ± 16.9% RH) (varieties: 'Northern Spy'; 'M1'; 'M9'; 'Loopspy'; 'Normandee'; 'Granny Smith'; 'Ottawa 3'; 'Saturn'; 'Pineapple Russet'; 'M54-1', binned together).

Heavily infected leaves and fruits were removed from plants with a scalpel. Infected plant material was transported to the laboratory in paper envelopes, taking care to retain as much mildew material as possible in transit. Whole leaves were placed directly into liquid nitrogen for later RNA extraction. For DNA, samples were placed into a two-litre glass beaker containing one litre of distilled water and stirred vigorously to wash off conidia. These conidial suspensions were then sieved into 50 ml falcon tubes which were centrifuged at 5000 × g for 5 min. Following this, supernatants were removed and discarded using a serological pipette, leaving pelleted conidia (500-2,000 µg). Washing of leaves was repeated into the same falcon tubes until all conidia had been dislodged and subsequent washes gave no increase in conidial yield. Any remaining water was then removed from the samples

by pipette following a final centrifugation at 5,000 × g for 5 min. Samples were then freeze-dried overnight, and the fully dried conidial powder was transferred to 1.5 ml Eppendorf tubes and stored at -80 °C.

3.2.1. DNA Extraction

DNA was extracted using broadly the same method as described above for apple scab; however fungal samples were combined with silicon dioxide sand, 50-70 mesh particle size, (1:1) and ground under liquid nitrogen using a mortar and pestle before being allowed to equilibrate to room temperature and being combined with lysis buffer in a 1:8 ratio. The quality of extracted DNA was initially assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific), provided these results were in the target range (OD260/230 = 1.8-2.0, OD260/280 = 1.8-2.0) then quantity and RNA contamination were further assessed via Qubit dsDNA High-sensitivity and Qubit RNA High-sensitivity assay kits with a Qubit 3.0 fluorometer (Life Technologies, Waltham, MA USA).

3.2.2. RNA Extraction

RNA was extracted for RNA-seq and gene annotation. RNA extractions were performed using 3% CTAB extraction buffer as described by Yu *et al.* (2012) with the following modifications: chloroform:isoamyl alcohol (24:1) washing was omitted; and precipitation was performed at -20 °C for four hours. The resulting RNA concentration and RNA Integrity Number (RIN) of samples were assessed using the Agilent RNA ScreenTape System with a 2,200 Tapestation (Agilent Technologies, Germany) according to the manufacturer's protocols. DNA contamination was assessed via Qubit dsDNA HS assay kit with a Qubit 3.0 fluorometer (Life Technologies, Waltham, MA, USA).

3.2.3. Internal Transcribed Spacer Sequencing

The ITS regions of isolates were amplified using serial dilutions of DNA samples. PCR was performed using 5 µL of Taq 5X master Mix (NEB), 16 µL water, 1 µL ITS-1 primer (TCCGTAGGTGAACCTGCGG), 1 µL ITS-4 primer (TCCTCCGCTTATTGATATGC) and 2 µL DNA dilutions. PCR was performed on a Veriti thermal cycler (Applied Biosystems) using the following cycling conditions: initial 95 °C for 3 min; 35 cycles of 95 °C for 20 s, 60 °C for 15 s, and 68 °C for 2 min; and final extension at 68 °C for 2 min. PCR products were visualised on a 1.5% agarose gel with GelRed (0.5 µL L^{-1}) before being purified using a Monarch® PCR & DNA clean-up kit (5 µg) following the manufacturer's instructions. Purified PCR products were quantified, diluted, and combined with forward and reverse primers in separate tubes before submission for Sanger sequencing via the Eurofins Genomics LIGHTrun tube service. The resulting sequences were

aligned to a reference ITS region downloaded from NCBI (GenBank accession no.: JQ999954 for *P. leucotricha* and KT359262 for *P. aphanis*) and analysed using Geneious V10.0.2.

3.2.4. DNA/RNA-seq Sequencing

DNA and RNA samples were submitted to Novogene, a commercial sequencing company. Sequencing was performed on an Illumina NovaSeq platform using PE150 chemistry.

3.2.5. Bioinformatic Analysis Pipeline

The mildew genome assembly pipeline used was as follows. Raw sequencing reads were trimmed, and adapters removed using Trimmomatic v0.39 (Bolger et al., 2014). Reads were subjected to a quality control check using FastQC v0.11.9, before and after trimming (Andrews, 2010). Kraken 2 v2.1.1 was used to taxonomically classify these trimmed reads (k-mer length = 31) (Wood et al., 2019). To do this, a custom database was constructed, including the standard Kraken 2 databases for archaea, bacteria, fungi, plants, protozoa, viruses, and vertebrate mammals, with the addition of each host plant genome and powdery mildew genomes publicly available in the NCBI database. Taxonomic classifications were visualised using Pavian v1.0 (Breitwieser and Salzberg, 2019). Coverage was estimated using SAMtools v1.1 'coverage' function and the K-mer Analysis Toolkit v2.4.2 function 'kat plot spectra-cn' (Li et al., 2009; Mapleson et al., 2017). Following this, reads were aligned to the respective host genome for each sample: apple, strawberry, or raspberry (Daccord et al., 2017; Edger et al., 2019; Wight et al., 2019). Alignments were also made to published powdery mildew genomes and transcriptomes using Bowtie 2 v2.4.2.

Those reads not aligning to the host genome were carried forward for *de novo* genome assembly using SPAdes v3.14.1 (Bankevich et al., 2012). The custom Kraken 2 database was then used to taxonomically classify contigs post assembly (visualised by Pavian) (Breitwieser and Salzberg, 2019; Wood et al., 2019). All contigs that were not assigned to the class Leotiomycetes were removed. Quality of the resulting genomes was assessed by searching for Benchmarking Universal Single-Copy Orthologues (BUSCO) with BUSCO v4.0.6 (Simão et al., 2015). For size estimation, 21-mers were counted using Jellyfish v2.2.3 (Marçais and Kingsford, 2011). Final assemblies were assessed for contamination using BlobTools: raw sequencing reads were Bowtie 2 aligned to final assemblies to generate coverage files; hit files were generated via a BLASTN search of assembled contigs against the NCBI 'nt' database (Laetsch and Blaxter, 2017). BLASTN classifications were performed to the phylum level, with powdery mildew belonging to the phylum *Ascomycota*.

3.3. Comparison of Powdery Mildew Effector Compliments

3.3.1. Collection of Genome Sequences

Analyses were performed utilising the three *P. leucotricha* (OGB2019, P112020, OGB2021) and three *P. aphanis* genome assemblies (SCOTT2020, DRCT72020, DRCT72021).

3.3.2. Identification of Repetitive Elements in Assembled Genomes

Repetitive elements within genome assemblies were predicted using RepeatModeler v2.0.2 (Flynn et al., 2020) and TransposonPSI (Haas, 2007), and the resulting library was masked using BEDTools v2.30.0 (Quinlan and Hall, 2010).

3.3.3. Gene Prediction and Annotation

RNA-seq was performed in order to provide evidence for gene prediction and transcriptome generation. Sequence reads from RNA-seq were subjected to a quality control check using FastQC v0.11.9. Sequences were trimmed and adapters removed using Trimmomatic v0.39. Reads were then aligned to the draft genome assembly using STAR v2.7.3 (Dobin et al., 2013). The BRAKER v1.9 pipeline was then used to make gene predictions using these alignments, and these predictions were supplemented by predictions made using CodingQuarry v2.0 in pathogen mode (Testa et al., 2015; Hoff et al., 2019). BRAKER gene models were used preferentially, adding CodingQuarry genes present in intergenic regions, as described by Armitage et al. (2018). Draft functional annotations were determined for gene models using Interproscan 5 v44-79.0 (Jones et al., 2014), as well as a BLASTP search against the Swiss-Prot database [downloaded September 2021] (Boeckmann et al., 2003).

3.3.4. Candidate Effector Identification

Investigation of candidate effector proteins was performed primarily by using the Predector v1.2.6 pipeline as well as BLASTP searches (Jones et al., 2021).

Secreted Proteins

Proteins were considered to be canonically secreted if they were predicted to contain <2 transmembrane domains and a signal peptide by any of the tools in the Predector pipeline: SignalP v3.0, SignalP v4.1g, SignalP v5.0b, SignalP v6.0g, DeepSig v1.2.5, TargetP v2.0, and Phobius v1.01 for signal peptide prediction; Phobius v1.01, and TMHMM v2.0c for transmembrane domain prediction (Krogh et al., 2001; Bendtsen et al., 2004b; Käll et al., 2004; Petersen et al., 2011; Savojardo et al., 2018; Almagro Armenteros et al., 2019a; Almagro Armenteros et al., 2019b; Teufel et al., 2022). The possibility of one transmembrane domain in the N-terminus was permitted, as this

could correspond to the signal peptide, given both are often predicted based on the presence of hydrophobic residues.

In addition to the analyses performed in the Predector pipeline, prediction of non-canonically secreted proteins was performed in the first instance using SecretomeP v1.0g with an NN-score cut-off of 0.6 (Bendtsen et al., 2004a). To reduce the possibility of false predictions, OutCyte v1.0 was subsequently used to screen putative non-canonically secreted proteins (Zhao et al., 2019). Proteins predicted to be non-canonically secreted by both tools have been reported in the main text.

Effector Prediction

CSEPs were reported where proteins were predicted to be canonically secreted and were found to have no homology to *Podospora anserina* (GCF_000226545.1) or *Neurospora crassa* (GCF_000182925.2) proteins by BLASTP searches, e-value 1×10^{-5}. Effector proteins are expected to be secreted and to lack homology in unrelated species.

The Predector pipeline also includes EffectorP v1.0, EffectorP v2.0, EffectorP v3.0, ApoplastP v1.0.1, and Deepredeff v0.1.1 which screen predicted proteins for effectors using a machine learning approach (Sperschneider et al., 2016; Sperschneider et al., 2018a; Sperschneider et al., 2018b; Kristianingsih and MacLean, 2021; Sperschneider and Dodds, 2022). EffectorP3 CSEPs effectors were reported where proteins were predicted to be canonically secreted, to have effector identity by EffectorP v3.0, and had no homologues to *P. anserina* or *N. crassa* proteins.

Small secreted cysteine-rich proteins were reported where proteins were predicted to be canonically secreted, were ≤300 residues in length, and contained ≥4 cysteine residues.

Effector Homologues

The Predector pipeline additionally includes database searches against a curated set of known fungal effector Hidden Markov Models (HMMs) [downloaded 30/07/2022, https://doi.org/10.6084/m9.figshare.16973665], PHI-base v4.13, which contains verified effector genes from many pathogens affecting both plants and animals, and the Pfam database [downloaded 30/07/2022] (Eddy, 2011; Finn et al., 2014; Steinegger and Söding, 2017; Urban et al., 2019; Jones et al., 2021). Simple regular expression matching was used to search for RxLR-like motifs.

Carbohydrate Active Enzymes and Transcription Factors

Secreted proteins were screened for carbohydrate-active enzymes by comparison with the dbCAN2 v10 database (Zhang et al., 2018). Transcription factors were identified by matching InterProscan and Pfam annotations of genes against a list of fungal transcription factor associated domains using

a custom Python script with further addition of X and Y domains, as described by Armitage et al. (2020)(https://github.com/eastmallingresearch/seq_tools/blob/master/feature_annotation/transcription_factors/interpro2TFs.py) (Shelest, 2017).

3.3.5. Orthology Analyses

Orthologue identification amongst predicted proteins was performed using OrthoFinder v2.5.4 (Emms and Kelly, 2015). Core orthogroups which were shared between all six assemblies were identified, as well as species-specific orthogroups which were shared between all three *P. leucotricha* assemblies but not found in any *P. aphanis* assembly, or *vice versa*. This analysis was repeated, excluding the raspberry powdery mildew assembly (SCOTT2020). Venn diagrams were created using the ggvenn R package v0.1.9 (Yan, 2021).

4. Results

4.1. Fungicide Resistance in Apple Scab

4.1.1. Progeny Isolates Segregated by Effective Tebuconazole Dose

The distribution of sensitivity values was continuous across the population, isolates could not be divided into discrete groups (Figure 1). Sensitivity to tebuconazole within the crossing population ranged from 0.015 mg L^{-1} to 2.63 mg L^{-1} (measured as ED_{50}). The fungicide sensitive parent (AF28) had an ED_{50} of 0.27 mg L^{-1} and ranked as the 24th least sensitive isolate. The resistant parent (Spartan 1) had one of the lowest sensitivities within the population (1.84 mg L^{-1}), second only to one progeny isolate; however, this sensitivity was higher than previously estimated for Spartan 1 (3.36 mg L^{-1}). Spartan 1 was the least sensitive isolate as measured by ED_{90}, however there were 16 isolates with higher ED_{10} values. AF28 had the 18th lowest ED_{10} value and 29th lowest ED_{90} value. Spartan 1 was always found to be less susceptible than AF28.

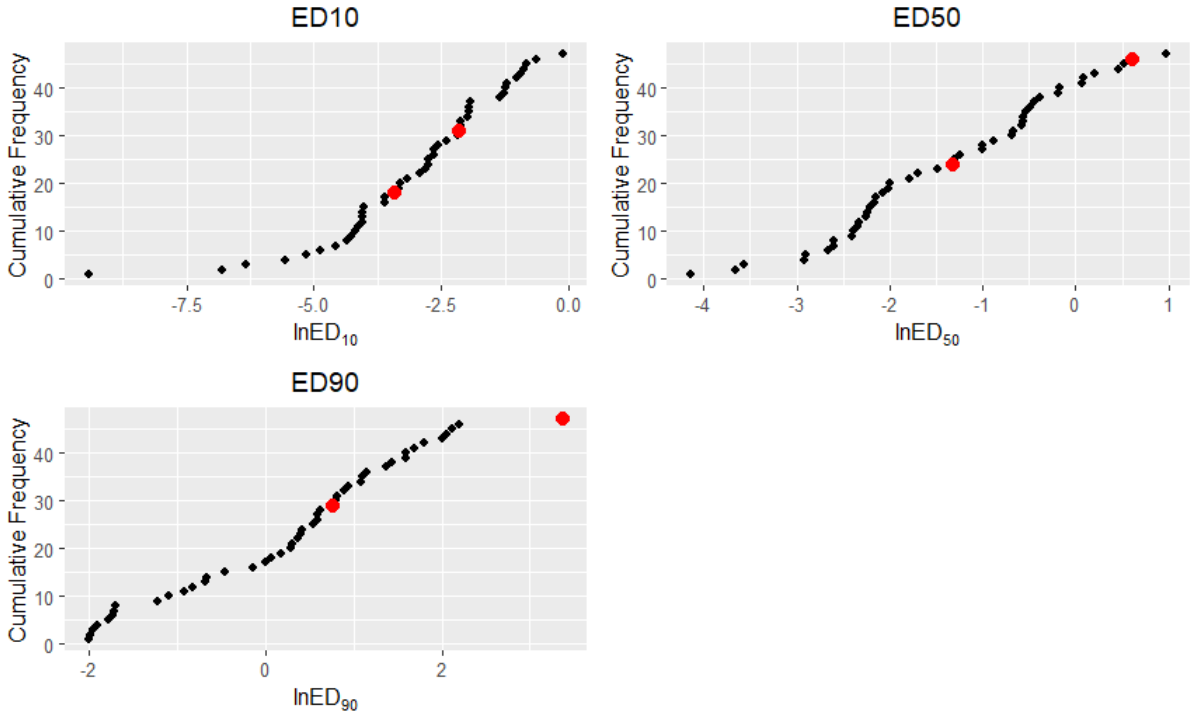

Figure 1. The cumulative frequency of *Venturia inaequalis* tebuconazole effective doses: for 47 apple scab isolates with reducing susceptibility to tebuconazole from left to right, susceptibility defined as the effective dose required to cause a 10, 50 or 90% reduction in mycelial growth: ED10, ED50, and ED90. Progeny isolates are shown in black; parent isolates are shown in red (Sensitive parent 'AF28' with lower effective dosages (left) and resistant parent 'Spartan 1' with higher effective dosages (right)).

4.1.2. Whole-Genome Resequencing Revealed Numerous Single Nucleotide Polymorphisms Across the Mapping Population

Whole-genome skim sequencing was performed for 56 apple scab isolates, including the two parents of the mapping population, AF28 and Spartan 1, and 54 progeny isolates. SNPs were called relative

to the reference assembly. Following filtering for quality, 46,727 SNPs were identified, an average of one SNP per 1,540 bp interval. These SNPs fell across 90 of the 238 contigs within the reference, these 90 contigs represented 64,327,219 of 72,310,420 bp, or 89% of the total assembly. Many of the 46,727 SNP markers segregated together, removal of SNPs with identical segregation patterns resulted in 861 informative SNP markers. These markers were used to generate a linkage map consisting of 11 groups for *V. inaequalis*, which has seven chromosomes.

4.1.3. Two Quantitative Trait Loci for Tebuconazole Sensitivity Were Identified

Two QTLs associated with reduced tebuconazole sensitivity were identified, these fell within linkage group one (p = 0.044) and linkage group seven (p = 0.0007). The linkage group seven (LG7) QTL was significant for sensitivity at both ED_{10} and ED_{50}, whilst the linkage group one (LG1) QTL was only significant at ED_{10} (Figure 2). Another putative QTL within linkage group four fell slightly below the threshold for significance (p = 0.059) at ED_{50}. The LG7 QTL spans ~1 Mb of a single contig within the reference *V. inaequalis* genome. Within this 1 Mb region is the CYP51 gene, which is located ~0.75 Mb from the most significant SNP for the QTL.

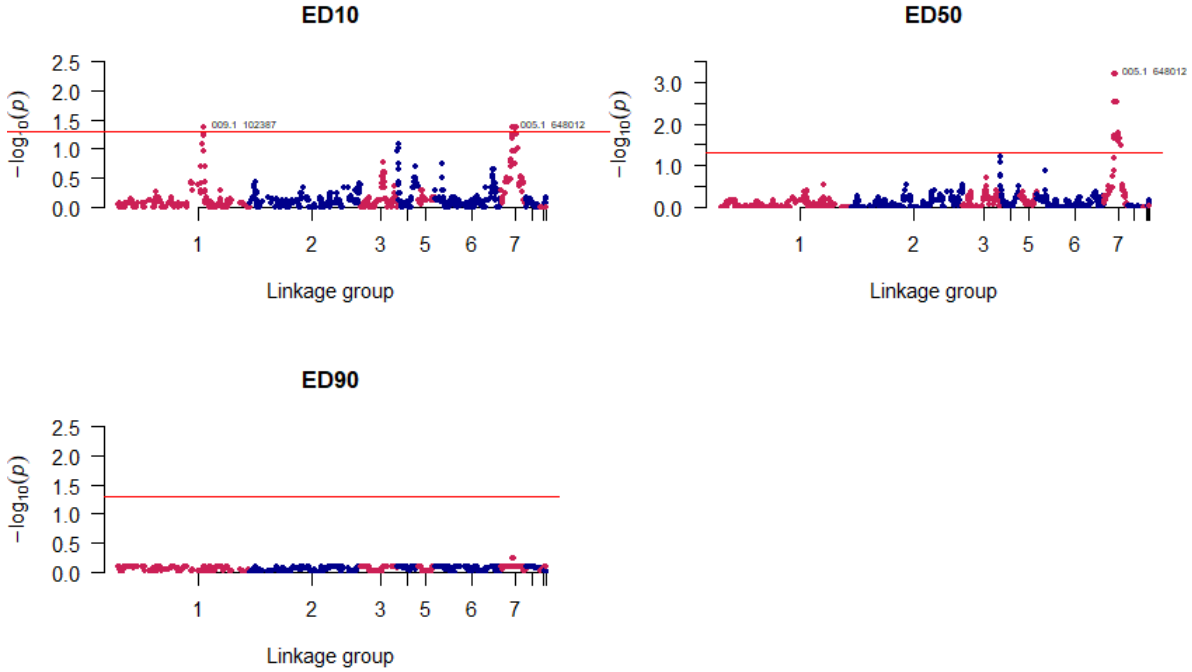

Figure 2. Association of apple scab polymorphic markers with tebuconazole sensitivity: 861 informative Single Nucleotide Polymorphism (SNP) markers are plotted by significance of their association at a given effective dose (ED_{10}, ED_{50}, or ED_{90}) against their estimated position within the *Venturia inaequalis* genome on one of the nine linkage groups. Significance of p = 0.05 is indicated by a red horizontal line, where SNP markers are below this threshold the identity of the most significant SNP marker is indicated.

The LG1 QTL had an effect size of -0.9 SD and could explain an estimated 34% of the observed variance in ED_{10}. The effect size of the LG1 QTL was calculated to be negative as the resistant parent carried a susceptible variant marker at this QTL. The LG7 QTL had an effect size of 1.36 SD and could explain 36% of the variance in ED_{10}; in contrast, 65% of variance in ED_{50} could be explained by the LG7 QTL which had an effect size of 2.18 SD, whilst the LG4 putative QTL had an effect size of 0.64 SD and could explain 26% of the variance. Given an overall ED_{50} range of 4.71 mg L^{-1} these QTLs were considered to have major effects.

4.2. Generation of New Genomic Resources for *Podosphaera leucotricha* and *Podosphaera aphanis*

4.2.1. The Filtered Mildew Assemblies Had High Completeness

Assembly statistics were assessed following the removal of potential contaminant contigs (Table 1). Comparison with the recently published *P. leucotricha* genome indicated that the apple powdery mildew assemblies were of good quality (Gañán et al., 2020). N50 measurements of assembly quality were more than double those reported by Gañán et al. (2020), ranging from 19,114 to 20,168 bp. Assembly sizes were between 49 and 52.6 Mb, larger than the published apple powdery mildew assembly, but far smaller than published *P. xanthii* assemblies utilising long-read sequencing data (Kim et al., 2021; Polonio et al., 2021). Based upon the distribution of DNA k-mer counts the size of the *P. leucotricha* genome was estimated to be ~160 Mb.

The *P. aphanis* genome assemblies were of comparable size to the *P. leucotricha* assemblies at 55.1 and 55.6 Mb for strawberry samples and 51.4 Mb for the raspberry powdery mildew sample. The size of the *P. aphanis* genome was also estimated to be ~160 Mb. N50 sizes were smaller for *P. aphanis* assemblies at an average of 10,821 bp; however, they were still comparable to published powdery mildew assemblies, such as the *P. leucotricha* genome at 8,371 bp. The largest contig within assembly DRCT72021 was substantially larger than any contig in the other assemblies at 559,432 bp. Alignments of trimmed reads against the final assemblies for each sample were used to assess sequencing coverage. The minimum coverage of an assembly was 72.5x for OGB2019, 'clean' samples P112020 and DRCT72020 each resulted in assemblies with >100x coverage.

The genome sizes for heavily contaminated samples were smaller following filtering, OGB2019 reduced from 283.7 Mb prior to filtering to 52.5 Mb after, a reduction of 81.5%. Prior to filtering, the OGB2019 assembly had a higher GC content (49.03%) than any other assembly, following filtering GC contents were similar within species with ~41.7% for *P. leucotricha* and ~43% for *P. aphanis*. Many of the largest contigs in the unfiltered assemblies represented contaminants; following filtering, the size of the largest contig in assembly OGB2019 decreased from 2.3 Mb to 136,346 nucleotides.

Table 1. Final genome assembly statistics for apple, strawberry and raspberry powdery mildew: *Podosphaera leucotricha* samples are presented alongside statistics for the *P. leucotricha* genome published by Gañán et al. (2020) (shaded) and *Podosphaera aphanis* samples from strawberry and from raspberry (shaded). Included are the number of contigs, total length of assembly, largest contig size, GC content, N50 and N75 sizes.

P. leucotricha				
ID:	**OGB2019**	**P112020**	**OGB2021**	**Gañán et al.**
Genome size	52,561,395	49,079,051	49,818,523	43,868,508
Contigs >500bp	8,040	6,805	7,325	8,921
Largest contig	136,346	100,493	108,534	60,133
GC%	41.71	41.76	41.54	43.69
N50	19,114	20,168	19,522	8,371
N75	9,119	1,568	9,576	4,117
Median depth	72.5	123.3	77.5	60

P. aphanis			
ID:	**DRCT72020**	**DRCT72021**	**SCOTT2020**
Genome size	55,613,046	54,152,750	51,418,734
Contigs >500bp	12,359	8,198	10,355
Largest contig	77,136	559,432	75,917
GC%	43.06	43.62	43.06
N50	11,409	12,165	8,889
N75	5,053	6,093	4,404
Median depth	432	74.4	84.5

The completeness of each assembly was assessed based on the presence or absence of BUSCOs. BUSCO analysis was also performed for each host plant species and several previously sequenced powdery mildew species for comparison. All the genome assemblies contained more than 97% of 1,706 ascomycete BUSCOs, with as high as 99.2% complete orthologues for SCOTT2020. This was a higher proportion than that found in the chromosome-level assembly of *Saccharomyces cerevisiae*, an ascomycete fungus, which contained 85.1% complete BUSCOs. Duplication of 7.3% of orthologues was once again observed in assembly DRCT72021 (Table 2).

Table 2. Ascomycete Benchmarking Single-Copy Orthologue (BUSCO) scores of powdery mildew genomes: for assembled genomes as well as host and published powdery mildew assemblies given in Table 4-1, the percentage and number of complete, duplicated, and missing BUSCOs are given.

Ascomycete BUSCOs						
Genome Assembly:	**Complete**		**Duplicated**		**Missing**	
P. leucotricha OGB2019	97.9%	1,670	1.3%	22	1.6%	27
P. leucotricha P112020	97.6%	1,665	0.2%	3	1.9%	32
P. leucotricha OGB2021	97.7%	1,667	0.2%	4	1.8%	31
P. aphanis DRCT72020	97.5%	1,664	0.3%	5	2%	33
P. aphanis DRCT72021	97.9%	1,670	7.3%	125	1.7%	30
P. aphanis SCOTT2020	97.6%	1,658	0.4%	6	1.9%	34
P. cerasi	97.4%	1,661	0.5%	8	1.8%	32
P. xanthii	96.5%	1,647	1.2%	21	3.1%	53
B. hordei	97.3%	1,660	0.7%	12	1.9%	33
E. necator	96.9%	1,653	0.1%	2	2.3%	40
P. leucotricha	97.4%	1,663	0.2%	4	1.7%	27
S. cerevisiae	85.1%	1,451	4.0%	68	13.8%	236

4.3. Comparison of Powdery Mildew Effector Compliments

Gene prediction was performed for the six genomes assembled, resulting in 15,178-17,569 genes predicted for *P. aphanis* and 18,304-19,673 genes for *P. leucotricha*. Orthology analysis was performed for proteins predicted within the six genomes in order to identify proteins unique to each species. Comparisons were made between orthogroups found within each of the three *P. aphanis* and *P. leucotricha* assemblies. The 49,054 *P. aphanis* gene predictions were resolved into 15,262 orthogroups, whilst the 56,533 *P. leucotricha* gene predictions were resolved into 16,958 orthogroups. A total of 47.9% (6,958) of all orthogroups found were common to both *P. aphanis* and *P. leucotricha* genomes. Only 11.7% (1,695) of orthogroups found in all *P. aphanis* genomes were unique to *P. aphanis*, whereas 40.4% (5,866) of orthogroups that were common across all *P. leucotricha* genomes, were not found in *P. aphanis* assemblies. These common orthogroups represented 2,048 *P. aphanis* unique proteins from the DRCT72020 genome and 5,057 unique *P. leucotricha* proteins from the P112020 genome.

Genes in orthogroups unique to *P. aphanis* and *P. leucotricha* were investigated in more detail (1,695 and 5,866 orthogroups), in order to identify unique effectors that may confer host range. Gene predictions from the DRCT72020 *P. aphanis* (Strawberry 2020) and P112020 *P. leucotricha* (Apple

2020) genomes were taken as best representatives of each species, as these assemblies contained the least contaminant sequences prior to filtering.

Predicted proteins were screened for sequence similarity to a set of 305 experimentally validated effectors compiled by Jones et al. (2021), as well as proteins in PHI-base. There was limited overlap between matches in these two sets of proteins. *De novo* prediction of effector genes was also performed. Putative secretomes for each genome were defined based upon predictions of proteins with signal peptides. Finally, Carbohydrate Active enZYme (CAZY) proteins from within the putative secretome of each genome assembly were identified. Of the 2,048 genes unique to *P. aphanis*, 49 were found to encode proteins bearing sequence similarity to experimentally validated effectors, whilst 295 unique effector homologues were identified in *P. leucotricha* (Table 3). Similarly, *de novo* effector prediction identified only 21-60 unique effectors in *P. aphanis,* but hundreds of CSEPs in *P. leucotricha*-only orthogroups. These *P. aphanis*-specific effectors presumably enable the pathogen to infect strawberry and represent good candidates for future functional validation experiments and use in diagnostic testing.

Table 3. *Podosphaera leucotricha* and *Podosphaera aphanis* specific and consensus proteome annotations: presented are gene annotations for the assembled apple powdery mildew genome P112020 and strawberry powdery mildew genome DRCT72020, differentiated by whether given predictions are within orthogroups common to both *P. aphanis* and *P. leucotricha* or unique to one species or the other. Included are the number of predicted genes, transcription factors, Carbohydrate Active enZYme (CAZY) proteins, and canonical and non-canonical secreted proteins. The number of predictions with orthologues in the Pfam database, with annotated virulence functions in the Pfam database, with orthologues in PHI-base, and the number orthologous to a collection of known effector proteins. Predicted numbers of Candidate Secreted Effector Proteins (CSEP), EffectorP3 predictions of effectors, and Small Secreted Cysteine-rich Proteins (SSCP) are given, and in brackets, the number of these without orthologues in PHI-base or the collection of known effector proteins.

	P. leucotricha **P112020**		***P. aphanis*** **DRCT72020**	
	Common	unique	Common	unique
Orthogroups	6,958	5,866	6,958	1,695
Predicted genes	9,557	5,057	10,649	2,048
Transcription factors:	646	164	785	151
Pfam IDed proteins:	6,303	988	6,842	598
Pfam virulence	74	1	72	2
PHI-base effector match:	158	2	112	2
Effector orthologues:	382	295	396	47
Canonical SPs:	645	379	670	72
SSCPs:	122 (76)	80 (46)	128 (80)	21 (17)
CSEPs:	199 (135)	346 (173)	226 (158)	60 (50)
Secreted EffectorP3	103 (57)	159 (78)	115 (67)	36 (31)
Secreted CAZY	82	1	81	1

5. Discussion

5.1. General Overview of Findings

This study addressed the sustainable management of horticultural crops. Fungal pathogens are a substantial burden on the horticultural sector in the United Kingdom (MacHardy, 1996; Yoder, 2000; Dean et al., 2012; Menzel, 2021). Recent developments in the field of genomics and sequencing allow high-resolution genetic description of an ever-increasing number of species. Here, economically important fungal pathogens affecting apple, strawberry and raspberry have been genetically characterised, namely *V. inaequalis, P. leucotricha* and *P. aphanis*. This research lays the groundwork for sequencing-based molecular diagnostics, allowing the detection of these pathogens and fungicide resistance. The following conclusions were reached:

- Polygenic resistance to tebuconazole occurs in *V. inaequalis* isolates.
- *P. aphanis* and *P. leucotricha* have large and highly repetitive genomes, similar to other powdery mildew species.
- *P. aphanis* and *P. leucotricha* carry unique compliments of effector genes.

Fungicide resistance in the apple scab pathogen *V. inaequalis* was investigated. Experiments were designed to test the hypothesis that resistance to DMI (DeMethylase Inhibitor) fungicides is a polygenic trait in *V. inaequalis* isolate 'Spartan 1'. DMI-resistant isolates were previously sampled from Kent orchards and used to create a mapping population segregating for resistance (Cordero-Limon et al., 2021). In this study, Single Nucleotide Polymorphisms (SNPs) were called and used to map Quantitative Trait Loci (QTLs) associated with reduced sensitivity to the DMI tebuconazole. Two QTLs were identified, indicating that multiple mechanisms may be involved in resistance. Specifically, one QTL was found to encompass the *CYP51* gene, which encodes the CYtochrome P450 monooxygenase protein target of DMIs. It is likely that this QTL reflects mutations upstream of *CYP51* and alterations in gene expression, as no differences were found in the CYP51 coding sequence (Cordero-Limon et al., 2018). A second QTL was found to be important only at lower fungicide concentrations. This second QTL was located on a separate linkage group to the *CYP51* gene. It is therefore hypothesised that this QTL reflects a *CYP51* independent mechanism of resistance (i.e. not altered expression or target site mutation), potentially upregulation of drug efflux transporters which are known to be involved in resistance, although trans-regulatory transcriptional regulation of *CYP51* cannot be ruled out (Nakaune et al., 1998; Hayashi et al., 2002; de Waard et al., 2006; Canon et al., 2009; Hulvey et al., 2012; Lendenmann et al., 2015). These findings confirmed the hypothesis that populations of *V. inaequalis* harbour polygenic DMI resistance.

Genomic resources have been generated for *P. aphanis* and *P. leucotricha*. These assemblies represent the first genomes available for *P. aphanis* and the first European samples assembled for *P. leucotricha*. These assemblies likely represent pan-genomes of multiple powdery mildew genotypes, having been assembled from mixed field samples collected in 2019, 2020, and 2021. However, given that only one mating-type locus was found in each assembly, it is likely that the powdery mildew populations sampled propagated clonally and that variation within each assembly is minimal (Asalf et al., 2013; Gañán-Betancur et al., 2021). Multiple contamination checks resulted in assemblies of equivalent quality to published powdery mildew genomes assembled from single-spored isolates (Wu et al., 2018; Gañán et al., 2020). Analysis of Benchmarking Single-Copy Orthologues (BUSCOs) revealed minimal duplication of orthologues, further confirming that the assemblies represent a single species. Good coverage of the *P. aphanis* and *P. leucotricha* gene space was also achieved, as indicated by high BUSCO completeness scores (>97.5% of ascomycete BUSCOs). The powdery mildew genomes generated displayed shared features with other published powdery mildew genomes, such as a large size and a high proportion of repetitive sequences. This work has resulted in the publication of a resource announcement paper describing the DRCT72020 *P. aphanis* genome assembly (Heaven et al., 2023).

The host ranges of plant pathogens are determined by effector genes, which are responsible for suppressing the host immune response (Schulze-Lefert and Panstruga, 2011). It was anticipated that, given their different hosts, *P. aphanis* and *P. leucotricha* would carry unique effector complements. The genome assemblies of *P. aphanis* and *P. leucotricha* were annotated, with particular attention paid to putative effectors. This analysis revealed a large number of secreted proteins (*P. leucotricha*: 861, *P. aphanis*: 610). Similar observations have been made for other powdery mildew species; for example, an assembly of *Erysiphe pisi* contained 622 secreted proteins (Sharma et al., 2019). Many proteins with sequence similarity to *Blumeria* spp. effectors were identified in the proteome of *P. leucotricha.* The expanded set of effectors seen in *P. leucotricha* is likely the result of an evolutionary arms race in the apple powdery mildew pathosystem; a number of major resistance genes against powdery mildew have been identified in apple and races of *P. leucotricha* are theorised (Krieghoff, 1995; Urbanietz and Dunemann, 2005; Lesemann, 2011). *P. leucotricha* and *P. aphanis* unique CSEPs identified here present good targets for functional validation as effectors through future host-induced gene silencing or transformation experiments (Christiansen et al., 1995; Chaure et al., 2000; Nowara et al., 2010; Vela-Corcía et al., 2015; Martínez-Cruz et al., 2017; Martínez-Cruz et al., 2018).

The set of effectors a pathogen possesses can be used to infer its host range and differentiate between various strains or races within the same species (Schulze-Lefert and Panstruga, 2011; Bourras et al., 2019). The ‘arms race’ between plants and pathogens, drives rapid coevolution of

pathogen effector and host resistance genes (Anderson et al., 2010). It is widely believed that effector repertoires, including both virulence and avirulence genes, are key determinants of the host range of a given pathogen strain (Godfrey et al., 2010; Spanu et al., 2010; Schulze-Lefert and Panstruga, 2011; Pedersen et al., 2012; Zhang et al., 2012; Wicker et al., 2013; Frantzeskakis et al., 2020). Many plant pathogen effectors display presence/absence or sequence features that are specific to different host species (Sonah et al., 2016). For example, effectors of pathogenic bacteria such as *Pseudomonas syringae* show considerable inter-strain variation, implying that they play a role in host specialisation (Guttman et al., 2002; Roden et al., 2004). An understanding how effector gene acquisition and/or avirulence gene loss/modification drives host range is crucial for developing effective disease control measures (Dodds et al., 2009; Sánchez-Vallet et al., 2018).

5.2. Future Prospects

5.2.1. Biologically Significant Genes Represent Good Diagnostic Markers

The objective of this study was to progress towards in-field diagnosis and monitoring of UK horticultural pathogens. The 2023-2028 plant biosecurity strategy for Great Britain, published by the UK government Department for Environment, Food and Rural Affairs (DEFRA), identifies 'surveillance, detection, and diagnostic tools' as a key area for research and development. Diagnosing fungicide resistance in pathogens allows growers to select the most effective agrochemicals; whilst monitoring avirulence gene diversity across a population can inform growers which Resistance (*R*) genes to deploy, and whether a deployed *R* gene is vulnerable to becoming ineffective.

Once mechanisms of fungicide resistance have been elucidated, fungicide-resistant genotypes can be rapidly identified through molecular techniques. For example, PCR primers have been designed that are able to distinguish between DMI-sensitive and resistant strains of *Penicillium digitatum* in citrus (Hamamoto et al., 2001; Gosoph et al., 2007; Sánchez-Torres and Tuset, 2011). Furthermore, Strobilurin resistance in powdery mildew, including *Blumeria hordei*, can be detected using a PCR-AFLP method (Sierotzki et al., 2000; Ishi et al., 2001; Bäumler et al., 2003).

A rapid and specific diagnostic for DMI insensitivity in apple scab would allow growers to utilise an alternative fungicide. A rapid and sensitive test able to detect scab and/or mildew before visible symptoms appear would enable a switch from calendar-based to targeted spray programmes. Additionally, in apple powdery mildew, a cryptic race structure exists wherein not all isolates are virulent against all varieties of apple (Urbanietz and Dunemann, 2005; Lesemann, 2011). A real-time sequencing approach that detects pathogens at the level of candidate effector genes, which are causative of host range, would inform growers which trees are susceptible. This would allow precise

targeting of pesticides or biological alternatives for mildew control, such as biocontrol or UV-C treatment (Kiss, 2003; Köhl et al., 2019; Zhu et al., 2019).

Until novel sequencing technologies are ready for use in on-site applications, a cost-effective alternative to PCR testing is isothermal amplification methods, such as helicase-dependent amplification, rolling circle amplification, recombinase polymerase amplification, or Loop-mediated isothermal AMPlification (LAMP) (Fire and Xu, 1995; Notomi et al., 2000; Vincent et al., 2004; Piepenburg et al., 2006; Lau et al., 2016). LAMP assays represent fast and reliable identification tools (Lau and Botella, 2017; Blaser et al., 2018). This method is highly specific as four (or six) primers are used, recognising six (or eight) distinct DNA regions of the target (Tomlinson et al., 2007). Internal primer pairs introduce self-complementarity into an amplification product, causing loops to form, whilst the extension of external primer pairs causes displacement of the extension products of the internal primers (Boonham et al., 2014).

An advantage of LAMP is the ability to conduct testing in the field, as no PCR machinery is required, amplification products can be directly visualised using SYBR green I, calcein plus $MnCl_2$, or hydroxy naphthol blue, or by measuring turbidity (Notomi et al., 2000; Wastling et al., 2010; Fukuta et al., 2014). A DNA target can be amplified in under one hour using only a heated block and can be used to detect as few as ten copies of target DNA at a cost of only $2-5 per sample (Chow et al., 2008; Tomlinson et al., 2010; Chen et al., 2012; Boonham et al., 2014; Wee et al., 2015). Isothermal methods have been used to identify plant-pathogenic fungi and oomycetes on seeds and from field samples including *Fusarium oxysporum* (Adb-Elsalam et al., 2011; Ahmadi et al., 2013; Zhang et al., 2013; Blaser et al., 2018). Accurate and timely identification of plant pathogens is the first step toward efficient mitigation of crop diseases (De Boer and Lopez, 2012). LAMP markers could be designed to target biologically important DNA regions carrying fungicide resistance mutations in *V. inaequalis* or host-specific effectors in *P. aphanis* and *P. leucotricha*; however, designing LAMP primers that work well is challenging (Lau and Botella, 2017).

5.2.2. Diagnosis-by-Sequencing has Great Potential to Improve Outcomes for Growers

DNA diagnostics and third-generation sequencing have been highlighted as emerging technologies (DEFRA, 2023). Diagnosis-by-sequencing of fungal plant pathogens is a powerful tool for identifying and characterizing fungal plant pathogens. This approach has become increasingly popular in recent years owing to advancements in sequencing technologies, which have significantly increased the speed and cost-effectiveness of sequencing. One key advantage of diagnosis-by-sequencing is that it allows for rapid and accurate identification of pathogens, even when they belong to complex or poorly characterised taxa. This is particularly important for plant pathogens, as many of them are difficult to identify based on morphological or agar-plate growth characteristics (Kellenberger, 2001;

Kang et al., 2010). Technically challenging and/or time-consuming detection methods are unlikely to confirm the presence of a pathogen before a disease outbreak develops. Therefore, rapid testing is a requirement for the timely deployment of control measures and biosecurity of perishable commodities.

Similar to LAMP assays, new sequencing technologies have the opportunity to move diagnostics away from centralised reference laboratories, providing data directly to end users either in the field or in remote or less well-equipped laboratories. In the near future, portable sequencing devices such as the MinION and Flongle from Oxford Nanopore could be used for this kind of diagnosis from mixed samples in the field (Bronzato Badial et al., 2018; Chalupowicz et al., 2018; Boykin et al., 2019). As nanopore technology can produce sequencing information in real time, it is possible to look selectively for specific sequences whilst ignoring, for example, host plant DNA. This is termed 'read until' sequencing (Loose et al., 2016). A given strand of DNA is checked against a reference database of sequences as it is sequenced and if there is no match, the current across the pore can be reversed and the nucleotide strand ejected. This 'Read-until' sequencing has been shown to be an important and useful tool for detecting specific DNA molecules within a mixed population. A technique known as 'What's In My Pot' (WIMP) analysis compares sequence reads to the known sequence database, assigns species taxonomy, and returns an assessment of taxon abundance in real time.

Oxford Nanopore technology is already being used in a plant pathogen diagnostic context. The Mobile And Real-time PLant disEase (MARPLE) diagnostic system (https://marple-diagnostics.org/) uses targeted re-sequencing with an Oxford Nanopore device to provide rapid point-of-care diagnosis of individual wheat rust strains within 48 hours of sampling, and has been deployed across East Africa and South Asia (Radhakrishnan et al., 2019). Boykin et al. (2019) were able to perform real-time on-farm genome sequencing of cassava plants with limited or no access to mains power, laboratory infrastructure, or internet connectivity, using a MinION device. Diagnosis for cassava mosaic begomovirus was delivered in under three hours and at a cost approximately 10 times lower than testing samples at a public certifying institute (Boykin et al., 2019). Boykin et al. (2019) estimate reagent costs per sample will drop to 4 USD with the 'Flongle' Oxford Nanopore device, plus 3.70 USD/sample for DNA extraction materials (MinION device = 1000 USD, high-specification laptop = 3000 USD) (Girgis et al., 2022).

The WIMP and 'read-until' method could be used as a general diagnostic tool to simultaneously address large numbers of potential pathogens. The ability to conduct assays for many pathogens and many key pathogen traits simultaneously would dramatically increase efficiency and allow more comprehensive data gathering. Chalupowicz et al. (2018) were able to identify plant pathogens within

1-2 hours, from a few as 42 reads using a 'what's in my pot?' workflow, and showed that simultaneous diagnosis of multiple pathogens is feasible using 3rd generation sequencing technology. It is possible to envisage a test using an Oxford Nanopore device which is precise enough to identify key pathogen characteristics such as resistance to certain fungicides and virulence on certain hosts. Such a diagnostic tool could be used to better inform management practices.

However, prior genomic knowledge of pathogen targets is necessary to use this approach effectively. Genomic information for plant-pathogenic fungi is lacking, the RefSeq database available to Chalupowicz et al. (2018) contained only 240 fungi, most of which were not plant pathogens, in contrast to 7,482 viral genomes and 5,083 bacteria (Federhen, 2015; Hawksworth and Lücking, 2017). To facilitate a comprehensive WIMP diagnostic tool, a database of sequences must be developed to allow the real-time lookup of target sequences. The assemblies generated in this study contribute to the development of such a database by producing some of the first genomic sequences for powdery mildews affecting horticultural crops. CSEPs have been identified, the presence/absence of these represent diagnostic targets for strain virulence, directly related to the biology of pathogen host range. Additionally, regions associated with fungicide resistance in apple scab were identified. These components may be used to construct a database for WIMP sequencing.

5.2.3. Pathogen Detection is Moving From Diagnosis to Surveillance

Pest and pathogen monitoring is key for efficient and early disease prevention and management. Most fields are too large for growers to monitor frequently. Remote sensing from satellite or drone imagery can be used to detect tell-tale signs of disease, such as increased fluorescence and heat emission (West et al., 2003; Mahlein et al., 2013; Berdugo et al., 2014; Yuan et al., 2016; Zarco-Tejada et al., 2018; Barbedo, 2019; Raza et al., 2020). For example, Yuan et al. (2016) demonstrated that *B. graminis* infections can be mapped at a regional level using broadband multi-spectral satellite image data. However, such detection methods are typically unable to identify individual pests and are considered non-diagnostic and non-actionable by most growers (Silva et al., 2021). Non-targeted high-throughput sequencing approaches hold promise for the broad-spectrum but precise surveillance of microbial communities, including the detection of known and unknown pathogens (Adams et al., 2018).

For many plant pathogens, including apple scab and powdery mildew, detection still relies upon the initial identification of visual plant symptoms, which can be time-consuming and subjective. By the time symptoms of infection are visible, plant tissues contain high pathogen levels, and damage to fruit may have already occurred (Agrios, 2005). Diagnosis-by-sequencing approaches have the

potential to provide pathogen detection prior to disease establishment and can be used to monitor changes in population structure (Hubbard et al., 2015). By tracking the movement and distribution of strains across the agronomic spectrum, and by gaining a deeper understanding of overall population genome diversity, disease management over the longer term can be improved, and patterns may be identified that facilitate pre-emptive countermeasures. Gutierrez-Vazquez et al. (2022) were able to use nanopore sequencing to profile field samples of *Zymoseptoria tritici* for *CYP51* mutations causing DMI resistance, they suggest that routine profiling of populations to enable better spray selection.

High-throughput sequencing technologies can be used to monitor biodiversity via environmental DNA (Qin et al., 2020; Leung et al., 2021; Clare et al., 2022; Lynggaard et al., 2022). Tremblay et al. (2018) were able to detect *Heterobasidion,* which causes root rot, via sequencing of spore trap samples, whilst Bérubé et al. (2018) were able to detect spores of the canker pathogen *Diplodia corticola* in Canada, prior to any symptomatic trees being reported in the country. These spore samplings were collected over a period of weeks (Tremblay et al., 2018). More recently Giolai et al. (2022) have demonstrated that ecosystems can be efficiently monitored via shotgun metagenomic profiling of airborne environmental DNA. Sampling three times a week they were able to quantify changes in pathogen abundance in an agricultural environment over a period of 1 ½ months, taxonomically classifying microbial species, including *B. hordei* (Giolai et al., 2022).

The use of diagnosis-by-sequencing in horticulture could allow for effective monitoring of the impact of agronomic practices and planting of host plants with new combinations of resistance genes. By combining this information with other data sources, precision agriculture approaches can be applied to enhance productivity, increase yields, and ultimately gain a competitive advantage for the industry.

5.3. Conclusion

In conclusion, the identification of genetic markers associated with fungicide resistance and the availability of new genomic resources for mildew offers promising targets for sequencing-based diagnostic approaches. Comparative genomic analyses have yielded new insights into mildew pathogenicity and evolution, as well as candidate effectors for screening host *R* genes to support effector-informed breeding strategies. Collectively, these findings support efforts to reduce the use of unnecessary fungicides, enhance disease monitoring and management capabilities, and breed horticultural crops with more durable resistance.

6. References

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