Towards the characterisation of the raspberry root rot complex

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Declaration

I declare that this thesis is my original work, that it has been written by myself and that it has not been accepted in any previous application for a degree. I have acknowledged all the sources of information which have been used in this thesis.

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Abstract

Raspberry root rot (RRR) caused by a consortium of oomycete species is an economically important disease of raspberries. Current management practices rely on an integrated program of chemical control via fungicides and cultural methods such as irrigation system sterilisation and planting resistant genotypes. The increasing deauthorization of fungicides with activity against *Phytophthora* species coupled with improving diagnostic technologies have prompted further research into the consortia of oomycete species involved in plant diseases like RRR. The aim of this study was to investigate the *Phytophthora* species present in the roots of plants exhibiting symptoms of root rot on nine commercial raspberry farms in the UK through traditional methods of isolation and molecular diagnostics such as lateral flow devices, polymerase chain reaction, and high throughput sequencing. Furthermore, the pathogenicity of the isolates obtained was assessed on a panel of commercially relevant raspberry varieties through detached leaf, root, and whole plant pathogenicity assays.

Four isolates of the *Phytophthora* species *P. citrophthora* and one isolate of *P. erythroseptica*, *P. cryptogea* and *P. pseudocryptogea* were recovered from symptomatic raspberry roots via isolation. Six isolates of the *Phytopythium* species *Pp. litorale* and one isolate of *Pp. vexans* were also recovered, a first report of these species in raspberry and the UK. High throughput Illumina sequencing revealed 41 distinct sequences amplified across sites corresponding to nine *Phytophthora* species, four *Globisporangium* species, three *Peronospora* species and three *Phytopythium* species. *P. rubi*, *P. cactorum*, *P. citrophthora,* and *P. bishii* - a relatively new introduction to the UK, were detected in 100% of samples. *Peronospora sparsa*, the causal agent of downy mildew in roses, and *P. rubi* were the two species with the highest abundance across all samples. Farm location was the most significant factor affecting the diversity and abundance of the species detected.

Subsequent pathogenicity testing on detached leaves and roots revealed plant genotype has a significant effect on the virulence of the isolates obtained in this study. The *Phytopythium* species *Pp. litorale* and *Pp. vexans* exhibited high and moderate pathogenicity on raspberry, resulting in fast-growing lesions on detached raspberry leaves and roots. Additionally, crown and root rot were observed in whole raspberry plants eight weeks after inoculation with zoospores of *Pp. litorale* and *Pp. vexans*.

This study has demonstrated the diversity of *Peronosporale* species associated with RRR in the UK, adding to our understanding of the disease. Furthermore, two new pathogens of raspberry are presented which are targets for further research.

Acknowledgments

My foremost thanks to my supervisory and advisory panel for their support, advice, and kind reviews throughout the past three years; Dr Charlotte Nellist, Professor Simon Edwards, and Felicidad Fernández-Fernández. I offer my heartfelt thanks to the staff of the Pathology department at NIAB East Malling, namely Dr Thomas Passey, Professor Xiangming Xu, Jennifer Kingsnorth, Georgina Fagg, Dr Matevz Papp-Rupar and Joyce Robinson for their excellent technical help and advice during moments of crisis and sampling woes. To Dr Chris Cook, Dr Sophia Bellamy, Katherine Stewart and Haidee Tang for their help and advice on bioinformatics, statistics, microscopy, and their friendship. Kind thanks to Andrea Guttierez-Fragra and Kathleen Carr for assistance with bioassay work. To Dr Kelly Ivors for sharing her unparalleled knowledge of *Phytophthora* species and isolation techniques with me, and for her continued encouragement and kindness as I begin my career in the fascinating world of oomycetes. To James Townsend for sharing his isolates and knowledge of *Phytophthora*. To Dr Suzanne Litthauer for providing training in molecular diagnostics. To Dr Greg Deakin for bioinformatic analysis. Thank you to Dr Eleanor Gilroy and Dr Aurelia Bezanger for sharing bare root raspberry plants and isolates of *P. rubi* from their collection at the James Hutton Institute. My thanks to the UK raspberry growers and farm managers who kindly allowed me to sample on their farms, and the Berry Gardens and FAST Agronomists who shared their knowledge of oomycete control and raspberry production, namely Rosie Houston, Piotr Peciak and Svilen Serafimov. My thanks to the CTP-FCR, AHDB and BSPP for funding this Ph.D.

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List of Abbreviations

ADONIS	Permutational Multivariate Analysis of Variance
ALFP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
ASV	Amplicon Sequence Variant
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
°C	Degrees Celsius
DEFRA	Department of Environment, Food and Rural Affairs
DNA	Deoxyribonucleic Acid
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
g	gram
H	Hour
H_2O	Water
HTS	High Throughput Sequencing
ITS	Internal Transcribed Spacer
L	Litre
LFD	Lateral Flow Device
m	milli
NCBI	National Centre for Biotechnology Information
NIAB	National Institute of Agriculture Botany
NMDS	Non-Metric Multidimensional Scaling
OTU	Operational Taxonomic Unit
PARP	Pimaricin, Ampicillin, Rifampicin, Pentachloronitrobenzene
PCA	Principal Component Analysis

PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RRR	Raspberry Root Rot
UK	United Kingdom
µ	micro
α	Alpha
β	Beta

Research Outputs

Browne, E. Y., Edwards, S. G., & Nellist, C. F. (2023). First report of *Phytopythium vexans* and *Phytopythium litorale* associated with root rot symptoms on red raspberry (*Rubus idaeus*). *New Disease Reports*, *48*(1). DOI: 10.1002/ndr2.12197

Browne, E. Y., Fernández, F.F., Edwards, S. G., Nellist, C. F 'Optimisation of a detached tissue method in the assessment of host resistance to *Phytophthora* and *Phytopythium* in red raspberry (*Rubus idaeus*)' (2023), *XIII International Rubus and Ribes Symposium*, 731, 89

Browne, E. Y., Edwards, S. G., Nellist, C. F. 'The virulence and diversity of *Peronosporales* species associated with root rot in red raspberry (*Rubus idaeus*)' (2023) *Plant Pathology* (in prep).

1 Introduction

1.1 The soft fruit industry

The term ‘soft fruit’ is not based on phylogeny or class, but one used primarily in post-harvest classification. Soft fruit refers to several commodities including strawberries, raspberries, blackberries and blueberries which have high metabolic rates, high levels of anthocyanins and are therefore usually red or blue in appearance (Manning, 1993). Due to the reported potent antioxidant activity of anthocyanins, fruit rich in these phenolic compounds are of high demand and thus commercially valuable (Wu et al., 2004). In 2021, the planted area of soft fruit in the UK was 10,450 hectares. Soft fruit value was £575.3 million in the same year and the number of holdings recorded as growing soft fruit was 10,450 in 2021 (DEFRA, 2020).

1.2 Red raspberry

European red raspberry (*Rubus idaeus*) is a woody perennial with a biennial cane habit as described by Hudson (1959). Raspberries produce new canes each year, called primocanes from the buds on plant roots. The plant produces flowers and fruit from the matured primocanes in the second year, which are then called floricanes, or long canes. After fruit production the floricanes naturally senesce and are cut back which allows new primocanes to emerge and the growing cycle commences again (Pritts, 2017).

Raspberry is a member of family *Rosaceae* which includes valuable commodity species which have been domesticated for human consumption such as *Malus* (apple), *Fragariae* (strawberry), *Prunus* (cherry) and *Pyrus* (pear).

The reported health benefits of raspberry consumption are numerous, and several studies note their beneficial effects in disease prevention and organ health. The absorbed phenolic flavonoids illicit antioxidant activities in the body, however, unabsorbed flavonoids also exhibit protective effects in the lower gastrointestinal tracts, particularly in the stomach and colon (Oldreive et al., 1998). The water-soluble pigment anthocyanin has been widely reported to lower rates of cardiovascular disease in humans (Cassidy, 2018). Raspberries are known for their high antioxidant and anthocyanin content and their associated health benefits. One such potent antioxidant present in red raspberries is the polyphenol ellagic acid, reported by Aiyer et al. (2008) to prevent the proliferation of breast cancer tumour cells. Similarly, Dobani et al. (2021) observed the polyphenolics present in red raspberries also reduced oxidative damage to DNA damage in the colon, reducing the risk of colorectal cancer. Raspberry-derived ellagic acid functions not only in reducing cancer proliferation, but also in improving brain function. Istas et al. (2018) performed a

triple-blind human study on ten healthy males given 400g frozen red raspberry drinks and a control group given water. Analysis of urinary and plasma metabolites 2 and 24 h post-ingestion noted those given the raspberry drink had significant improvements in flow-mediated dilation, a measure of endothelial function in the brain. These health benefits coupled with their sweet taste and attractive colouring make raspberries a valuable commodity.

Selective breeding of raspberries for better shelf life has been instrumental in the global uptake of the fruit and has enabled growers to produce highly profitable yields, which grew from a farm gate price of £8686 per tonne in 2018 to £9311 per tonne in 2020. Production value followed a similar positive trend, increasing from £137 million in 2018 to £140 million in 2020 (DEFRA, 2020). The planted area, production, value and total supply of raspberries in the UK has steadily increased over the last decade, see Figure 1.1 below (DEFRA, 2020).

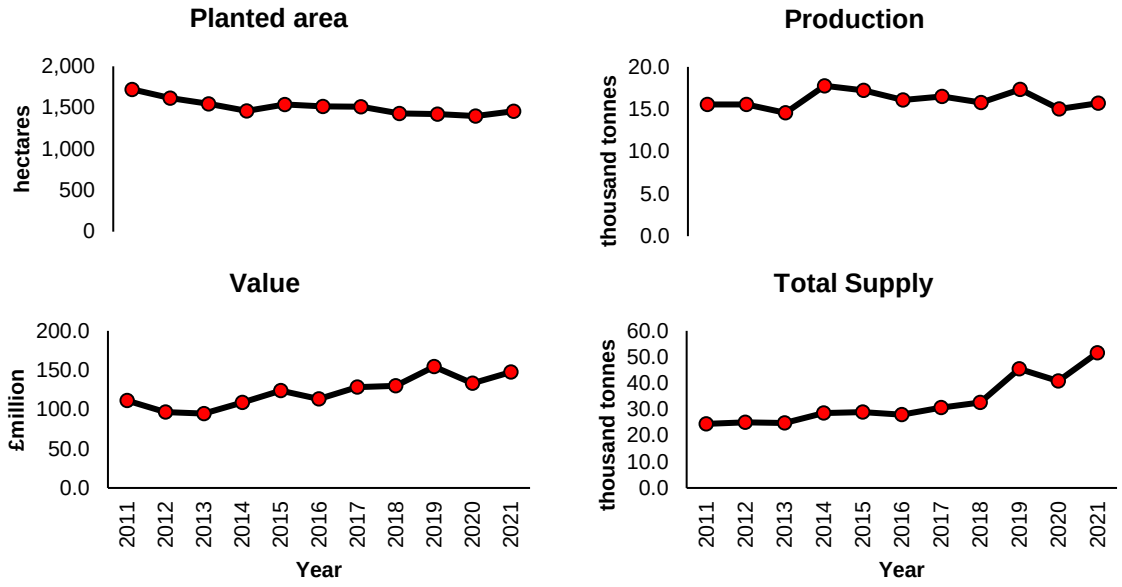


Figure 1.1: The planted area, production, value, and total supply of raspberries in the UK from 2011-2021 (DEFRA, 2020). Figures published for 2021 are preliminary figures.

The development of early-harvesting cultivars with good disease resistance increased the overall yield of fruit produced in the UK. Growers are increasing moving to solely pot-based cultivation (DEFRA, 2020). The move from soil to pot-based production also allows for heavy fruiting varieties which may be more vulnerable to disease to be grown in tunnels.

1.3 Food pathogens and global food security

Oomycete and fungal pathogens can rapidly decimate crop populations, causing up to 40% losses in crops per year, particularly in developing nations (Pitt *et al*., 2009). Rapid sporulation and mycelial spread can infect vast host areas in a relatively short time frame due to spore movement via rain splash and through waterways and irrigation systems.

The impacts of oomycete and fungal pathogens on humanity are recorded throughout history. Perhaps the most well-known example is the Irish Potato Famine (1845–1849), wherein the oomycete *Phytophthora infestans* decimated the Irish potato crop, resulting in widespread food crisis exacerbated to famine by British government-imposed exportation of food to Britain during these years (Woodham-Smith, 1991). In 1942, over 2 million people in Bengal, India died from starvation when their main food source, rice, was destroyed by the Ascomycete fungus *Cochliobolus miyabeanus* (formerly *Helminthosporium oryzae*). Arguably the most devastating epidemic in the plant pathological literature, 91.2% yield losses were observed compared to pre-famine figures. Remaining rice stores were exported to feed British troops in Europe, causing economic collapse and widespread disease (Padmanabhan, 1973).

1.4 Description of raspberry root rot

Root rot of the European red raspberry (*Rubus idaeus*), caused by a consortium of *Phytophthora* species, is a recurring and destructive disease of this commodity fruit. Raspberry root rot is among the three most devastating diseases of raspberry, including spur blight (*Didymella applanata*) and cane *Botrytis* (*Botrytis cinerea*). Raspberry root rot was first noted in the UK in 1980 (Duncan et al., 1987). The disease is most frequently observed during persistent periods of high rainfall and humidity (late Autumn/Winter) and when the crop is in high productivity. This timing corresponds with the most economically important stage of raspberry growing, thus severely impacting a grower's ability to profit from this work-intensive crop. As such, raspberry root rot is a significantly limiting factor in UK raspberry production.

Over ten species of *Phytophthora* have been recorded in raspberry crops; *Phytophthora citricola* (syn. *P. pini*), *P. citrophthora*, *P. cryptogea*, *P. cactorum*, *Phytophthora gonapodyides*, *P. rubi*, *P. idaei, P. erythroseptica*, *P. bishii (*syn*. P. bisheria)*, *P. cambivora*, *P. drescheri* including some heterothallic and homothallic species yet unidentified (Duncan et al., 1987; Burlakoti et al., 2023; Stewart et al., 2014; Wilcox, 1989; Wilcox et al., 1993; Wilcox & Latorre, 2002). *P. rubi* was first reported by Wilcox et al. (1993) as a highly virulent species causing root rot of raspberry in the UK, Germany and the US. Kennedy & Duncan (1995) first described *P. idaei* as the slow-growing causal agent of root rot on Scottish raspberry plants in 1995. *Phytophthora erythroseptica* and *P.*

cactorum were first described in raspberry in the UK by Duncan et al. (1987). Wedgewood et al. (2020) detected *P. rubi*, *P. citrophthora*, *P. bishii*, *P. citricola*, *P. cactorum*, *P. plurivora*, *P. alni* and *P. idaei* in the canes, roots and leaves of raspberry plants exhibiting root rot through nested PCR.

Whilst there is a diversity of *Phytophthora* species involved in raspberry root rot, *P. rubi* was reported as the most prevalent (Stewart et al., 2014). Man In 't veld (2007) investigated isozyme profiles of both *P. fragariae* and *P. rubi* and noted specific differences in restriction enzyme patterns of mitochondrial DNA, genes, pathogenicity and amplified fragment length polymorphisms (AFLPs) which constitute closely related, but separate species.

Phytophthora historically were thought of as fungi, however phylogenetic and taxonomic analyses showed distinct differences in true fungi and oomycetes, and that oomycetes are more closely related to brown algae and diatoms (Gunderson et al., 1987). Oomycetes like *Phytophthora* and *Pythium* possess tubular cristae, have cellulose cell walls and can synthesize the amino acid lysine via the diaminopimelic acid pathway which is contrasting to true fungi (Powell et al., 1985). Thus, true fungi and oomycetes have evolved similar mechanisms of life, but separately.

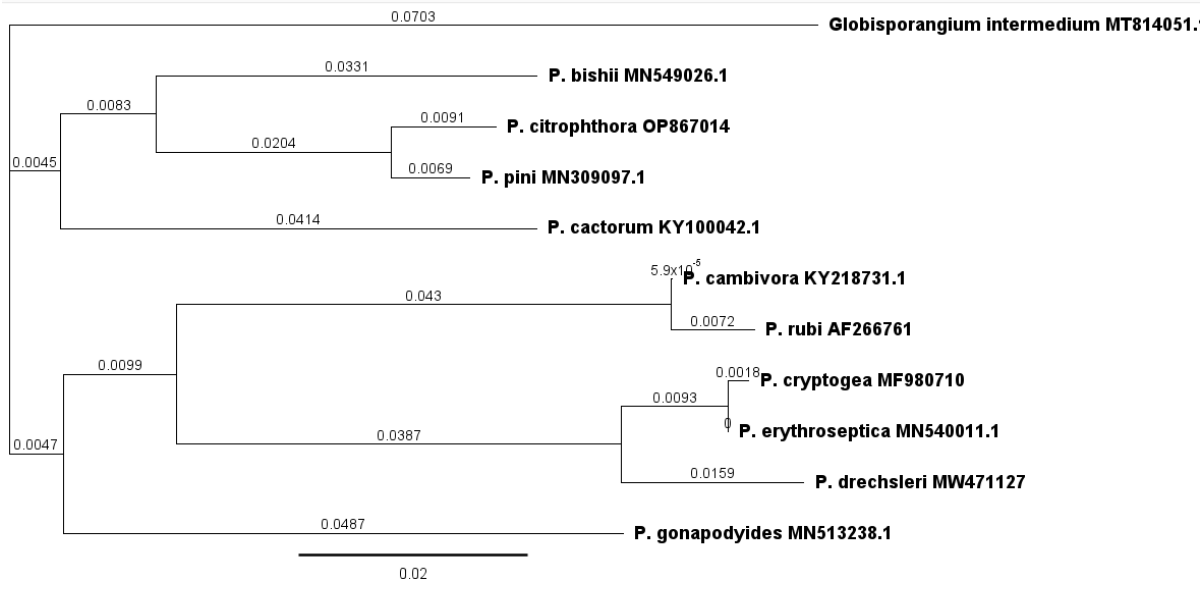


Figure 1.2: Maximum likelihood phylogenetic tree of *Phytophthora* species reported in raspberry based on the rDNA ITS region. *Globisporangium intermedium* is used as an outgroup. Bootstrap probability values are displayed at nodes. The phylogenetic tree was generated using the neighbour-joining consensus method with a bootstrap test (1,000 replicates) in the Geneious v2023 software.

1.5 Symptoms of raspberry root rot

While raspberry root rot begins as an infection of the plant root system, symptoms observed above-ground include premature chlorosis, leaf wilt, red-brown cane necrosis, floricane death and stunted primocane growth (Wilcox, 1989). Infected plants have sparse foliage with few emerging primocanes. Leaves of infected canes are bronzed and striped with scorching at margins. Red lesions can be observed along canes.

Figure 1.3: Raspberry cv. Maravilla leaf exhibiting characteristic leaf bronzing and scorched margins associated with *Phytophthora* root rot.

Below ground, fine lateral roots of infected plants are characteristically red/brown and are soft and easily crushed (Figure 1.4). These plants can produce feeder roots, but they are weak and cannot absorb the nutrients needed to sustain growth and fruiting (Stewart et al., 2014).

Figure 1.4: Raspberry cv. Maravilla root ball in coir showing root deterioration characteristic of raspberry root rot.

Raspberry plants infected with *Phytophthora* species are commonly found in clusters, or disease pockets, in low lying areas with poor drainage. These infection hotspots are noted primarily in late autumn and winter in the UK, when temperatures are low (<19°C) and humidity is high (>70%) (Jeff Layton, formerly Senior Agronomist, Berry Gardens Growers Ltd, pers. comm.). Infected floricanes typically die before harvest or cannot produce fruit due to the vascular tissue damage caused by *Phytophthora* hyphae, resulting in significant economic losses for growers.

1.6 *Phytophthora* mechanism of infection

The success of the *Phytophthora* genus' global colonisation is in part due to the ease in which they can infect and proliferate in a multitude of plant tissues. Species such as *P. citricola* have multiple host species, whilst some species are specialised to one host species with *P. rubi* being a perfect example of such pathogen-host specificity (Jung et al., 2005; Stewart et al., 2014). Developing management strategies to raspberry root rot and improving our understanding of the disease requires an in-depth knowledge of the disease cycle.

Phytophthora infects a susceptible host primarily through their motile biflagellate zoospores (Beakes et al., 2012) (Figure 1.5). The flagellar on the anterior section of the spore allows for directional movement, making them remarkable mobile, some of which can swim 25-35 mm in waterlogged soils (Duniway, 1976). Phenolic compounds, sugars and amino acids found in root exudates act as chemoattractants to *Phytophthora* zoospores (Cahill & Hardham, 1994; Khew, 1973). The zoospores of some soilborne *Phytophthora* species selectively swim towards the root elongation zone instead of the root cap (Hardham, 2001). Hardham & Blackman (2018) posits this specificity may be due to higher number of attractant molecules in this rapidly growing root section. The root elongation zone may also have more easily penetrable primary cell walls. This selectivity enhances the pathogens chances of survival.

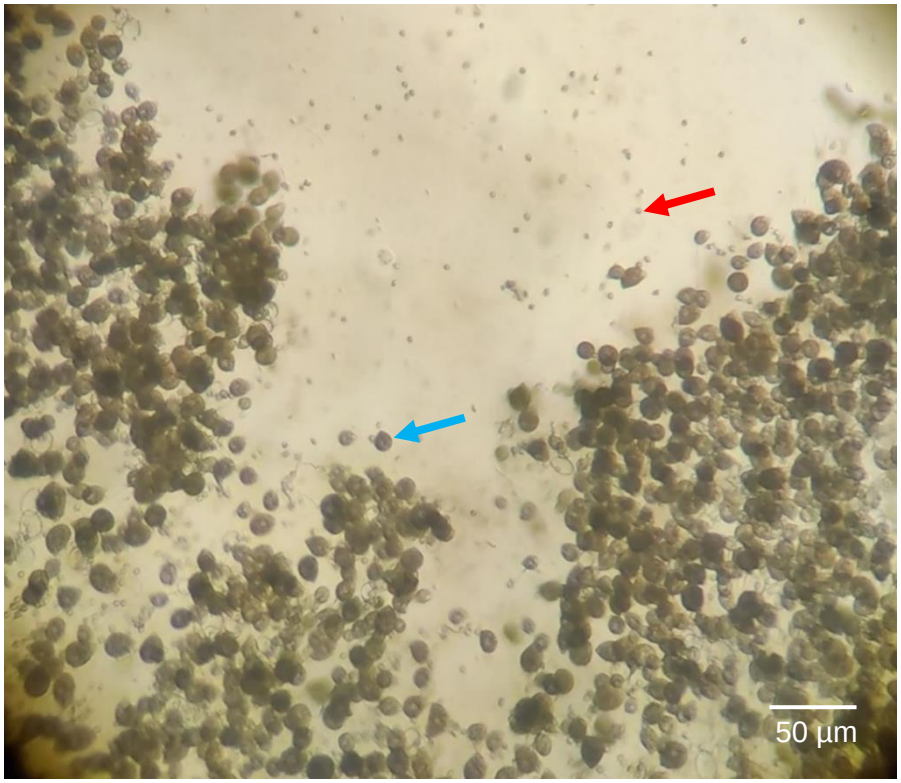


Figure 1.5: A *Phytophthora* species producing the reproductive structures sporangia (blue arrow) and motile, biflagellate zoospores (red arrow) visualised under x25 magnification on a confocal light microscope.

Once attached to the root, the flagellae detach and the zoospore differentiates to form a cyst with a cell wall in a process called encystment. This cell wall formation allows enough turgor pressure to build up within the spore for its germ tube to penetrate through the root cell wall. In addition, the cysts produces many cell wall degrading enzymes which target and degrade plant cell wall polysaccharides such as cellulose and hemicellulose (Judelson & Ah-Fong, 2019). Under adequate conditions of nutrient and calcium availability in the rhizosphere, the cyst can germinate and produce a germ tube which enters membranes of the cell, the plant is now infected. *Phytophthora* also have the ability to produce a myriad of cytoplasmic and apoplastic effectors into host cells, suppressing

host immune system and ensuring almost total, unchecked parasitism (Petre & Kamoun, 2014).

1.7 The microbiome of the rhizosphere

The microbiome of the rhizosphere supports critical processes for plant health such as decomposition of organic matter, macro and micronutrient solubilisation and biocontrol of soil borne pathogens (Dahlstrom et al., 2020; Mahmud et al., 2021; Nysanth et al., 2022). The collective genome of the community of bacteria, rhizobia, archaea, viruses and fungi in the rhizosphere microbiome is so large it is commonly referred to as the plant's second genome (Li et al., 2021). Microbes in the rhizosphere can exhibit direct antagonism and resource competition with pathogens (Berendsen et al., 2012). Volynchikova & Kim, (2022) hypothesize the inhibitory effect of rhizosphere-associated microorganisms on the growth of *Phytophthora* spp. is related to their ability to produce anti-microbial compounds. These compounds can inhibit mycelia growth, sporulation and the production of zoospores and can trigger the plant defence response in their host (Volynchikova & Kim, 2022).

1.8 Approaches to prevent raspberry root rot-induced decline in raspberry crops.

1.8.1 Chemical control

Methyl N-(methoxyacetyl)-N-(2,6- xylyl)-DL-alaninate, commercially known as Metalaxyl, and copper nitrate were introduced as chemical treatments for raspberry root rot in the 1980s following high incidences of the disease being recorded (Duncan & Kennedy, 1989). Phosphorous salts such as phosphites and phosphonates have long been posited as a mitigator of *Phytophthora*-induced root disease. Phosphonates are reduced phosphorous compounds which exhibit varying inhibitory activity on fungi and oomycetes (Martínez, 2016). Barchietto et al. (1988) suggested phosphonate-mediated inhibition of *Phytophthora* was due to its ability to alter the pathogens elicitation of the host defence system. Jee et al. (2002) also noted the inhibitory effects of phosphonates on *Phytophthora* species. Their study found that the addition of potassium phosphonate into hydroponic culture at 99.9 mg/L strongly inhibited *Phytophthora drechsleri* growth in lettuce. No phytotoxic effects were observed on lettuce leaves at this concentration of KH_2PO_3. A similar reduction of *Phytophthora* species on plants treated with KH_2PO_3 (phosphite) occurred in field trials; the infection rate of treated plants was 2% whilst control plants which received no treatment had a 70% infection with *Phytophthora.* Historically, soil sterilisation with chemical fumigants such as methyl bromide was used to

control *Phytophthora*, however the technique was banned in the EU in 2005 due to its deleterious effects on the ozone layer (Ristaino & Thomas, 1996).

1.8.2 Cultural control

Current control strategies employed for raspberry root rot rely on cultural practice due to the lack of fungicide efficacy and the ubiquitous nature of the pathogen in soil. Infection prevention is employed through securing clean planting material, maintenance of freely draining soil and sterilising irrigation lines. Plants testing positive for *Phytophthora* infection are immediately destroyed to prevent further spread. Infection risks have led to ~70% of UK raspberry growers moving from field to pot-based cultivation involving the almost-yearly replenishment of stock which increases the cost and labour involved in raspberry production (Bezanger, 2019).

Reducing extended root contact with standing water is one of the first actions taken by growers trying to reduce raspberry root rot infection. Through planting crops on ridges, colloquially known as 'hilling', soil and roots have increased aeration and better drainage which reduces the standing water available for *Phytophthora* zoospore movement (Stewart et al., 2014). Knowledge exchange between researchers and growers is critical in the mitigation and management of raspberry root rot. Those producing the crop must be aware of the methods of *Phytophthora* spread, both via their irrigation systems and in run off from infected fields and tunnels causing further crop losses. It has been noted, however, that the most important means of spreading root rot is in the transport of plant materials from propagation (J. Graham et al., 2011). Thus, growing plants with genetic resistance, and testing material both before and after transport from propagator to grower is critical in controlling *Phytophthora* spread.

1.8.3 *Phytophthora*-resistant breeding

In the UK, several cultivars have been developed with resistance to raspberry root rot. A marker for *Phytophthora*-resistance, named Rub118b, has been discovered in varieties such as Glen Mor, Glen Fyne and Latham (Graham et al., 2011). Graham et al. (2011) performed Quantitative Trait Loci (QTL) mapping and discovered the Simple Sequence Repeats (SSR) marker Rub118b allele from the resistant cultivar Latham was present in resistant germplasm; the marker was not present in susceptible germplasm. They noted that there has not been any resistant cultivars of commercial importance developed in Europe, thus, the need for breeding programmes to develop resistant cultivars with strong saleable qualities i.e., large berries with good shelf life and quick and resilient primocane growth.

1.8.4 Traditional diagnostics

Phytophthora species can be detected by the plating of symptomatic tissue onto agar which has been amended with a specific cocktail of antibiotics and fungicides which allow the growth of *Phytophthora* but not of any competing bacteria, fungi or other oomycetes. The most commonly used media is PARP, so named for its ingredients pimaricin - a fungicide, ampicillin and rifampicin – antibiotics with activity against gram positive and negative bacteria and the fungicide pentachloronitrobenzene (PCNB) (Jeffers and Martin, 1986). The resultant isolate can then be identified via morphological or DNA analysis via PCR and Sanger sequencing and comparison to known species.

Baiting is a common technique used for detecting *Phytophthora* which involves floating a piece of plant tissue which is susceptible to *Phytophthora* on top of a soil and water solution which contains the suspected *Phytophthora* (Erwin and Riberio, 1996). The plant tissue, or float, attracts the zoospores of the *Phytophthora* species via chemotaxis which swim through the soil and water solution to infect the bait. The *Phytophthora* species can then be recovered from the bait via isolation as outlined above.

While traditional isolation and baiting techniques are the only way to obtain a physical isolate, they can only be performed by skilled pathologists with experience working in oomycete detection. Furthermore, baiting and subsequent infection can take many weeks to gain an isolate, particularly slower growing *Phytophthora* species such as *P. rubi*. As such, the efficiency of *Phytophthora* detection from baiting can range from 0-90%. Factors such as host tissue and the time of year the sample is taken can affect the detection efficiency of *Phytophthora* (O'Brien et al., 2009).

1.8.5 Molecular diagnostics

Molecular testing for *Phytophthora* has progressed significantly in the past decade following the development of Lateral Flow Devices (LFD) which can be either genus or clade specific. The availability of a quick, handheld device with minimal solvent requirements has allowed for agronomy and plant health teams to rapidly assess the health of crops before planting. Whilst lab-based, the Polymerase Chain Reaction (PCR) technique is more specific and can detect individual species present in soil and plant tissue samples within a reasonable time frame.

PCR is a quick and efficient method of detecting *Phytophthora* species in environmental samples. Genus specific primers have been developed which can rapidly detect *Phytophthora* in a sample. A process known as nested PCR, wherein the product of a first-round of a PCR reaction is used in the second round, can utilize species-specific

primers to further increase the specificity of detection. Most of the primers used to detect *Phytophthora* amplify a region of the internal transcribed spacer (ITS), however this region is not always sufficient to separate between *Phytophthora* taxa which are closely related (Schena et al., 2006; Schena & Cooke, 2006). *Phytophthora* genus- and species-specific primers have been developed from the ras-related protein gene *Ypt1,* β-tubulin, the mitochondrial genes *cox1* and *cox2* and the adp9 and nad9 gene regions (Schena et al., 2008; Schena & Cooke, 2006; G. Bilodeau et al., 2009; Martin et al., 2004; Bilodeau et al., 2014). Species-specific primers been developed for some *Phytophthora* species present on raspberry such as *P. rubi*, *P. citrophthora*, *P. citricola* and *P. cactorum* (Schwenkbier et al., 2015; Ippolito et al., 2002; Bilodeau et al., 2014).

In addition to a nested PCR and Sanger sequencing, DNA can be extracted and sent for High Throughput Sequencing (HTS). HTS is a comprehensive term for sequencing DNA and RNA rapidly and can be used to investigate many facets of a target genome or environmental sample. HTS has been used extensively as a diagnostic and investigative tool in *Phytophthora* research. Català et al., (2015) identified 13 *Phytophthora* species in two Spanish forestry sites using 454 pyrosequencing. In pyrosequencing the products of the enzymatic incorporation of target nucleotides such as proton or pyrophosphate release are detected after being converted into a light signal by the enzyme luciferase. Detection results in sequence reads of variable lengths (Knief, 2014). Prigigallo et al. (2015) noted that pyrosequencing gives higher resolution than cloning and Sanger sequencing on *Phytophthora* species. Burgess et al. (2019) used metabarcoding with the nested ITS primer pairs covering ITS1, 5.8S and ITS2 regions from Scibetta et al., (2012) to analyze the diversity of *Phytophthora* from 640 soil samples from forests across Australia, finding 64 distinct phylotypes including 21 novel taxa and 25 species which had previously been undescribed in the country. Ilumina sequencing provides more reads of higher quality and has lower error rates than 454-pyrosequencing (Riddell et al., 2019).

1.9 Identification of *Phytophthora*; conventional vs high throughput technology

High throughput sequencing technologies are often compared with traditional isolation and baiting methods of *Phytophthora* species. Vannini et al. (2013) detected 13 *Phytophthora* species in Italian forest soils compared with just four from traditional baiting. Similarly, analysis by Bose et al. (2018) on native and non-native South African forests discovered 25 new taxa using metabarcoding, compared with just five taxa recorded from baiting. However, the drawbacks associated with HTS are important to note. Conventional methods of baiting and isolation onto selective media yield pure cultures which are crucial for analyzing the morphology, taxonomy and pathogenicity of isolates (Bose et al., 2018). However, these methods are time-intensive and can be affected by many factors including

host tissue and time of sampling. Thus, determining the diversity and pathogenicity of a *Phytophthora* populations in nature requires a combination of conventional and advanced identification and isolation methods.

HTS technologies are often compared with traditional isolation and baiting methods; Vannini et al. (2013) detected 13 *Phytophthora* species in Italian forest soils compared with just four from traditional baiting. Similarly, analysis by Bose et al., (2018) on native and non-native South African forests discovered 25 new taxa using metabarcoding, compared with just five taxa recorded from baiting. However, the drawbacks associated with HTS are important to note. Conventional methods of baiting and isolation onto selective media yield pure cultures which are crucial for analyzing the morphology, taxology and pathogenicity of isolates (Bose et al., 2018). Thus, determining the diversity and pathogenicity of *Phytophthora* communities in nature requires a combination of conventional and advanced identification and isolation methods.

The increasing losses, both, economic and environmental associated with *Phytophthora* disease warrant rapid and efficient methods of detection. Metabarcoding using HTS has become well established as research method for diagnostics and biomonitoring in the global fungal and oomycete scientific community. The high cost associated with HTS (£50 per sample for Illumina sequencing) is a limiting factor in its uptake as a diagnostic method for pathogens, however, costs are steadily reducing, making the technology more accessible for *Phytophthora* detection, improving our knowledge of the diversity, spread and host preferences of newly discovered and well-studied species alike.

A study by Sarker et al. (2021) investigated the impact of sporangia production and zoospore release on the recovery of *Phytophthora* species from baiting. They noted that species with faster zoospore production were isolated most frequently, thus those with slower reproductive rate, or those which did not produce zoospores at all, are classed as low frequency/not present in the environment. Additionally, some species may not be culturable *in vitro*, and whilst they are very much present in the environment and may be major causal agents of disease, are not recorded as present via plating techniques (Santiago Català et al., 2015).

In Sarker et al. (2021) study, one species took 5-7 days to produce sporangia (*Phytophthora constricta*) whilst others such as *Phytophthora nicotinae*, *Phytophthora multivora* and *Phytophthora thermophila* released spores within 1-2 days of baiting. This study has wide-ranging impacts on tradition *Phytophthora* disease diagnostics, much of which uses a standardized baiting protocol of set timings for float submergence, disease tissue recovery, and plating. This standardized timing for baiting thus is not efficient or accurate as a means of detecting many slower sporulating species such as *Phytophthora heveae* and *Phytophthora versiformis*, which are causal agents of macadamia decline in

Hawaii and Eucalypt decline in Western Australia and thus highly commercially relevant (Paap et al., 2017; Sugiyama et al., 2020). Sarker et al. (2021) suggest species diversity shown in previous studies which relied on baiting alone cannot be fully representative of oomycete populations present and thus highlights the importance of multiple methods of *Phytophthora* isolation and identification to glean broader and more accurate insights into their populations. This in turn allows for a more tailored management and mitigation procedures to be employed in disease prevention.

The increasing losses, both economic and environmental associated with *Phytophthora* diseases warrant rapid and efficient methods of detection; this alone justifies the higher costs associated with HTS. In addition, processing costs are gradually reducing, making the technology more accessible for *Phytophthora* detection, improving our knowledge of the diversity, spread and host preferences of newly discovered and previously described species alike.

1.10 High Throughput Sequencing for determining Phytophthora species diversity in environmental samples.

Recent innovations in HTS have facilitated more efficient, sensitive, and reliable sequencing of *Phytophthora* from environmental samples. Furthermore, this rapidly advancing technology allows the detection of all species in a sample, include those not yet described (Mendoza et al., 2014). Metabarcoding is a molecular technique wherein specific genomic regions are affixed with molecular barcodes corresponding to certain samples i.e., environmental DNA samples which can then be sequenced.

HTS has potential for rapid identification of *Phytophthora* diversity and, as noted by Riddell et al. (2019), can be used to detect pathogens at the early stages of infections. Multiple species may be detected simultaneously (Mendoza et al., 2014). This allows for suitable management decisions to be made by growers, whether through biocide application or plant destruction. The bioinformatic processing of HTS data clusters sequence reads of 97% similarly into operational taxonomic units (OTUs). Due to the nature of oomycete ribosomal internal spacer 1 (ITS1) sequences, OTUs that differ by more than 1% in sequence identity from their closest reference sequences are most likely a different species than the one assigned by the software and exact matches do not always indicate individual species (Redekar et al., 2019).

1.11 Is there more to raspberry root rot than *Phytophthora?*

More recent reports on raspberry root rot note *Phytophthora* is not the only genus involved in the disease. Sapkota et al. (2022) suggested a complex of oomycetes, fungi, viruses,

bacteria, and nematodes, working in tandem, are responsible for the plant collapse observed in the field. Further interdisciplinary work is needed to assess the impacts of this consortium of pest and pathogens on raspberry in the UK. Disease complexes, such as apple replant, are well characterized in the literature (Tewoldemedhin et al., 2011). The condition causes root rot in apple trees planted in the same location where other apple trees previously grew, and is reported to be caused by a complex of fungi, oomycetes, nematodes and bacteria (Tewoldemedhin et al., 2011).

Aims

The aim of this project was to identify the *Phytophthora* species present on commercial UK raspberry farms to improve our understanding of the disease and identify new potential targets for genotype resistance screening. The following research questions were addressed in this project:

1. What management practices are UK growers using to mitigate the effects of RRR?
2. What *Phytophthora* species are present on English and Scottish raspberry farms, and what factors affect their diversity?
3. What is the pathogenicity of UK raspberry isolates and does genotype affect isolate virulence?

2 Isolation and identification of *Phytophthora* species associated with root rot of red raspberry in the UK.

2.1 Introduction

Root rot of the European red raspberry (*Rubus idaeus*), caused by a yet-unknown consortium of oomycete species, is a recurring and destructive disease of this commodity fruit. Raspberry root rot (RRR) was first noted in the United Kingdom in 1980 (Duncan et al., 1987). The disease is most frequently observed during persistent periods of high rainfall and humidity and when the crop is in high productivity. While RRR begins as an infection of the plant root system, symptoms observed above-ground include premature chlorosis, leaf wilt, red-brown cane necrosis, floricane death and stunted primocane growth (Wilcox, 1989). Infected plants have sparse foliage with few emerging primocanes. Leaves of infected canes are bronzed and striped with scorching at margins.

Below ground, fine lateral roots of infected plants are characteristically red/brown and are soft and easily crushed. These plants can produce feeder roots, but they are weak and cannot absorb the nutrients needed to sustain growth and fruiting (Stewart et al., 2014). This timing corresponds with the most economically important stage of raspberry growing, thus severely impacting a grower's ability to profit from this work-intensive crop. As such, RRR is a significantly limiting factor in UK raspberry production.

Historically, *Phytophthora* root rot has been attributed to more species than just *Phytophthora rubi*. Duncan *et al*. (1987) reported the pathogenicity of *Phytophthora megasperma*, *Phytophthora erythroseptica* and *Phytophthora dreschleri*, which is considered synonymous with *Phytophthora cryptogea*, on red raspberry in the UK (Cline, *et al.*, 2008). Wilcox (1989) also investigated the pathogenicity of *P. megasperma*, *P. cryptogea*, *Phytophthora cactorum, Phytophthora citricola and Phytophthora fragariae* var. *rubi* (now known as *Phytophthora rubi*) on raspberry in New York, US. Wilcox and Latorre (2002) observed *P. cryptogea*, *P. citricola*, *P. rubi*, *P. megasperma* and *P. gonapodyides* in Chilean raspberry plants. Additionally, *Phytophthora bisheria* (now known *as Phytophthora bishii*) was reported in red raspberry in Australia (Abad *et al.*, 2008). An investigation into the *Phytophthora* species present in raspberry in England was conducted by Wedgewood et al. (2020). The study analysed 79 cane, 89 root and 12 leaf samples from raspberry plants exhibiting root rot symptoms. The study identified eight *Phytophthora* species via nested PCR: *P. rubi*, *P. citrophthora*, *P. bishii*, *P. citricola* (*P. pini*), *P. cactorum*, *P. plurivora*, *P. alni* and *P. idaei*.

Due to deauthorisation of many chemical control agents, current disease management relies on prevention through cultural practice and growing resistant cultivars. Infection prevention is employed through securing clean planting material, maintenance of freely draining soil as well as growing resistant cultivars. Breeding programmes typically use *P. rubi* to test plant susceptibility to RRR, however, changing species diversity in the RRR complex may impact the reliability of these genotypes in the field. This project sought to explore the hypothesis that there are more species than *P. rubi* responsible for RRR in the UK. This study aims to evaluate the species of *Phytophthora* present in red raspberry plants exhibiting symptoms of RRR in the UK.

2.2 Methods

2.2.1 Questionnaires, sampling, and processing

A questionnaire was developed following consultation with a senior raspberry agronomist who noted the most important factors involved in mitigating root rot: fungicide application, irrigation sterilisation, growing resistant cultivars and replacing plants regularly.

Questionnaires were distributed via e-mail to UK raspberry growers in 2020 (See Figure A1 in the Appendix section of this thesis) prior to sampling to assess the strategies and growing regimes used to mitigate root rot on farms. Raspberry plants exhibiting symptoms of RRR were sampled in

Figure 2.1: Approximate location of farms sampled as part of this study.

October 2020 and September 2021. Samples were collected from two sites October 2020 (III-1V) and seven sites in 2021 (1-7), see Figure 2.1.

Raspberry root and cane samples were taken from eleven raspberry varieties and one blackberry (*Rubus subgenus rubus*) variety in England and Scotland. The varieties sampled in this study are proprietary and are thus coded A-K, E is a blackberry variety. Four samples were taken from Farms I-IV in 2020. An average of 10-15 samples were taken per site in 2021 depending on the incidence of root rot observed. Five blackberry roots samples were taken from Farm 5 only, at the grower's request. Circa 100 g of roots with rhizosphere or substrate soil attached were taken and placed in 1 L plastic bags. Trowels, secateurs, and handsaws were thoroughly disinfected using 70% ethanol between samples. Samples were placed in coolers during transport and held in a 4°C cold store until processed.

2.2.2 Isolation of *Phytophthora* species from raspberry tissue

Isolation was performed according to a modified version of the method outlined in Stewart *et al.* (2014). Diseased roots, i.e., those which had significant browning or apparent lesions, were placed in a sieve and rinsed in running tap water to remove soil/substrate. The roots were cut into 10 mm sections and transferred to 70% ethanol for 1 minute and rinsed twice in sterile distilled water. After rinsing, roots were placed on sterile filter papers to dry for 30 seconds. Five root sections per sample were aseptically transferred to 9 cm Petri dishes containing CMA (Difco Cornmeal Agar) amended with pimaricin (0.4 mL/L of a 2.5% (w/v) stock), ampicillin (250 mg/L), rifampicin (0.1 mg/L) and pentachloronitrobenzene (PCNB; 5 mL/L); CMA-PARP (Jeffers and Martin, 1986). The plates were incubated in the dark at 20°C until mycelial growth was observed (5-7 days). The hyphal tips of growing colonies were routinely transferred onto fresh CMA-PARP. Cultures were transferred to CMA media to ensure no contaminating fungi were present. The isolates were tested using the Agdia ImmunoStrip® for *Phytophthora* (Phyt) (Agdia, United States). Five, 4 mm plugs from a putative *Phytophthora* isolate were added to the buffer bag and processed according to manufacturer's instructions.

2.2.3 Identification of *Phytophthora* isolates

To determine if the isolates in question were oomycetes, plugs of each isolate were placed in a Petri dish containing 20 mL of a 50% (v/v) sterile soil extract (100g soil in 1L distilled water, left to settle overnight and filtered through three layers of Muslin cloth, the filtrate was autoclaved at 120°C for 15 minutes, cooled and diluted 1:1 with distilled water) and incubated at room temperature on a bench top for 48 h to promote the production of sporangia and oospores. After 48 h, sections of the submerged plugs were observed under a microscope. Isolates exhibiting sporangia with morphological similarity to

Phytophthora were selected for molecular identification via Sanger sequencing (Figure 2.2)

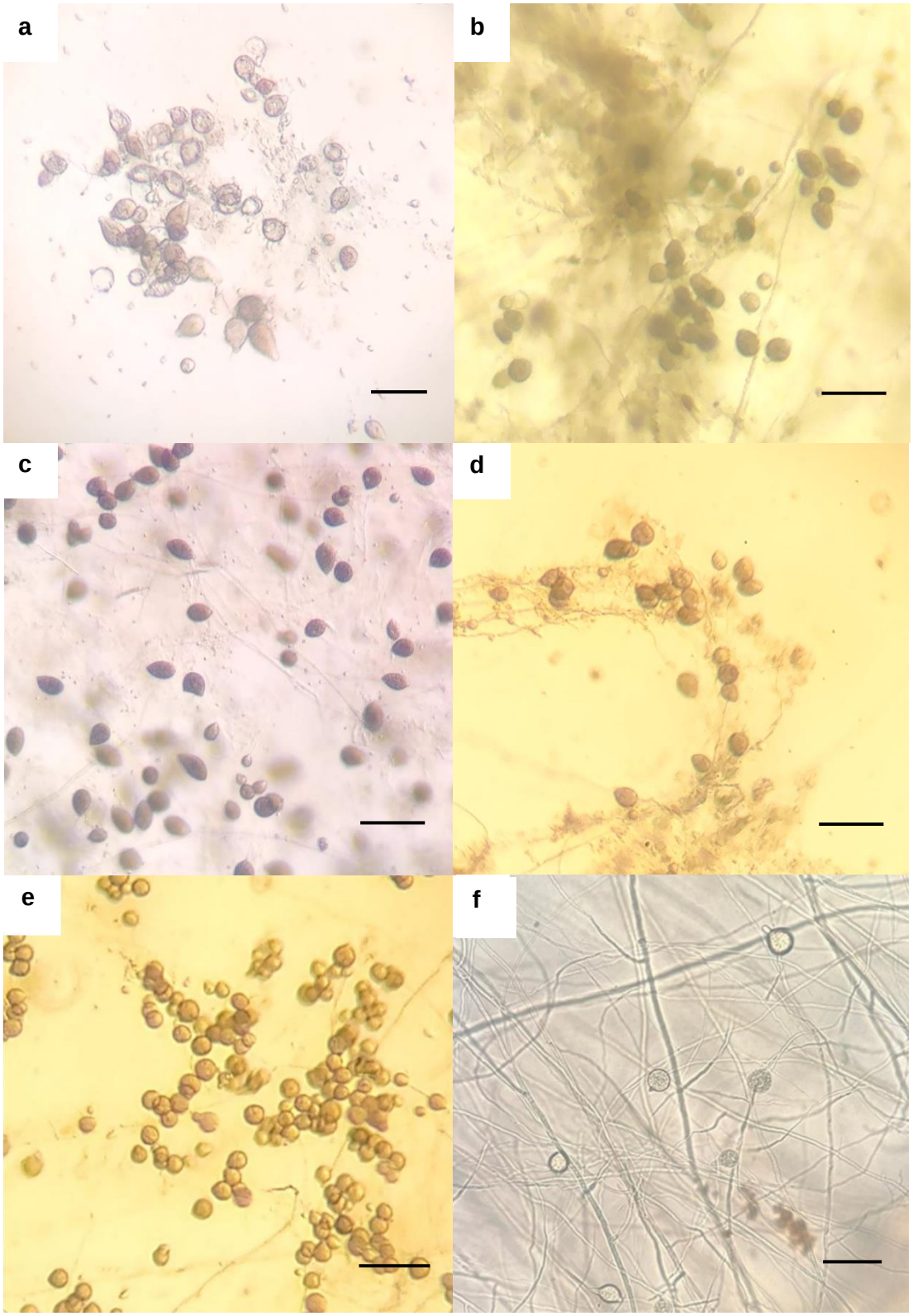


Figure 2.2: Diversity of sporangia of *Phytophthora* and *Phytopythium* isolates grown on V8 media recovered from UK raspberry grower sites. a) *Phytophthora citrophthora* b) *Phytophthora cryptogea*, c) *Phytophthora erythroseptica*, d) *Phytophthora pseudocryptogea*, e) *Phytopythium vexans* and f) *Phytopythium litorale*. The bar in each panel represents 50 µm.

To determine the identity of cultures, a rapid fungal DNA extraction was performed using the Sigma-Aldrich Extract-N-Amp™ Plant extraction and dilution buffers (Sigma-Aldrich, U.K), following the manufacturers protocol. The extracted DNA was stored at -20°C in preparation for downstream analysis. For Sanger sequencing, the ribosomal internal transcribed spacer 1 and 2 (ITS1 and ITS2) and the 5.8S ribosomal unit region (>900 bp) was amplified from the DNA using the ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (White *et al*., 1990). Sterile MilliQ water was used as a negative control. Genomic DNA extracted from stock cultures of *Phytophthora fragariae* (isolate BC16 from NIAB East Malling isolate collection) and *Cladosporium cladosporioides* (isolate 100, NIAB East Malling isolate collection) were used as oomycete and fungal positive controls, respectively.

PCR amplifications were carried out in 25 µL reaction volumes. Each reaction tube contained 2.5 µL of both primers at 10 µM, 2.5 µL of sterile MilliQ water, 12.5 µL of 2x PCR MyTaq Red Mix (containing 5 mM dNTPs and 15 mM $MgCl_2$) (Bioline), and 5 µL of a 2 µM DNA template or MilliQ H_2O. Thermo-cycling reactions were carried out in a BioRad C1000 Touch Thermal Cycler with the following programme; 34 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s, and a final step at 72 °C for 10 min. The PCR amplification products were separated by electrophoresis in 1.5% agarose gels stained with GelRed (Biotium) in 1x TAE (40 mM Trisacetate pH 8.0, 1 mM EDTA) buffer at 100 V for 1 h and visualised under a UV trans-illuminator (Bio-Rad ChemiDoc™ MP Imaging System). Images were taken with Image Lab™ (version 5.2) image acquisition and analysis software. Amplification product size was determined by comparison with 500 bp hyper DNA ladder (Bioline). Sanger sequencing of the resultant PCR product using both forward and reverse primers was performed by Eurofins Genomics, Ebersberg, Germany. The forward and reverse untrimmed reads were combined via *de novo* assembly using the Geneious Bioinformatics software v. 2021. The consensus sequence identified using the BLASTN (Altschul et al., 1990) search tool. A similarity of >97% was accepted as identification.

2.2.4 Molecular analysis of *Phytophthora* species in symptomatic raspberry material

Symptomatic roots were thoroughly washed with tap water to remove residual soil or substrate. Canes were cut into 10 x 10 mm sections using secateurs and scalpels which were disinfected with 70% ethanol between samples. Between 1-2 g of root sample were placed into 2 mL Eppendorf tubes and frozen at -80 °C and freeze-dried using the Telstar LyoQuest -55 prior to DNA extraction using the Qiagen PowerSoil Pro Kit (Qiagen) as per the manufacturer's instructions. DNA was quantified by spectrophotometry (Nanodrop, Thermo Scientific) before downstream analysis. Five microliters of each DNA sample

(undiluted and ten times diluted) was amplified using the *Phytophthora* genus-specific primer pair YPh1F (5'-CGACCATKGGTGTGGACTTT-3') and YPh2R (5'-ACGTTCTCMCAGGCGTATCT-3') which amplified a portion of the *Ypt1* gene (Schena *et al*., 2008). Amplification conditions were: 1 cycle of 95°C for 2 min; 35 cycles of 94 °C for 30 s, 58 °C for 45 s, 72 °C for 30 s; and a final cycle of 72 °C for 10 min. Five milliliters of Sterile MilliQ water was used as a negative control and 5 µL of gDNA of *P. fragariae* isolate BC-16 (DNA extracted using the Sigma-Aldrich Extract-N-Amp™ Plant extraction and dilution buffers (Sigma-Aldrich, U.K), following the manufacturer's protocol) as a positive control. The PCR amplification products were observed by electrophoresis on a 1.5% gel and sent for Sanger sequencing as above. The forward and reverse untrimmed reads were combined via *de novo* assembly using the Geneious Bioinformatics software v. 2021. The consensus sequence identified using the BLASTN (Altschul et al., 1990) search tool. A similarity of >99% was accepted as identification.

2.3 Results

2.3.1 Grower Questionnaires

Completed questionnaires were obtained from 18 raspberry growers in the UK in 2020. A total of 44% chose coir as their main substrate for pot-based production, while 17% grew in compost. Six percent of growers maintained a soil-based production and 33% grew in both soil and coir (Table 2.1). Plants were maintained for up to six years in soils, with most substrate-grown plants destroyed after three years of production.

Table 2.1: UK raspberry grower survey results detailing growing media used in commercial production. All data is presented as a percentage of the total 18 responses.

Growing media	Substrate		Soil	Coir & Soil
	Coir	Compost		
Percentage of 18 respondents	44%	17%	6%	33%

Growers employ a variety of methods to mitigate the effects of RRR. All the growers surveyed applied the fungicide dimethomorph (Paraat) developed to control *Phytophthora* root rot in soft fruit. One grower applied methyl N-(2,6-dimethylphenyl)-N-(methoxyacetyl)alaninate (Metalaxyl-M) which has activity against the order *Peronosporales*. All growers surveyed chose growing a root rot-resistant cultivar as their primary method of disease mitigation (Table 2.2).

Table 2.2: UK raspberry grower responses detailing the control methods used to mitigate the impact of raspberry root rot. All data is presented as a percentage of the total 18 responses.

Control method	Fungicide		Additional control methods		
	Paraat	Metalaxyl-M	Resistant cultivar	Chemical water sterilisation	UV water sterilisation
Percentage of 18 respondents	100%	4%	100%	13%	5%

Sixteen percent of growers noted that root rot had no impact on their production, while 29% were severely impacted by the disease. The incident of RRR on the growers farms was relatively low in 2019, with just 12.5% reporting the disease in over 25% of their crops (Table 2.3).

Table 2.3: UK raspberry grower survey results detailing the impact of raspberry root rot on their fruit production and the incidence observed on their farms in 2019. All data is presented as a percentage of the total 18 responses.

Impact of RRR on production				Incidence of RRR in 2019					
None	**Negligible**	**Moderate**	**Severe**	**>5%**	**10%**	**15%**	**25%**	**50%**	**100%**
16%	33%	21%	29%	58%	12.5%	8.3%	12.5%	0%	0%

2.3.2 Sampling

Samples were collected from plants exhibiting symptoms of RRR, sites had varying levels of disease meaning 5-15 samples were taken per farm with an average of 10 samples per farm. Two samples were taken from asymptomatic plants on Farms 1, 2 and 4 at the growers request. Due to variation in cultivar susceptibility to RRR, and some growers having a primary cultivar with an additional smaller crop of a secondary cultivar, cultivars were not sampled equally. The isolation of symptomatic tissue onto selective agar resulted in 29 isolates which produced *Phytophthora*-like sporangia. All isolates were recovered from farms in the West Midlands and Scotland (Table 2.4).

Table 2.4: Locations of farms in which *Phytophthora* and *Phytopythium* isolates were recovered.

Code	Farm location
4	Herefordshire, England
5	Perthshire, Scotland
6	Perthshire, Scotland
7	Perthshire, Scotland

No isolates were recovered from blackberry material. *Phytopythium litorale* was the most frequently isolated species, with six isolates obtained, four of which came from cane material and two were from roots. Out of the nine farms sampled, isolates came from only four sites which showed high RRR disease incidence (over 70% incidence reported by the grower).

Sanger sequencing confirmed the identity of 12 isolates as five species of *Phytophthora* and seven isolates of two *Phytopythium* species by sequencing of the ribosomal *Ypt* gene region (Table 2.5).

Table 2.5: Sanger sequencing results from the amplification of the ITS region of DNA extracted from isolates obtained from UK raspberry farms using the ITS4 and ITS5 primer pair.

Species	Similarity (%)	Number of isolates	Variety code	Farm code
Phytophthora citrophthora	99.5	2	A	4
Phytophthora cryptogea	100	1	C	7
Phytophthora pseudocryptogea	98.7	1	C	4
Phytophthora erythroseptica	98.2	1	B	6
Phytopythium litorale	99.8	6	A,C,E	4,5,6,7
Phytopythium vexans	99.6	1	D	6

Table 2.6: Sanger sequencing results from amplification of the *Ypt* gene region of DNA extracted from symptomatic raspberry roots using the Yph1F and Yph2R primer pair.

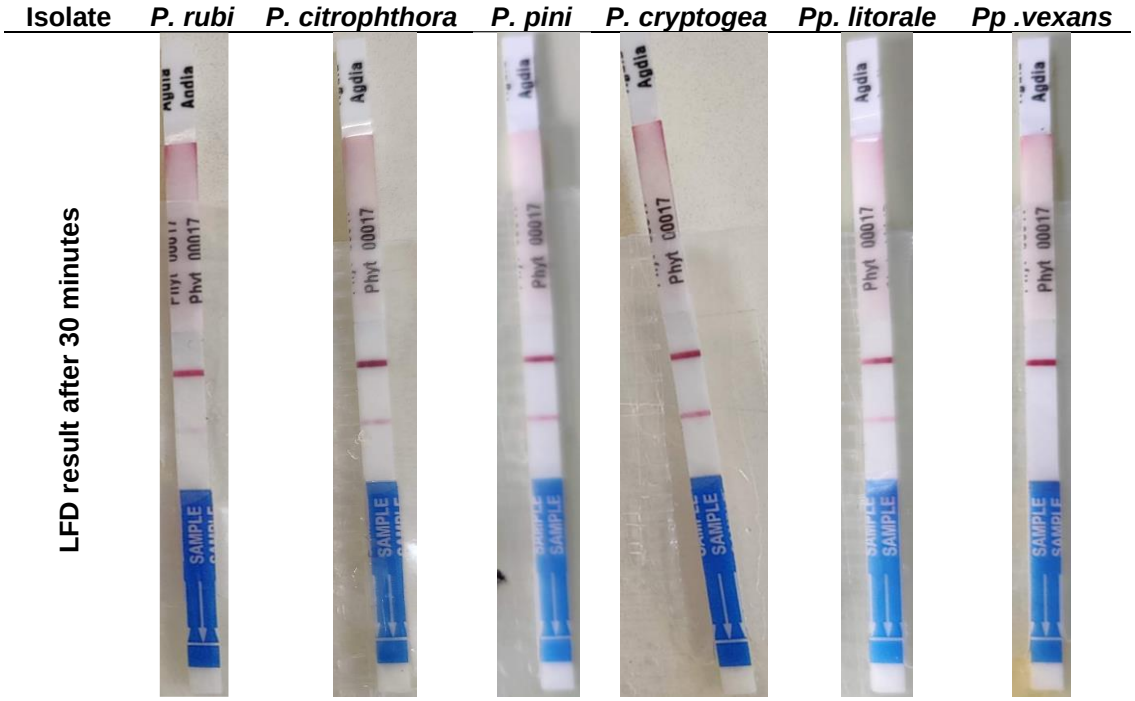
***Phytophthora* species**	**Number of samples**	**Variety code**	**Farm code**	**Country**
P. citrophthora	4	A	4	Scotland
P. plurivora	3	A,D	2,4	England
P. idaei	2	B	2,6	England
P. rubi	2	B, E	1,5	Scotland
P. pseudocryptogea	1	D	4	Scotland
P. hedraiandra	1	C	7	Scotland
P. meadii	1	A	5	England
P. ilicis	1	B	6	Scotland

Molecular analysis via amplification and sequencing of the ITS region directly from diseased plant samples using the detected nine *Phytophthora* species in 16 samples from seven farms (Table 2.6). *Phytopythium litorale* was the most frequently detected species. Notably, *P. rubi* was detected in just one raspberry sample (Variety B) and this site had very low reported occurrence of RRR. *Phytophthora rubi* was also recorded in one blackberry sample (Variety E). No *Phytopythium* species were detected in raspberry roots as *Phytophthora*-specific primers were used for these samples, as *Phytopythium* was previously unreported in raspberry.

2.3.3 LFD testing

All isolates except *Pp. vexans* were detected using the Agdia immunostrip, see Figure 2.3: Results of *Phytophthora* immunostrip testing of agar plugs of *Phytophthora* and *Phytopythium* isolates. The strips have a control (top red line) and a test (bottom red line). Two lines indicate a positive result, one indicates a negative result.

Figure 2.3: Results of *Phytophthora* immunostrip testing of agar plugs of *Phytophthora* and *Phytopythium* isolates. The strips have a control (top red line) and a test (bottom red line). Two lines indicate a positive result, one indicates a negative result.



2.4 Discussion

There was a high level of disease noted in the Scottish farms sampled as part of this study, while root rot in Kent farms was low. Scottish growers all noted a higher incidence of root rot in 2019 than growers in the West Midlands and Kent. All Scottish growers sampled as part of this survey maintained a mixed substrate production i.e., soil and coir, however, two out of three Scottish growers noted their intentions to grow in pots only in the following year. In the chemical control of RRR, most growers opt for the use of fungicides with both curative and protective actions such as dimethomorph and mefenoxam. All growers surveyed applied dimethomorph (sold as Paraat) to their crop, while only one farm applied mefenoxam (sold as Ridomil Gold). Paraat is a systemic morpholine fungicide which inhibits sterol synthesis, critical for cell wall formation in *Phytophthora* (Rekanović et al., 2012). Mefenoxam is a systemic phenylamide fungicide with deleterious effects on protein and nucleic acid synthesis through the inhibition of RNA polymerase in *Peronosporales* species. These fungicides are a critical prophylactic method of controlling oomycete disease in raspberry, however, reports of developing fungicide resistance in *Phytophthora* populations affect the reliability of such measures. A study by Bézanger (2021) in 2021 investigated the sensitivity of ten *P. rubi* isolates to the fungicides dimethomorph, mefenoxam, fluazinam, fluopicolide and propamocarb. The study measured the growth of isolates on media which had been amended with doses of each fungicide ranging from 0.1 ppm to 10 ppm and for mefenoxam only, an additional dose of 100 ppm was screened. Dimethomorph exhibited the highest inhibitory effect on all *P. rubi* isolates in the study, with a 0% of the control growth at 1 ppm, the representative field application dose. Mefenoxam exhibited an isolate-dependent inhibitory effect while the other fungicides tested had intermediate responses. Stewart et al. (2014) investigated the potential of *P. rubi* isolates to develop resistance to mefenoxam *in vitro* through assessing the growth of isolates on fungicide-amended PDA. The EC_{50}, the concentration needed to reduce mycelial growth by 50%, was compared to resistant and susceptible *P. infestans* isolates. They noted no differences in the EC_{50} frequency distributions among their isolation populations, concluding the isolates did not express potential for resistance to mefenoxam.

Numerous reports on *Phytophthora* in red raspberry note *P. rubi* as the dominant species in various countries including the US, Serbia and the UK (Benedict et al., 2018; Gigot et al., 2013; Koprivica et al., 2009; Pattison et al., 2004; Stewart et al., 2014; Tabima et al., 2018; Wedgewood et al., 2020; Wilcox et al., 1993). However, *P. rubi* was detected in just one sample from the West Midlands in raspberry and one blackberry sample from Scotland using the methods described here. A limitation of these methods is the lack of consistent replication in the isolation and molecular analysis of the samples. Isolations

were performed on four root sections per sample, increasing the number of isolations per sample may have increased the number of isolates obtained. Additionally, DNA extraction and subsequent PCR with the *Ypt* primers was performed without replicates for each sample which may limit the detection of *Phytophthora* species in the samples.

The findings in this study hints at a more diverse raspberry *Phytophthora* species composition in the UK. A communication from industry agrees that species such as *P. citrophthora*, *P. pini* and *P. bishii* are increasingly being isolated from cultivated raspberry crops in the UK (Dr Kelly Ivors, Pathologist, Driscoll's Global Plant Health; Pers. Comm.).

Many factors can influence the species composition of *Phytophthora* in agricultural environments. Ruiz Gómez *et al.* (2019) noted the impact of disease symptoms on the diversity of oomycete species in holm oak decline areas in the Iberian Peninsula with species diversity being significantly lower in highly defoliated zones than in areas with less severe symptoms. Changing climatic conditions, management practices, plant cultivars, growing systems, agrochemical and plant transport may influence the diversity of *Phytophthora* communities in raspberry plants. The detection of *Phytophthora* species such as *P. hedraiandra*, and *P. ilicis*, which are previously unreported in raspberry, and *P. meadii*, unreported in the UK, is of note. *P. hedraiandra* was formally described in 2004, having been isolated from *Viburnum* plants (de Cock et al., 2015). Moralejo et al. (2009) note the species is spreading through nursery trade, specifically ornamental nurseries in southern and northern Europe, which is a major trade route for raspberry and could explain its detection in this study. A study by Prigigallo et al. (2015) further reinforces the theory that plant nurseries are an important source of the introduction and spread of non-native *Phytophthora* species into environments. The same study reported *P. meadii* in the soils surrounding mint (*Menta* sp.) and mandarin (*Citrus reticulata*) plants and in the roots of several ornamental species (Prigigallo, Mosca, Cacciola, Cooke, & Schena, 2015).

Two Phytophthora species, *P. rubi* and *P. bishii* were detected in blackberry samples showing symptoms similar to RRR. To our knowledge this constitutes the first report of either species in blackberry, however, further studies are needed to confirm their prevalence and pathogenicity. Aghighi et al. (2016) reported multiple *Phytophthora* species, including *Phytophthora bilorbang* and *Phytophthora cryptogea* as pathogens of European blackberry (*Rubus anglocandicans*) in Australia. A study by Duncan et al. (1987) reported the resistance of the blackberry cultivar Aurora to five *Phytophthora* species which caused significant disease in raspberry. This may explain the apparent absence of other *Phytophthora* species in the blackberry samples analysed in this study.

Two species of *Phytopythium*, *Pp. litorale* and *Pp. vexans,* were recovered from symptomatic raspberry roots. The cane and root tissue from which they were isolated exhibited similar symptoms to those from which *Phytophthora* was isolated.

To our knowledge, there are no published reports investigating the presence of *Phytopythium* species in red raspberry. *Phytopythium* has recently been reported as pathogenic on other members of the Rosaceae family such as strawberry, apples and pears (Tewoldemedhin et al., 2011; Moein et al., 2019; Jabiri et al., 2020; Mert et al., 2020; Pánek & Střížková, 2021; Ibañez et al., 2022; Zhou et al., 2022)

The site in which *Phytopythium* isolates were recovered in this study notably used water from a local river to irrigate their plants. Furthermore, *Pp. vexans* and *Pp. litorale* were isolated from sites at a low elevation and residual pooling of drip irrigation around the pots was observed. Open water systems such as lakes and rivers can be a significant source of oomycete pathogens such as *Phytopythium*, with many representatives of this genus detected in freshwater and flooded environments (Nam & Choi, 2019; Redekar et al., 2019). *Phytopythium* such as *Pp. irregulare*, *Pp. ultimum* and *Pp. sylvaticum* were reported to have higher disease severity on apple seedlings under higher irrigation regimes (Moein et al., 2019). Benfradj *et al.* (2017) and Jabiri *et al.* (2021) noted a higher prevalence of *Pp. vexans* in drip irrigation systems versus submersion irrigation in citrus trees and apple and pear orchards. Furthermore, over 30 species of *Phytophthora* have been described in water systems around the world (Scibetta et al., 2012; Redekar et al., 2019). Thus, the management of irrigation systems to prevent overflooding and regular disinfection of irrigation lines may reduce the spread of these pathogens in raspberry crops.

The potential of *Phytopythium* species to be a major contributor to RRR has yet to be determined, and to our knowledge there have been no reports on the pathogenicity of this genus on red raspberry. The work outlined in this study describes a more diverse oomycete species composition in red raspberry than previously reported. These findings highlight the importance of regular surveying and sampling of cultivated raspberry crops as a method of disease management. Awareness of the changing patterns of oomycete diversity in raspberry allows researchers to identify changes in life cycles, disease symptoms and pathogenicity of species of interest, which serves to inform growers and breeders. The detection of *Phytopythium* species which have not been identified in raspberry previously is of note, and their respective threat to UK production remains unknown.

3 Diversity of *Phytophthora* species present on selected UK raspberry grower sites.

3.1 Introduction

The management of raspberry root rot requires the integration of cultural and chemical practices due to the efficiency in which oomycetes such as *Phytophthora* can infect roots and survive in the soil. Growing resistant cultivars, planting on hills (known as ridging), ensuring adequate drainage, and carefully managing moisture levels is critical for prophylactic soil-borne disease control. Growers supplement these husbandry practices with the application of fungicides such as dimethomorph and mefenoxam with preventative and curative activity on oomycetes to reduce the impact of root rot on their crops. The *Phytophthora* species composition in UK raspberry has changed over the past 40 years (Duncan et al., 1987; Wedgewood et al., 2020). These changes may still be taking places and as such, biomonitoring of *Phytophthora* species in raspberry is a critical step in mitigating the effects of root rot, and identifying new species which may pose a threat to UK production. Additionally, it is still not clear how factors such as variety, location or substrate type can impact the diversity of *Phytophthora* in red raspberry.

To identify the causal *Phytophthora* species in a plant exhibiting the symptoms of root rot, pathologists sample symptomatic populations and perform lab-based isolations or molecular analysis on single samples. However, these techniques usually only detect a single species within a sample, and only those which have previously been reported. The optimization of high throughput sequencing as a diagnostic tool has enabled researchers to identify a greater number *Phytophthora* species present in a symptomatic plant, both known *Phytophthora* species and those which are yet undescribed (Riddell et al., 2019). High throughput sequencing (HTS) through metabarcoding has been particularly well utilized in studying *Phytophthora* species present in environmental samples and identifying factors which influence their diversity (Prigigallo et al., 2015; Burgess et al., 2017; Bose et al., 2018; Redekar et al., 2019; Riddell et al., 2019; Vélez et al., 2020; Landa et al., 2021; Sarker et al., 2023).

Many factors can influence the pathogen populations within a plant root. Location can have a significant influence on both *Phytophthora* species composition, detection frequency, and abundance (Català et al., 2017;Bose et al., 2018; Sapp et al., 2019; Gyeltshen et al., 2021). The source of environmental DNA (eDNA) can also have a significant effect on *Phytophthora* species richness. Tissue type; whether soil, root, or leaves can affect the *Phytophthora* species detected via metabarcoding. Bose et al.

(2018) reported a significant difference in the species richness and community composition of *Phytophthora* species detected in samples taken from the roots and soil of *Eucalyptus grandis* and *Acacia mearnsii*. The study reported a higher abundance of *Phytophthora* species present in composite soil than in root samples. Marčiulynienė et al. (2021) reported a significant difference in the oomycete species abundance between tree species in roots, but not in rhizosphere soil samples from bare root seedlings of oak, alder, spruce, beech and pine trees.

Metabarcoding in *Phytophthora* studies, both as a diagnostic tool, and an exploration of new species, has been more extensively studied in rhizosphere soil samples from symptomatic plants. Riddell et al. (2019) identified 23 known *Phytophthora* species, including *Phytophthora pseudosyringae* and *Phytophthora austrocedri*, both of which are highly virulent to trees, in the soils of woodlands and public gardens in the UK in 2019, and a further 12 oomycete species with no match to *Phytophthora*. Furthermore, four quarantine-regulated pathogens and three yet-undescribed *Phytophthora* species were detected. A review of this study, published by Green et al. (2020) noted geographic location, soil type, host family or host health status did not affect the *Phytophthora* species detected by Riddell et al. (2019). Bose et al. (2018) compared the *Phytophthora* species associated with non-native trees in South Africa with natural plantations via metabarcoding. Thirty-two *Phytophthora* species were identified, with 14 being new reports of the species in South Africa. Species clustered according to vegetation type in this study (Bose et al., 2018). Legeay et al. (2020) noted a low diversity of *Phytophthora* species present in samples taken from trees of ten taxonomic groupings in the Amazonian rainforest. The study detected just six *Phytophthora* species with a cryptic species genetically similar to *Phytophthora heavae*, making up 97% of the reads, including a new species from clade 10. In this study, host plant family, location and environment had significant impacts on the *Phytophthora* community composition.

The first report of using HTS as a tool in assessing the *Phytophthora* species associated with root rot in red raspberry was recently published by Sapkota et al. (2022). In this study, 128 oomycetes isolated in British Columbia from plants exhibiting root rot were identified using multiplex targeted sequencing on the Ilumina platform with primers for the heat shock protein 90, elongation factor 1α and β- tubulin genes. Eighty-five percent of isolates were identified as *Phytophthora rubi*, while the remaining 15% were *Phytophthora gonopodyides,* a species with moderate virulence on raspberry (Wilcox & Latorre, 2002).

High throughput sequencing has yet to be investigated as a method of elucidating the complexity of the raspberry root pathosphere in the UK. The findings of the previous chapter hint at a more diverse *Peronosporales* species composition in raspberry, thus

metabarcoding using the ITS region on the Illumina NovaSeq platform was utilized in this study to investigate the identity, abundance, and diversity of *Phytophthora* species present in the roots of raspberries displaying root rot symptoms from commercial UK raspberry farms. This work seeks to understand the impact of substrate, plant variety, location, and plant health status (symptomatic vs asymptomatic of root rot) on the diversity and abundance of *Phytophthora* species in commercial red raspberry plants. Furthermore, this study gives further insight into the factors affecting *Phytophthora* diversity and abundance in red raspberry potentially providing better management strategies for root rot in the long term.

3.2 Materials and Methods

3.2.1 Root collection and preparation

Four commercial raspberry farms in the UK were sampled in Autumn 2020 (I-IV). Nine farms were sampled in Autumn 2021 (1-9). Ten-fifteen root samples were collected from plants displaying symptoms of root rot disease such as wilting, cane lesions and blackened roots at each site in 2021, two-four samples per farm were taken in 2020. Two samples were taken from asymptomatic plants on Farms 1, 2 and 4 at the growers request. More samples were taken from sites with higher incidences of root rot, less from farms with lower disease. For each sampling point the variety and substrate type was recorded. Each individual sample was taken from ~5-10 cm from the crown of the infected plant. Each sample was approximately 100 g and stored in individual 1L grip-seal™ polyethylene bag. Root samples were stored at 4°C and processed within one week of collection. The root samples were rinsed thoroughly under running water to remove rhizosphere soil and freeze-dried using the Telstar LyoQuest -55 for 24 h prior to downstream processing. A preliminary DNA extraction and PCR was performed on each sample to ensure the presence of *Phytophthora*.

DNA extraction on 100 mg of freeze-dried roots were performed using the Qiagen PowerSoil Pro Kit (Qiagen) as per the manufacturer's instructions. DNA was quantified by spectrophotometry (Nanodrop, Thermo Scientific) before downstream analysis. PCR amplifications were carried out in 25 µL reaction volumes. Each reaction tube contained 2.5 µL of both primers at 10 µM, 2.5 µl of sterile MilliQ water, 12.5 µl of 2x PCR MyTaq Red Mix (containing 5 mM dNTPs and 15 mM $MgCl_2$) (Bioline), and 5 µL of 2 µM DNA template or MilliQ H_2O. Five microliters of each DNA sample (undiluted and ten times diluted) was amplified using the *Phytophthora* genus-specific primer pair YPh1F (5'-CGACCATKGGTGTGGACTTT-3') and YPh2R (5'-ACGTTCTCMCAGGCGTATCT-3') which amplify a portion of the *Ypt1* gene (Schena *et al*., 2008). Amplification conditions

were: 1 cycle of 95°C for 2 min; 35 cycles of 94°C for 30 s, 58°C for 45 s, 72°C for 30 s; and a final cycle of 72°C for 10 min. Sterile MilliQ water was used as a negative control and *Phytophthora idaei* DNA as a positive control. The PCR amplification products were separated by electrophoresis in 1% (w/v) agarose gels stained with GelRed (Biotium) in 1 X TAE (40 mM Trisacetate pH 8.0, 1 mM EDTA) buffer at 100 V for 1 h and visualised under a UV trans-illuminator (Bio-Rad ChemiDoc™ MP Imaging System). Images were taken with Image Lab™ (version 5.2) image acquisition and analysis software. Amplification product size was determined by comparison with 500 bp hyper DNA ladder (Bioline). Sanger sequencing using both forward and reverse primers was performed by Eurofins Genomics, Ebersberg, Germany.

3.2.2 DNA extraction and amplicon sequencing

Freeze-dried root samples weighing 50 mg each were sent to Novogene (UK) Ltd for DNA extraction, Library Prep and ITS (internal transcribed spacer) amplicon sequencing. Amplicon PCR was conducted using the *Phytophthora*-specific nested primer pairs 18Ph2F and 5.8S-1R in the first round and ITS6 and 5.8S-1R (Table 3.1) in the second round according to the protocol of (Scibetta et al., 2012). Sequencing was performed via synthesis technology on the Illumina NovaSeq 6000 platform.

Table 3.1: Primers used in the amplicon sequencing of raspberry roots exhibiting symptoms of root rot.

Primer	Region	Position	Fragment length (bp)	Sequences (5'-3')
18Ph2F	ITS1	Forward	24	GGATAGACTGTTGCAATTTTCAGT
5.8S-1R	ITS1	Reverse	20	GCARRGACTTTCGTCCCYRC
ITS6	ITS1	Forward	21	GAAGGTGAAGTCGTAACAAGG

3.2.3 ASV generation

The bioinformatic analysis of the data was performed by Dr Greg Deakin, bioinformatician at NIAB. The sequence data from each sample was processed and sequences with less than a single nucleotide difference were clustered into amplicon sequence variants (ASV) using the USEARCH 10.0f pipeline (Edgar, 2013) and a representative sequence generated for each ASV. Sequences were quality checked using UPARSE (Edgar, 2010) quality filtering and primer sequences removed. Unique sequences were identified and sequences with <4 reads were discarded. Unique sequences were ordered by decreasing read frequency and ASVs generated by clustering at 97% similarity and a unique sequence for each ASV was generated. All sequence reads were mapped to the ASV

representative sequences, and an ASV frequency count table was produced for statistical analysis. The ASVs were aligned to a reference database using the MetaFunc package v0.1.0 (Sulit et al., 2023) in R. Taxonomy was assigned to each ASV using the SINTAX algorithm (Edgar, 2016). Oomycete ASVs were compared to the nearest matching sequences in the GenBank nt database using the BLASTN+ program for identification (Altschul et al., 1990).

3.2.4 Statistical analysis

The statistical analysis of the bioinformatics data was also performed by Dr Greg Deakin. Rarefaction was used to normalize count data and remove outliers in the ASV table using the Vegan package v2.6-4 (Dixon, 2003) in R. The alpha (α) diversity of the rarefied counts were subsequently analysed using the Chao1, Simpson and Shannon indices using the Phyloseq package in R (McMurdie & Holmes, 2013). The LmPerm v2.1.0 (Torchiano, 2022) package was used to conduct a permutation multivariate analysis of variance (permANOVA) and significance analysis of the α diversity indices to assess the interaction between farm location, variety and substrate type.

Beta (β) diversity between farm location, variety and substrate type was subjected to principal components analysis (PCA) and non-metric multidimensional scaling (NMDS) using Bray-Curtis distances in the Vegan package. A permANOVA was conducted on the β diversity indices using the Adonis2 function in the Vegan package to assess the effects of farm location, variety, and substrate type on *Phytophthora* diversity. Principal components analysis (PCA) was used to reduce the dimensionality of the dataset into four PCs, capturing more variance and allowing the data to be more easily interpreted. ANOVA analysis was conducted on the first four PCs to determine the contribution of each experimental factor: location, variety, and substrate type to the total variability of each PC. Differential analysis was conducted to normalise the data, as the quantity of DNA differed between samples, and determine which ASVs differed between the experimental factors, i.e., the effect each factor has on individual taxa, using the DESeq2 package v1.38.3 (Love et al., 2014) in R. The *p*-value threshold for differential analysis was adjusted to 0.1. A SINTAX confidence score of ≥ 0.65 at the lowest assignable taxonomic rank was assigned to each ASV using the DEseq2 package.

3.3 Results

3.3.1 Sequencing data analysis

From a total of 134 root samples taken, 87 yielded a PCR product with the *Ypt1* primers. Sixty two out of these 87 samples sent to Novogene for DNA extraction and amplicon sequencing yielded ITS PCR products. A summary of sequencing data can be seen in Table 3.2 below.

Table 3.2: Summary of filtered amplicon sequence variants (ASV) generated using a 97% sequence similarity.

Total raw reads	Total number of ASVs	Number of ASVs per sample		Number of reads per sample		Number of reads per ASV	
		Min	Max	Min	Max	Min	Max
5,416,190	23	1,435	149,153	4,133.4	673,749	2,626	1,389,673

Samples from Farm I and Farm II did not produce any *Phytophthora* PCR product using the 18Ph2F/5.8S-1R and ITS6/5.8S-1R primer set, however, these samples did produce a PCR product with the *YpT1* gene primers (Table 3.3).

Table 3.3: Number of samples per farm which produced a PCR product using the YpT1 primers and were sent for sequencing, and those which produced high quality clustered reads through metabarcoding using ITS primers.

Farm	Location	# of samples taken	# of samples positive for *YPt1*	# of samples positive for ITS
I	Kent, England	7	2	0
II	Kent, England	7	2	0
III	Kent, England	5	2	3
IV	Kent, England	5	4	4
1	Stafford, England	15	10	10*
2	Ludlow, England	15	10	4*
3	Hereford, England	15	5	1
4	Stafford, England	17	10	9*
5	Perth, Scotland	10	10	7
6	Aberdeen, Scotland	15	16	13
7	Perth, Scotland	15	10	5
8	Kent, England	4	2	2
9	Kent, England	4	4	4
Total number of samples		**134**	**87**	**62**

*Two asymptomatic samples each from Farms 1, 2 and 4 were sent for amplicon sequencing to assess the effect of plant health on the diversity and abundance of *Peronosporales* species.

Plants of Variety B were most frequently sampled as this variety has reported *Phytophthora* resistance and is thus chosen as a primary cultivar by many UK growers. Variety E is a Blackberry variety. Varieties C and G-K are experimental varieties and 2020 was their first year of commercial trial (Table 3.4).

Table 3.4: Number of samples per variety which produced a PCR product using the YpT1 primers and were sent for sequencing, and those which produced high quality clustered reads through metabarcoding using ITS primers.

Variety	# of samples taken	# of samples positive for *YPt1*	# of samples positive for ITS
A	5	5	5
B	98	56	31
C	8	6	6
D	5	6	6
E	4	4	4
F	5	3	3
G	4	2	2
H	2	1	1
I	2	2	2
J	1	1	1
K	1	1	1
Total # of samples	**134**	**87**	**62**

3.3.2 Taxonomy

One hundred percent of ASVs were assigned to the genus rank with an 80% confidence value. The percentage of reads assigned to species taxonomic rank at 50%, 65% and 80% confidence value were 10.17%, 6.74% and 6.7%, respectively. Farm 6 had the highest total reads.

BLAST analysis identified 41 ASVs. A summary of these ASVs and their identities can be found in Table A1 in the Appendix. Nine *Phytophthora* (11 phylotypes), three *Phytopythium* (23 phylotypes), four *Globisporangium* (4 phylotypes), two *Pythium* (two phylotypes) and one *Elongisporangium* (one phylotype) species were detected. Phylotypes of the downy mildew species *Peronospora medicaginis-minimae*, *Peronospora* cf. *fagopyri*, *Hyaloperonospora parasitica* and *Pseudoperonospora urticae* were also detected. The top ten ASVs, i.e., those with the highest counts, are outlined in Table 3.5 below. ASVs with 97% similarity to the *Phytophthora* species *P. rubi, P. cactorum, P. bishii* and the downy mildew species *Peronospora sparsa* were detected in 100% of samples. Two ASVs with sequence similarity to *Phytophthora citricola* were detected in 74% and 56% of samples. The *Phytopythium* species *Pp. litorale and Pp. citrinium* were detected in 62% and 44%, respectively. The phylotype corresponding to *Phytophthora rubi* had the highest number of counts, followed by *Peronospora sparsa* and *Phytophthora*

cactorum. The most prevalent *Phytophthora* species in the samples, i.e., the top 10 ASVs, come from within clade 1, 2 and 7.

The percentage of total reads for *Phytophthora* was higher than *Peronospora* and *Phytopythium* for soil, compost and coir and for all of the varieties (Figure 3.1 and Figure 3.2). *Phytopythium* species had high reads in samples of Variety B, G and H, but a lower percentage of total reads in the other varieties (Figure 3.2).

ASVs corresponding to the Genus *Peronospora* comprised 100% of the total reads in asymptomatic samples (Figure A1).

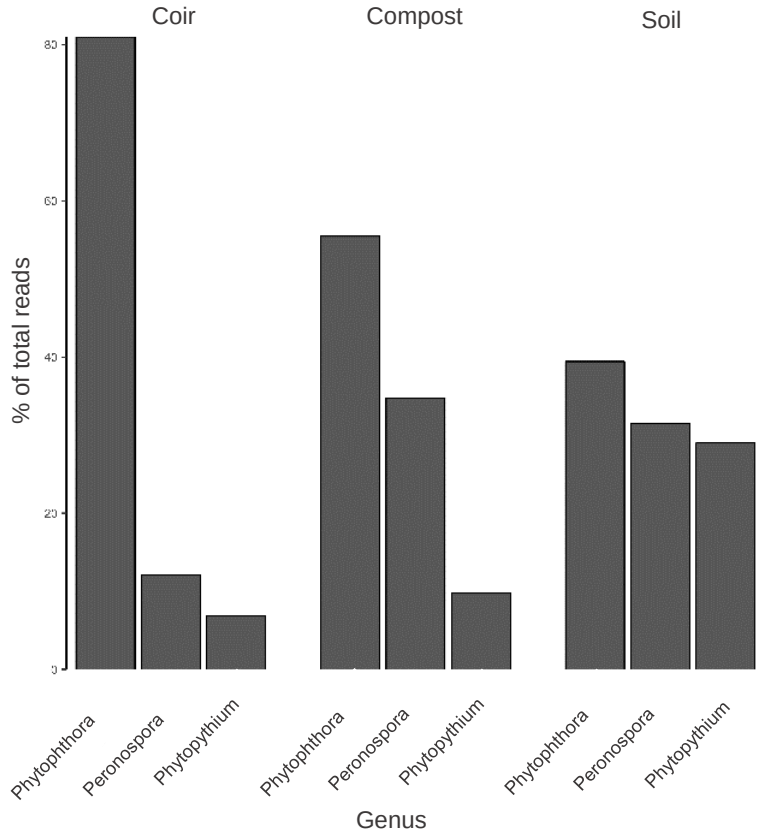


Figure 3.1: Percentage of total reads of *Phytophthora, Peronospora* and *Phytopythium* in three substrate types – Coir, Compost and Soil.

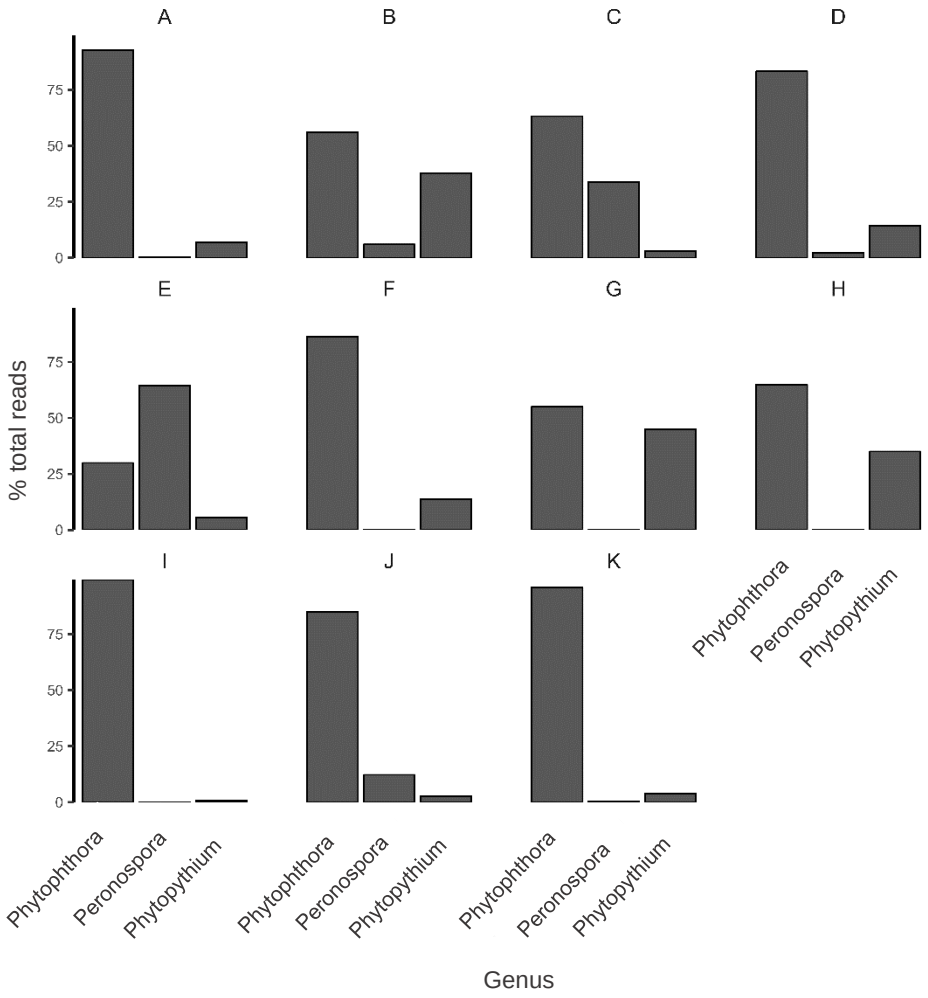


Figure 3.2: Percentage of total reads of *Phytophthora*, *Peronospora* and *Phytopythium* in eleven *Rubus* varieties (A-K), E is a blackberry variety, all other varieties are red raspberry.

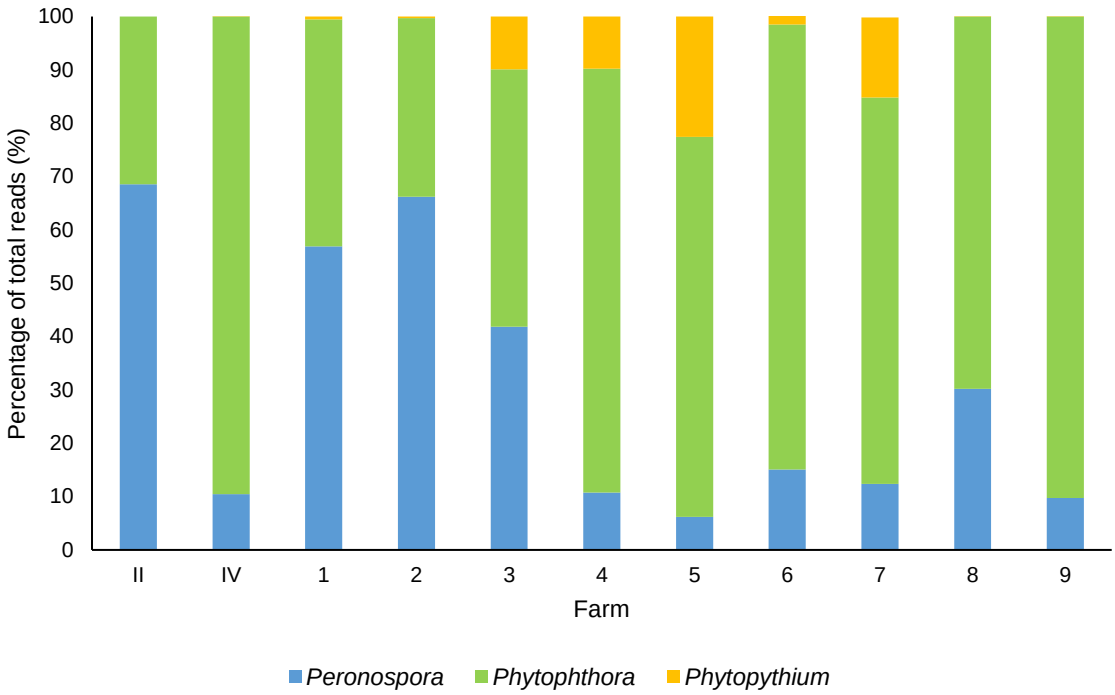


Figure 3.3: Percentage of total reads of the *Peronospora*, *Phytophthora* and *Phytopythium* species present on nine commercial raspberry farms in 2021.

Table 3.5: Summary of the top 10 amplicon sequence variants (ASV) generated using a 97% sequence similarity. ASVs sequences were compared to the GenBank nt database using the BLASTN+ for species identification.

Species	Similarity(%)	Clade	% of samples	% of total reads	Counts	Proportion of total counts
Phytophthora rubi	100	Subclade 7a	100	32	1,734,373	0.2109
Peronospora sparsa	100	N/A	100	28.9	1,563,729	0.1901
Phytophthora citrophthora	100	Subclade 2a	56	28.8	1,559,085	0.1896
Phytophthora cactorum	100	Subclade 1a	100	19.9	1,078,735	0.1312
Phytopythium litorale	100	N/A	62	12.9	700,131	0.0851
Phytophthora citricola	100	Subclade 2c	56	10.2	549,753	0.0668
Phytophthora cactorum	100	Subclade 1a	100	5.8	315,854	0.0384
Phytophthora bishii	100	Subclade 2c	100	4.3	230,353	0.0280
Phytophthora citrophthora	100	Subclade 2a	50	3.3	178,424	0.0217
Phytopythium citrinium	100	N/A	44	1.8	97,645	0.0119

A summary of the *Phytophthora* species detected in each farm in this study is outlined in Table A2 in the Appendix. ASV analysis revealed *P. rubi* as the most frequently detected

species across all farms, the species was detected in 100% of samples and comprised 32% of the total reads for all samples. Farm 1 in the West Midlands had a consortium of eight *Phytophthora* species present, while Farms 8 and 9 in Kent had just *P. rubi*, *P. cactorum* and *P. citrophthora*. *P. pini*, *P. crassamura* and *P.* sp1 were not detected in samples taken on farms in Kent. *Phytophthora cryptogea* and *P. pseudocryptogea* were detected in one sample on Farm III and one sample on Farm IV in Kent (Table 3.6).

Table 3.6: *Phytophthora* species detected on 11 commercial raspberry farms via metabarcoding.

			Number of samples with reads								
Farm	**Region**	**Total # of samples**	***P. rubi***	***P. cactorum***	***P. citrophthora***	***p. pini***	***P. bishii***	***P. cryptogea***	***P. pseudocryptogea***	***P. crassamura***	***Phytophthora sp. 1***
III	Kent	3	3	3	2	0	3	0	1	0	0
IV	Kent	4	4	4	1	0	4	1	0	0	0
1	W. Midlands	10	10	10	7	7	10	6	4	7	6
2	W. Midlands	4	4	4	3	3	4	3	2	2	1
3	W. Midlands	1	1	1	1	1	1	1	1	0	0
4	W. Midlands	9	9	9	8	8	9	4	6	2	0
5	Scotland	7	7	7	7	7	7	4	3	1	1
6	Scotland	13	13	13	13	8	13	9	9	2	0
7	Scotland	5	5	5	1	0	5	5	4	0	0
8	Kent	2	2	2	1	0	2	0	0	0	0
9	Kent	4	4	4	1	0	4	0	0	0	0
	Total # of samples	**62**	**62**	**62**	**45**	**34**	**62**	**33**	**30**	**14**	**8**

P. rubi, *P. cactorum* and *P. bishii* were detected on all varieties sampled. *P. citrophthora* was only detected on samples from Variety A, B, D and E. *P. crassamura* was detected on samples from Variety A, B and D. *Phytophthora* sp1 was detected on Variety B and F only (Table 3.7).

Table 3.7: *Phytophthora* species detected on 11 commercial raspberry farms via metabarcoding. Variety E is a blackberry variety

			Number of samples with reads								
Variety	**# of samples**	**Total**	***P. rubi***	***P. cactorum***	***P. citrophthora***	***p. pini***	***P. bishii***	***P. cryptogea***	***P. pseudocryptogea***	***P. crassamura***	***Phytophthora sp. 1***
A	5	5	5	5	5	5	5	4	2	0	0
B	31	31	31	28	22	31	21	18	9	8	13
C	6	6	6	2	0	6	4	4	0	0	0
D	6	6	6	6	5	6	2	3	1	0	0
E	4	4	3	3	3	3	2	0	0	0	0
F	4	4	4	2	0	4	0	2	0	1	1
G	2	2	2	1	0	2	1	0	0	0	0
H	1	1	1	0	0	1	0	0	0	0	0
I	2	2	2	0	0	1	0	0	0	0	0
J	1	1	1	0	0	1	1	0	0	0	0
K	1	1	1	0	0	1	1	0	0	0	0
Total number of samples	62	59	59	44	34	61	37	31	12	9	14

Samples from Farm 7 and Farm IV had the highest percentage of *P. rubi* with 61.8% and 50.5% of reads in each farm, respectively. Sequencing revealed the presence of a phylotype corresponding to *Phytophthora crassamura* in 14 samples and 0.006% of total reads, this species had the highest read in a sample taken from a soil-grown raspberry plant in Farm 1. A phylotype corresponding with a *Phytophthora* species collected from Holm Oak in Spain by Català et al. in 2017 was detected in 14 samples and 0.1% of total reads (Table 3.6).

Three *Phytopythium* species were detected; *Pp. litorale*, *Pp. citrinium* and *Pp. vexans* and Farm 6 had the highest number of reads for all three *Phytopythium* species; 2.1%, 1.7% and 0.4% of total farm reads, respectively. Samples from Scottish farms had the highest detection rates for all three *Phytopythium* species. *Pp. citrinium* and *Pp. vexans* were not detected in Kent and were most frequently detected on samples from Scottish farms (

Table 3.8).

Table 3.8: *Phytopythium* species detected on 11 commercial raspberry farms via metabarcoding.

Farm	Region	Number of samples with reads			
		Total	***Pp. litorale***	***Pp. citrinium***	***Pp. vexans***
II	Kent	3	0	0	0
IV	Kent	4	1	0	0
1	W. Midlands	10	4	5	2
2	W. Midlands	4	1	1	0
3	W. Midlands	1	1	1	1
4	W. Midlands	9	7	7	2
5	Scotland	7	6	5	5
6	Scotland	13	12	8	9
7	Scotland	5	5	0	0
8	Kent	2	1	0	0
9	Kent	4	1	0	0
Total number of samples		**61**	**39**	**27**	**19**

Pp. litorale was present in all varieties except Variety H and Variety F. *Pp. vexans* and *Pp. citrinium* were only detected in varieties A, B, D and E (Table 3.8).

Table 3.8: *Phytopythium* species detected on 11 commercial raspberry varieties via metabarcoding, variety E is a blackberry variety.

Variety	# samples	Number of samples with reads		
		Pp. litorale	***Pp. citrinium***	***Pp. vexans***
A	5	3	5	2
B	31	18	14	10
C	6	5	0	0
D	6	5	5	5
E	4	4	3	2
F	3	0	0	0
G	2	1	0	0
H	1	0	0	0
I	2	1	0	0
J	1	1	0	0
K	1	1	0	0
Total number of samples	62	38	27	19

3.3.3 Alpha diversity

The α-diversity of a sample indicates the richness and the evenness of species in a sample. The Chao1 index was used as this accounts for species of a low abundance in a sample. The Simpson index accounts for the number of taxa and the abundance and

typically gives more weight to dominant taxa. These α-diversity indices were used to assess the effects of location and variety on *Phytophthora* diversity within samples. Farm and plant variety had the largest effect on α-diversity on the Chao1 index, $p<0.002$ and $p< 2x10^{-16}$, respectively, indicating the species richness and evenness of *Phytophthora* communities differ between site and plant variety (Table 3.9).

Table 3.9: Permutation-based ANOVA on the alpha diversity index variability accounted for by farm location, plant variety, the interaction between location and variety. Values in bold indicate a *p*-value <0.05.

Indices	*P*-value			
	Farm location	**Variety**	**Interaction**	**Residuals**
Chao1	**0.002**	**$<2x10^{-16}$**	0.073	672
Simpson	0.8824	0.7562	0.8824	13757.2

Due to the nested nature structure of the experimental design, the statistical effect of substrate on both the α- and β- diversity could not be calculated. However, the α-diversity of the samples from the three substrate types can be visualised in Figure 3.4. The α-diversity was similar in samples from plants grown in coir, compost, and soil on both the Chao1 and Simpson indexes (Figure 3.4).

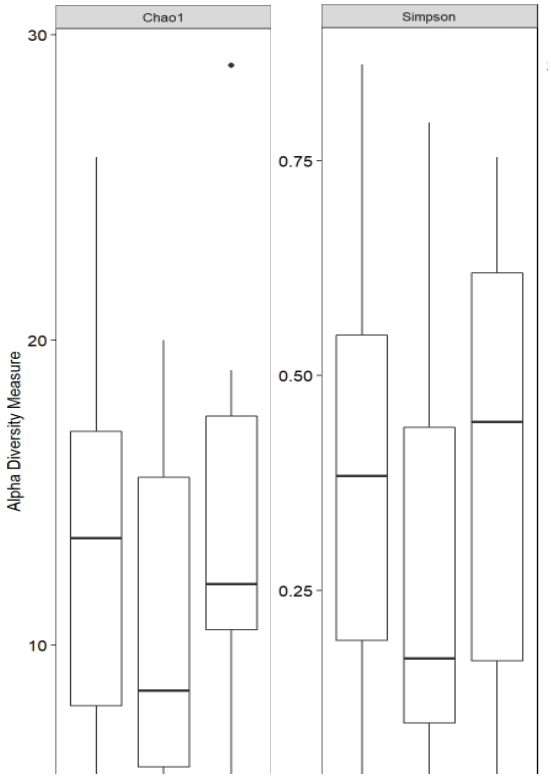


Figure 3.4: Boxplot of the alpha diversity measures, Chao1 and Simpson of the *Peronosporale* species associated with raspberry roots grown in three substrate types. The x-axis indicates the substrate type the sample was taken from. The bold line intersecting the boxes indicates the median alpha diversity measure. The lines on the top and bottom of each box represent the upper and lower quartile. The bold black point indicates an outlier. The boxplot was made using R Studio v1.4.05.5.

Alpha diversity was significantly lower in samples from farms 8, 9, II and IV than the other samples on the Chao1 index. There were no significant differences in the α-diversity between farms on the Simpson index (Figure 3.5)

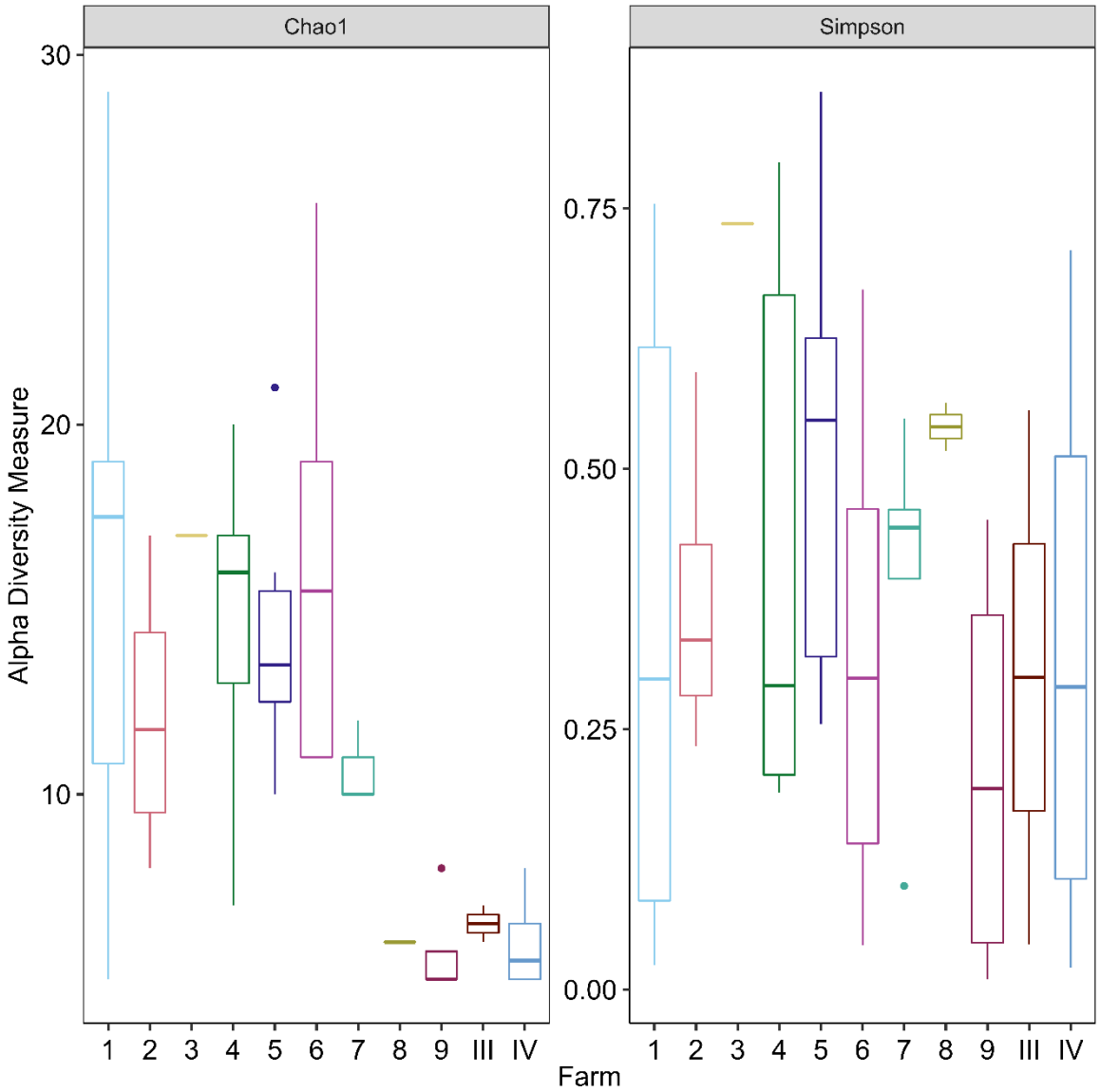


Figure 3.5: Boxplot of the alpha diversity measures, Chao1 and Simpson index for *Peronosporale* species present in the roots of red raspberry on UK farms. The x-axis indicates the farm number. The bold line intersecting the boxes indicates the median alpha diversity measure. The lines on the top and bottom of each box represent the upper and lower quartile. The bold black point indicates an outlier. The boxplot was made using R Studio v1.4.05.5.

3.3.4 Beta diversity

The Beta diversity indicates the diversity between samples. Principal Differences in *Phytophthora* populations between samples were calculated using principal coordinates analysis (PCA) and permutational multivariate analysis of variance (PERMANOVA) using the Bray-Curtis distance index. PCA showed the significant effects of farm location and variety effect on PC1 $p = 3.611x10^{-11}$ and $p = 0.003$, respectively (Figure 3.6). This farm effect was also detected in PC3 ($p = 0.0006$) and PC4 ($p = 0.0005$). Farm location had the highest effect on β-diversity through PCA, accounting for 47% of variance. Variety contributed to 11% of the variance observed. This can be seen in the clustering of points by colour gradient in Figure 3.6. The percentage variation in the first 4 PCs were 38.1%, 14.1%, 10.4% and 9.5%, respectively.

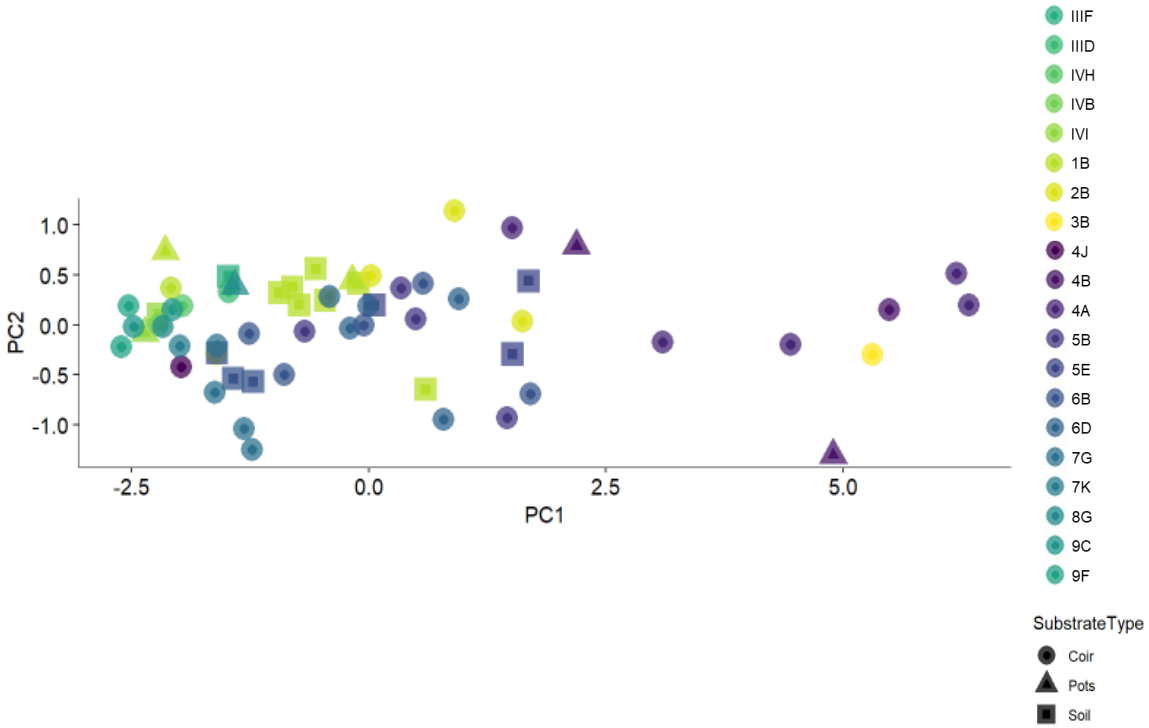


Figure 3.6: PCA plot of PC1 vs PC2 showing the effect of factors farm location, variety, and substrate type on the β-diversity of *Phytophthora* in each sample. Farm and variety are separated by colour, substrate type is separated by shape.

ADONIS analysis of the Bray-Curtis index also highlighted the significant effect of farm location on β-diversity ($p = 0.001$); (Table 3.10). There did not appear to be a high diversity of *Phytophthora* between varieties. This farm effect can be seen in the NMDS analysis in ADONIS analysis of the β-diversity using the Bray-Curtis distance similarity index showed farm location had a significant effect on the β-diversity of samples (Table 3.10). Similar to the PCA plot in Figure 3.6, samples from the same farm cluster together on the NMDS plot (Figure 3.7). Samples did not cluster by the substrate type, suggesting this factor does not affect the β-diversity of *Phytophthora* species. However, a clustering

was visible in farms in the West Midlands and Kent, suggesting samples from these regions have a similar β-diversity on the Bray-Curtis index. Samples from farms in Scotland had similar β-diversity to the other samples, with no apparent clustering (Figure 3.7).

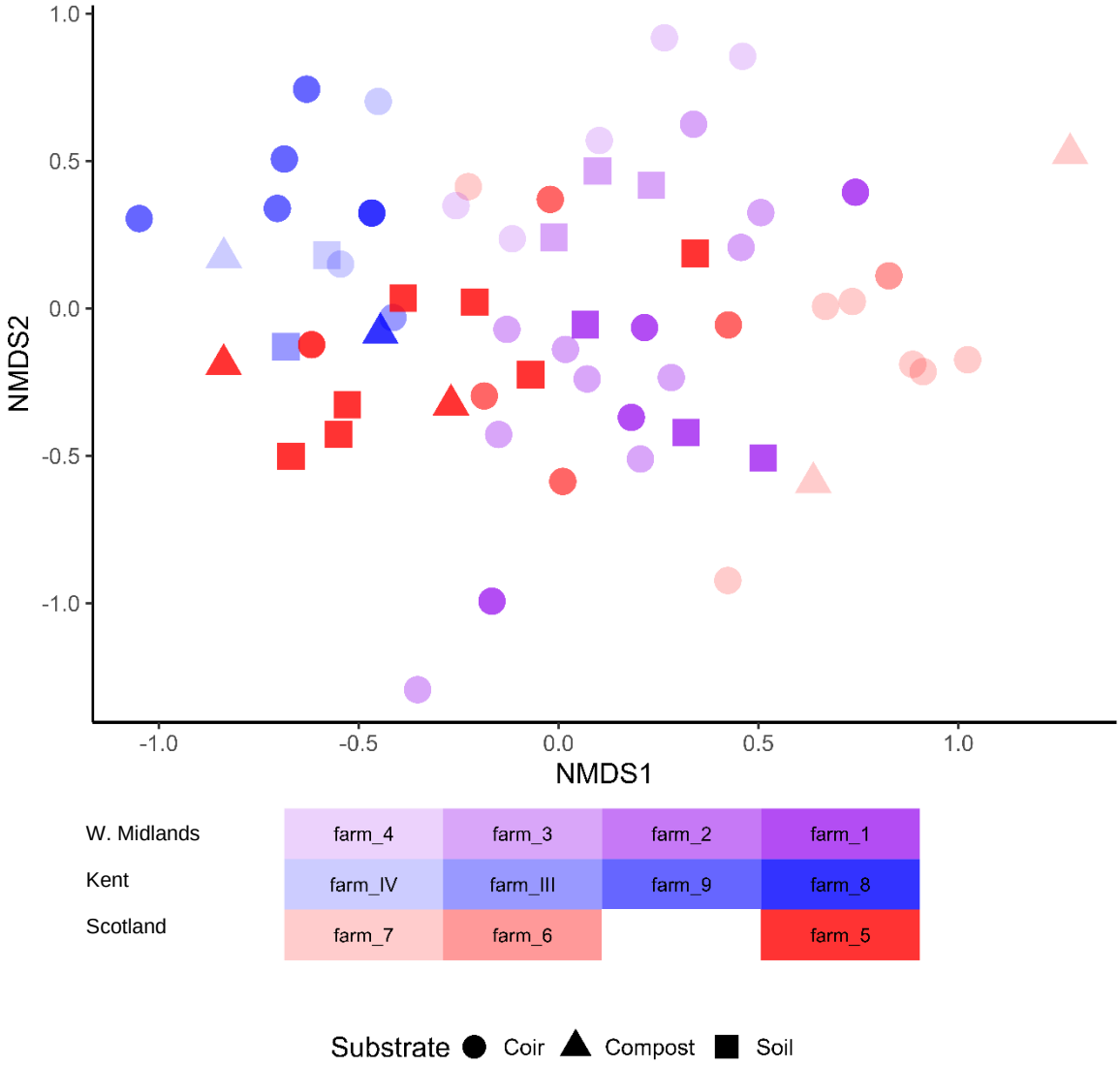


Figure 3.7: Non-metric multidimensional scaling (NMDS) analysis of the Bray-Curtis similarity index showing β-diversity of *Phytophthora* in each sample. Distance between points equates to dissimilarity. The effect of factors farm, location and substrate type on diversity are shown. Farm regions are separated by colour, different shapes indicate the three substrate types assessed.

Table 3.10: ADONIS analysis of the effect of farm location, plant variety and their interaction with substrate type on the Bray-Curtis index. Values in bold indicate a *p*-value <0.05.

Index	*P*-value				
	Farm location	**Variety**	**Variety: Substrate**	**Farm:Variety:Substrate**	**Residuals**
Bray-Curtis	**0.001**	0.213	0.640	0.969	10.808

3.3.5 Differential analysis

There were a number of confounding effects between farm, variety and substrate type. Samples from Variety J and K were unreplicated, and four varieties were completely confounded with farm effect. The DeSeq2 model used in the differential analysis of these samples is not suited to confounded data, thus the effect of these factors on the abundance of taxa in the samples cannot be reliably concluded.

However, the DeSeq2 model ran effectively on the effect of plant health status ie., symptomatic vs asymptomatic of root rot (Table 3.11). Two ASVs corresponding to *P. citrophthora*, and one ASV corresponding to *P. citricola* and *P. cactorum* and were significantly more abundant in symptomatic samples. One ASV corresponding to *P. cactorum* was of higher abundance in the asymptomatic samples.

Table 3.11: DESeq2 results for the differential analysis on the *Peronosporales* present in raspberry plants asymptomatic and symptomatic of root rot. The number of ASVs indicates the number remaining after ASV filtering. Log Fold Change (LFC) > 0 indicates the ASVs are higher in the first treatment i.e., symptomatic, than the second; asymptomatic. The reverse is true for LFC < 0.

DESeq2 model	# of ASVs	LFC > 0 (higher)	LFC < 0 (lower)	Low Counts
Symptomatic vs Asymptomatic	22	4, (18%)	1, (4.5%)	1, (4.5%)

3.4 Discussion

This study assessed the prevalence, abundance, and diversity of *Phytophthora* species on commercial red raspberry farms in the UK through ITS metabarcoding. Raspberry plants of 11 cultivars, grown in soil, coconut coir and compost exhibiting symptoms of root rot were sampled from 11 commercial farms. The aim of this study was to further our understanding of the raspberry *Phytophthora* pathosphere and how these factors can affect its composition and diversity. To this aim, HTS was applied to identify the *Phytophthora* species present in diseased roots of commercial red raspberry farms in the UK and their diversity.

Metabarcoding in the current study revealed multiple ASVs with the same identity, indicating there are multiple strains of the same species, particularly *Phytopythium* species. Eleven ASVs corresponding to *Phytopythium citrinium*, four *Pp. vexans* and two *Pp. litorale* ASVs were detected. This highlights the need for future study into the pathogenicity of multiple isolates of *Phytopythium* species, as intraspecies pathogenicity can vary significantly (Bezanger, 2021; Pánek & Střížková, 2021).

P. rubi was the *Phytophthora* species with the highest read number across all farms in this study. This finding corresponds with previous reports of the dominance of *P. rubi* in the raspberry root rot complex (Duncan et al., 1987; Wilcox et al., 1993; Gigot et al., 2013; Stewart et al., 2014). The findings of this study are in agreement with Wedgewood et al. (2020) who identified the *Phytophthora* species present in 89 raspberry root tissue, 79 cane tissue samples and 12 raspberry leaf samples exhibiting symptoms of root rot from England, UK, though nested PCR with the same primers used in the current study. *P. rubi* was detected in 43% of samples in Wedgewood et al (2020)'s study and was the most detected *Phytophthora* species in all tissue types.

P. citrophthora, *P. bishii*, *P. citricola*, and *P. cactorum* were the most prevalent *Phytophthora* species on the farms sampled in this study. Wedgewood et al. (2020) reported a similar species composition in English raspberry samples wherein *P. citrophthora*, *P. bishii*, *P. citricola*, and *P. cactorum* were detected in 8.9%, 6.1%, 2.8%, and 1.7% of samples. *P. citrophthora*, was first reported as a highly virulent pathogen of raspberry in Chile by Latorre (1993) in 1993. Wilcox (1989) reported *P. citricola* as an extremely virulent pathogen to whole plants of raspberry in the United States, while *P. cactorum* was mildly virulent. Prior to Wedgewood et al. (2020) report, *P. bishii (syn P. bisheria)* had only been noted in raspberry in Australia and the virulence of this species is unknown (Z. G. Abad et al., 2008).

A single phylotype with a 100% sequence similarity to *P. crassamura* was detected in 14 samples across five farms. This is a noteworthy observation as *P. crassamura* has not been reported in raspberry or the UK. *P. crassamura* was first reported in 2015 as a causal agent of root rot in *Juniperus phoeniceae* in the Mediterranean basin (Scanu et al., 2015). The *P. crassamura* ASV had the highest reads in seven soil samples from Farm 1 in the West Midlands, however, the species was also detected in two coir samples from this region in lower abundance. The species was detected with very low reads in five coir samples in Scotland and *P. crassamura* was not present in Scottish soil samples.

Three *Phytopythium* species; *Pp. litorale*, *Pp. citrinium* and *Pp. vexans* were detected in this study. The genus *Phytopythium* was first described by Bala et al. (2010) with the type species *Phytopythium sidhum*, isolated from declining banana trees in Pakistan. This pathogen was categorized as part of a new genus as the isolate produced ovoid, internally proliferating sporangia like *Phytophthora*, but the mechanism of zoospore discharge via cell differentiation in a vesicle outside of the sporangium, was more similar to *Pythium*. de Cock et al. (2015) describe *Phytopythium* as morphologically and phylogenetically intermediate between *Phytophthora* and *Pythium*. The genus was resolved into three phylogenetic clades using the *cytochrome oxidase I* and *II* (*coxI* and *coxII*) gene and the rDNA ITS region by Baten et al. (2014).

Phytopythium was first reported as a pathogen in the Rosaceae family in 2011 by Tewoldemedhin et al. (2011) who noted the pathogenicity of *Pp. vexans* and *Pp. litorale* on apples in South Africa. The isolates were obtained from the roots of trees with M793 apple rootstocks which exhibited root and collar rot. Subsequent inoculation of the growing media of 4-week-old apple seedlings with mycelial discs of the isolates showed *Pp. vexans* to be highly virulent, while *Pp. litorale* had low virulence after three months incubation.

Phytopythium has also been reported as a pathogen in strawberry (*Fragaria* × *anannassa* Dutch), another soft fruit member of the Rosaceae family. A group in the Czech Republic isolated *Pp. vexans, Pp. litorale* and *Pp. mercuriale* from plants suffering strawberry decline in 2021 (Pánek & Střížková, 2021). The group confirmed the pathogenicity of each isolate on detached leaflets of five strawberry varieties. None of the *Phytopythium* isolates had consistent virulence i.e., high total number of lesions on more than one variety. *Pp. litorale* was the only *Phytopythium* species reported as highly virulent in the study contrary to reports from apple (Tewoldemedhin et al., 2011). A recent report by Ibañez et al. (2022) on the oomycete species recovered from strawberry crowns and roots in Argentina included *Pp. vexans*, *Pp. litorale* and *Pp. mercuriale*. The pathogenicity of these species was confirmed via inoculation and subsequent re-isolation of strawberry plants cv. Festival

with mycelial plugs. At the time of writing there are no reports of *Phytopythium* species associated with raspberry root rot, furthermore, the genus has not been reported in the UK.

Phylotypes corresponding to species of *Pythium* and *Globisporangium* were also detected in this study. *Pythium and Globisporangium* are oomycetes and members of the order Pythiaceae. *Pythium* has been implicated in replant disease in raspberry (Schilder & Gillett, 2007). *Globisporangium* has yet to be reported in raspberry but has been reported to cause root rot disease in strawberry (Pánek et al., 2022).

Peronospora sparsa was detected in 100% of samples in this study. *P. sparsa (syn. Peronospora rubi)* is the causal agent of downy mildew in rose and has been reported to infect leaves, flowers, fruits and stems of raspberry (Williamson et al., 1995). The virulence of *P. sparsa* to raspberry is unknown, therefore the threat it poses to UK production cannot be assessed. In the study conducted by Wedgewood et al. (2020), *P. sparsa* was detected by nested PCR in one out of 89 root samples and three out of 79 cane samples, and the species was not detected in symptomatic leaf samples tested by Wedgewood et. al. (2020). *P. sparsa* was detected by PCR in the leaves of arctic bramble (*Rubus arcticus subsp. arcticus*) and cloudberry (*R. chamaemorus*) exhibiting symptoms of downy mildew, noted as angular, carmine-red leaf lesions and dehydrated berries, in Finland by Koponen et al. (2000). *P. sparsa* has been shown to overwinter in the belowground parts of arctic bramble (Lindqvist et al., 1998). This may explain why *P. sparsa* was so readily detected in this study, as the species can survive harsh cold weather such as that experiences by bareroot plants in a cold store. Additionally, as bareroot plants raspberry plants are typically lain on top of one another in a cold store during the winter. Plants with *P. sparsa*-infected leaves may have been in close proximity to the roots of the plants sampled in this study, causing cross-contamination, which may explain its omnipresence amongst the samples analysed. Random sampling of raspberry bare root plants in cold storage for *P. sparsa* would be needed to confirm this.

Farm location had the most significant effect on the abundance and diversity of the oomycete species detected in this study. This finding is in agreement with previous reports of location being the most significant factor in *Phytophthora* abundance and diversity (Català et al., 2017; Bose et al., 2018; Sapp et al., 2019; Gyeltshen et al., 2021).

Growers typically chose to grow a variety as a primary crop which has noted resistance to root rot and produces good quality fruit. Variety B was the primary crop on 7 out of 11 farms sampled in this study. This variety is reported to have field resistance to *Phytophthora*. However, resistance of raspberry varieties to *Phytophthora* is usually tested against, or reported in fields containing *P. rubi* (Nestby & Heiberg, 1995; Graham et al.,

2011; Kempler et al., 2012). Plants from Variety B had high read numbers of *P. rubi, P. cactorum* and *P. bisheria* (100% of samples). The resistance of raspberry varieties to *P. rubi* is reportedly linked to a larger root density and diameter (Graham et al., 2011). To this end, the more vigorous root growth associated with *Phytophthora*-resistant varieties may reduce the ability of pathogens to encyst on the root. The resistant QTL underlying root vigour and resistance is found in a similar region to genes involved in transcription factors implicated in plant defence in other species (Graham et al., 2011). Graham et al. (2011) hypothesised that resistance may be linked to a plant's ability to trigger root differentiation and growth when attacked by a pathogen such as *Phytophthora*. *Phytophthora* has a dual-phase life cycle, firstly biotrophic and when conditions are optimum for infection, a necrotrophic phase which results in plant disease. Rapid root growth may enable a plant to survive *Phytophthora* infection through the production of new roots which can maintain nutrient and water uptake. It is possible that some oomycete species found in the samples may be unable to grow as quickly on the plants as the highly virulent *P. rubi*, *P. citrophthora* and *P. citricola* due to the resistant plant's ability to 'outgrow' some slower growing species.

Samples were not evenly collected from each variety due to growers trialing a few plants of newer varieties C and G-K, and just three samples were taken from the blackberry variety E, therefore a robust statistical analysis of the effect of plant genotype on *Phytophthora* community diversity and abundance was not possible. Alpha-diversity estimated using the Chao1 index revealed plant genotype has a significant effect on the species richness and evenness of *Phytophthora* species in the samples. However, β-diversity analysis using the Bray-Curtis index indicated variety does not have a significant effect on the diversity of *Phytophthora* species in raspberry roots. Therefore, the impact of genotype cannot be fully inferred from these results. Beta diversity analysis relies more on the abundance of the *Phytophthora* groups, while α-diversity is a measure of species richness and evenness, thus variety may affect the richness of Peronosporales species, but not their overall abundance. Future work should analyse an even number of samples from multiple genotypes to assess their effect on the diversity and abundance of Peronosporales species associated with root rot in raspberry.

The high relative abundance of *P. rubi* and *P. cactorum* in the samples taken from all of the asymptomatic plants is of note. The presence of these species in the potting coir or irrigation systems on the farms may explain their detection in the roots of healthy plants, however, it is more likely that these plants were in the preliminary stages of disease, as symptomatic plants were present in the same tunnels.

The research outlined in this study offers more insight into the understudied field of the raspberry *Phytophthora* complex, presenting a more diverse oomycete community in the crop than previously thought. The prevalence and abundance of *Phytopythium* species is of note as this genus has not been reported previously in raspberry or in the UK. This study adds to the growing body of work on metagenomics as a diagnostic tool for *Phytophthora* and as a method to identify potential targets for pathogenicity studies and resistance screening of emerging raspberry breeding lines.

4 Pathogenicity of *Peronosporales* species on red raspberry

4.1 Introduction

Identifying the symptoms of a plant disease is the first step in determining its potential impact on both the plant itself and the surrounding crop. *Phytophthora* root rot symptoms include leaf reddening, chlorosis of the leaves and cane, cane dieback, wilt and blackened roots which are easily crushed. The next stage in identifying the causal agent(s) involved in the disease is direct isolation from the symptomatic tissue i.e., root or cane material onto *Phytophthora*-specific media such as PARP, a media amended with specific concentrations of antibiotics and fungicides designed to promote the growth of *Phytophthora* but exclude fungi and bacteria (Jeffers and Martin, 1986). When a clean isolate has been obtained, both morphological analysis and molecular diagnostics such as PCR are used for identification. In order to ascertain the potential risk of an isolate to a crop species, pathogenicity testing is a critical step in plant pathogen epidemiology. Pathogenicity assays involve infecting a healthy plant or detached plant tissue with an isolate of interest, recording the resultant symptoms, and re-isolating the species from the diseased tissue. This process is known as proving Koch's postulates. Koch's postulates is a measure of scientific rigour and establishes the etiology of a disease through analysis of the relationship between a putative pathogen and its host (Bhunjun et al., 2021). Proving Koch's postulates thus confirms that an isolate is a pathogen and can be a target for further study.

Assessing the pathogenicity of a *Phytophthora* species is performed on either whole plants or detached plant tissues. Detached leaf assays have shown to be useful laboratory assays in preliminarily determining pathogen species of interest for further field-based assays. Indeed, detached leaves are widely used by plant pathologists as baits to isolate *Phytophthora* and *Pythium* species from suspect soils. Due to the economic and historical relevance of *Phytophthora infestans*, there are many reports of the successful application of detached leaf assays to determine the virulence of this species on potato (*Solanum tuberosum*); (Akhtar et al., 2012; Sharma et al., 2013; Michalska et al., 2016; Brylińska & Śliwka, 2017; Njoroge et al., 2019; Karki & Halterman, 2021). Detached leaf assays have also been used in *Phytophthora* virulence studies on strawberry (*Fragaria x ananassa*; Orsomando et al., 2001; Toljamo et al., 2017; Pánek & Střížková, 2021; Macan et al., 2022). The detached leaf method has not been reported for determining *Phytophthora* pathogenicity on raspberry. The AHDB report SF158 did report the isolation of *Phytophthora rubi* from leaves of raspberry plants exhibiting root rot symptoms, the

authors hypothesise that the *Phytophthora* had not travelled through the plant, but it is more likely that the leaves were touching infected soil material during transit form the sampling sites.

The isolates used in this study encompass previously reported species on raspberry and two new species which are yet to be described on this host, see Table 4.1. It must be noted that due to the significant variability of *Phytophthora* species virulence amongst isolates (Bézanger, 2021; Pánek & Střížková, 2021), the virulence recorded is reflective of the isolates tested in each study rather than the species as a whole.

Table 4.1: *Phytophthora* species reported in red raspberry (*Rubus idaeus*). First descriptions of the species and first reports of their presence and virulence in raspberry are shown.

Species	First reported	Pathogenicity on raspberry	Reported virulence on raspberry
Phytophthora rubi	Wilcox et al., 1993	Wilcox et al., 1993	Highly virulent
Phytophthora idaei	Kennedy & Duncan, 1995	Kennedy & Duncan, 1995	Moderate to low virulence
Phytophthora citrophthora	Leonian, 1925	Latorre, 1993	Highly virulent
Phytophthora citricola/pini	Leonian, 1925	Latorre, 1993	Highly virulent
Phytophthora cryptogea	Pethybridge & Lafferty, 1919	Boesewinkel, 1982	Highly virulent
Phytophthora erythroseptica	Pethybridge, 1913	Converse & Schwartze, 1968	Moderately virulent
Phytophthora megasperma var. megasperma	Drechsler, 1931	Duncan et al., 1987	Moderately virulent
Phytophthora bisheria	Abad et al., 2008	No reports	N/A
Phytophthora gonopodyides	Buisman, 1927	Wilcox & Latorre, 2002	High to low virulence

Phytophthora rubi was first described as *Phytophthora fragariae* var. *rubi* as the species shares a very similar isozyme profile to its sister taxa *Phytophthora fragariae* which causes red stele rot in strawberry (Man In ’t veld, 2007). *P. rubi* is widely reported as the primary causal agent of root rot in raspberry and studies report it as highly virulent (Wilcox et al., 1993; Stewart et al., 2014; Graham et al., 2021). Prior to the description of *Phytophthora fragariae* var. *rubi* by Wilcox et al., (1993), isolates were often described as part of a heterogenous collection of subgroups and often assigned *Phytophthora erythroseptica*. The first designation of raspberry isolates to the *Phytophthora fragariae* taxon was by Wilcox (1989), after which raspberry isolates previously designated as *Phytophthora megasperma* and *Phytophthora erythroseptica* were re-classified as *Phytophthora fragariae* var. *rubi* by Wilcox et al., (1993).

Phytophthora citrophthora and *Phytophthora pini* were first described in citrus in 1925 by Leonian (1925). Both species were reported as pathogens of raspberry by Latorre (1993). The study reported the pathogenicity of both species showing comparably high pathogenicity on raspberry cvs Willamette and Heritage. The race of *Phytophthora citricola* found in raspberry is more commonly referred by its synonym *Phytophthora pini* (pers comm, Dr Kelly Ivors, Senior Plant Pathologist, Driscoll's; Waterhouse, 1963)

Phytophthora cryptogea was first described in tomato in 1919 by Pethybridge & Lafferty (1919). More recent studies have noted *P. cryptogea* to be part of a species complex (Safaiefarahani et al., 2016). Mitochondrial and genomic sequencing has shown *P. cryptogea* to be a member of the major *Phytophthora* clade 8a, which is also comprised of *Phytophthora erythroseptica*, and *Phytophthora pseudocryptogea*. *P. cryptogea* was first reported as a pathogen on raspberry in 1982 by Boesewinkel (1982). Washington (1988) investigated the pathogenicity of *P. cryptogea* isolates on twelve raspberry cultivars with resistance noted in cultivars Chilcotin, Nootka, Haida and Puyallup. *P. erythroseptica* was first described in 1913 as the causal agent of pink tuber rot on potatoes (Pethybridge, 1913). *P. erythroseptica* was first reported to be associated with raspberry root rot by Converse & Schwartze in 1968. A further study by Vrain & Pepin (1989) confirmed the pathogenicity of this species on raspberry. *P. pseudocryptogea* was first described as a distinct species causing pink tuber rot in potato in 2015 by Safaiefarahani et al. (2015). Prior to this, *P. pseudocryptogea* was classed as part of a species complex of *P. cryptogea*. To date there has been no reported pathogenicity studies of *P. pseudocryptogea* on raspberry. Furthermore, there are no reports of *P. pseudocryptogea* in the UK.

Pythium litorale (now reclassified as *Phytopythium litorale*) was first isolated from lake water in Germany in 2006 by Nechwatal & Mendgen (2006). There are no pathogenicity reports of this species on raspberry, however, this species showed high virulence on strawberry (Pánek & Střížková, 2021). *Pythium vexans* (now *Phytopythium* vexans) was described in 2014 by Abad et al. (2014). Like *Pp. litorale*, there are no reports of this species on raspberry, however, the species was noted to have moderate pathogenicity on strawberry by Pánek & Střížková (2021).

The aim of this study is to investigate the pathogenicity of *Phytophthora* and *Phytopythium* species isolated from red raspberry plants via detached leaf, root and whole plant virulence analysis. Additionally, whole plant host resistance of five commercial raspberry varieties to *Phytophthora* and *Phytopythium* species will be assessed.

4.2 Materials and Methods

4.2.1 Detached leaf inoculation

A detached leaf assay was modified from Loyd et al. (2014). A panel of *Phytophthora* and *Phytopythium* isolates were chosen for pathogenicity testing (Table 4.2). The *P. pini* and *P. rubi* isolates were obtained from James Townsend of Berry Gardens Growers Ltd. and the James Hutton Institute, respectively. The isolates and a plain agar control were grown on V8 agar (Difco cornmeal agar, 17 g/1 L of deionized water, pH adjusted to 7.0 using 9 mL of 1M KOH) in the dark at 20°C for two weeks. Raspberry leaflets from five varieties (Table 4.3) were collected from the newest, fully expanded leaf. Briefly, the raspberry leaves were surface sterilized in sodium hypochlorite (0.25% available chlorine) for 3 minutes, briefly submerged in 70% (v/v) ethanol and then rinsed three times in sterile distilled water and dried on blotting paper in a laminar air flow cabinet.

A 10% (v/v) sterile soil extract was produced using the method outlined in Abad et al. (2019) wherein 100 mg of soil was added to 1 L of distilled water, left to settle overnight at 4°C and filtered through three layers of cheesecloth. The filtrate was retained and autoclaved at 120°C for 15 minutes and cooled to room temperature prior to use. One hundred mL of the cooled filtrate was added to 900 mL sterile distilled water to make a 10% (v/v) sterile soil extract solution.

Six agar plugs cut from the leading edge of 10-day old *Phytophthora* cultures using a sterilized 7 mm cork borer were submerged into a 500 mL plastic container with 200 mL of the 10% (v/v) sterile soil extract. Raspberry leaves of five raspberry varieties (Table 4.3) sterilised as above were then floated on top (see Figure 4.1). Four replicate leaves per

isolate were used. The containers were sealed and stored at 22°C in an incubator in the dark and observed daily for signs of infection. The leaves were incubated for seven days.

Figure 4.1: Experimental set up of detached leaf float assay showing a sterile raspberry leaflet floating in a 10% soil extract infected with agar plugs of *Peronosporales* isolates

Lesion area and disease percentage for each leaf was calculated using the American Phytopathological Society (APS) Assess 2.0 software (Lamari, 2002). The percentage disease of each leaf was analysed using a one-way and two-way analysis of variance (ANOVA) and Tukey's HSD (honestly significant difference) test using R statistics software. Seven days after inoculation, leaves exhibiting lesions were removed from the soil extract, sterilised, and placed on *Phytophthora*-specific media. Re-isolation from the diseased leaf lesions was performed according to the method outlined in Stewart et al. (2014). Diseased leaves, i.e., those which had significant browning or apparent lesions, were cut into 10 mm^2 sections, soaked in sodium hypochlorite (1.2% available chlorine) for 2 minutes and rinsed in sterile distilled water three times. Two pieces of leaf per sample were carefully submerged in 9 cm Petri dishes containing V8 amended with pimaricin (0.2 mL of a 5 mg/L solution), ampicillin-Na (250 mg/L), rifampicin (0.4 µL of 2.5% aqueous solution), and pentachloronitrobenzene (PCNB 5 mg/L) (V8-PARP). The plates were sealed with Parafilm and incubated in the dark at 18°C until mycelial growth was observed (5-7 days after isolation). The hyphal tips of growing colonies were transferred onto fresh V8-PARP. Cultures were routinely transferred to V8 media to ensure no contaminating fungi were present which could affect pathogenicity and sequencing results.

To determine the identity of cultures, a rapid fungal DNA extraction and PCR amplification was performed using ITS 4 and ITS5 primers (White et al., 1990) and samples were sent for Sanger sequencing using the same method outlined in Chapter 2.

Table 4.2: *Phytophthora* and *Phytopythium* isolates used in pathogenicity assays.

Farm code/Isolate code	Species	Source
5	*Phytopythium litorale*	Aberdeen, Scotland
4	*Phytophthora citrophthora*	Herefordshire, England
ICO45	*Phytophthora pini*	James Townsend, Kent, England
7	*Phytophthora cryptogea*	Herefordshire, England
6	*Phytopythium vexans*	Perth, Scotland
6	*Phytophthora erythroseptica*	Perth, Scotland
4	*Phytophthora pseudocryptogea*	Perth, Scotland
SCRP339	*Phytophthora rubi*	James Hutton Institute, Dundee, Scotland
SCRP1213	*Phytophthora rubi*	James Hutton Institute, Dundee, Scotland

Table 4.3: Raspberry varieties used in pathogenicity assays.

Variety Code	Variety name	Reported resistance
Pilot Trial	Proprietary variety	Reported resistant
1	Tulameen	Susceptible
2	Valentina	Resistant
3	Latham	Resistant
4	Proprietary variety	Unknown
5	Proprietary variety	Unknown

To assess the ability of raspberry *Phytophthora* and *Phytopythium* isolates to infect raspberry tissue, an initial pilot trial of the leaf float assay was conducted using leaves of a proprietary commercial variety. This variety was grown by 90% of the growers surveyed in Chapter 2 of this thesis. Preliminary pathogenicity testing on this single variety was

conducted to assess if the method worked with raspberry. The experiment was repeated three times with four leaves as biological replicates per isolate treatment.

To determine if raspberry cultivar can impact the pathogenicity of the isolates assessed in this study, leaves from five varieties were used in a float assay with the same method outline above. Four leaves per isolate/genotype treatment were used per assay as biological replicates. The experiment was set up according to a randomised complete block design and repeated three times.

4.2.2 Detached root assay

Inoculation was performed according to a modified version of the method outlined in Pathrose et al. (2010). Detached roots from visually healthy (straw-coloured roots with good tissue integrity and no lesions present, strong leaf and cane growth) one year old primocane raspberry plants of varieties 1, 2, 3, 4 and 5 were placed in a sieve and rinsed in running tap water to remove soil/substrate. Roots were chosen based on morphological similarities. The roots were submerged in 70% (v/v) ethanol for one minute and rinsed twice in sterile distilled water. After rinsing, roots were placed on sterile filter papers to dry for 30 seconds. Ten replicate roots per variety and isolate were individually and aseptically transferred onto a sterile filter paper which had been sprayed with 0.5 mL of sterile water into 90 x 15 mm Petri dishes. The cut end of the root was suspended in 500 µL sterile water in a 1.5mL Eppendorf tube sealed with Parafilm™ to maintain hydration throughout the course of the experiment (Figure 4.2). The apical portion of the root was wounded using a sterile inoculation needle and 5 µL of a $1x10^4$ zoospores/mL suspension was pipetted onto the wound. The petri dish was sealed with Parafilm™ to maintain humidity and incubated at 23 °C in the dark for 14 days. Each isolate:variety treatment was replicated three times.

Figure 4.2: Detached root assay experimental set-up. The root was inoculated with zoospores of isolates of *Phytophthora* and *Phytopythium* and incubated for 14 days. The inoculation point is indicated by the red circle.

To re-isolate the oomycetes, the Petri dish was split into four sections (Figure 4.3), each zone represented the spread of the infection via tissue necrosis through the root. Zone 1 included the inoculation point at the apical end of the root, Zone 2 was mid-apical zone of the root, Zone 3 was the mid-basal zone of the root and Zone 4 was the basal end of the root segment i.e., the end growing closest to the plant crown, see Figure 4.3. This zonation was to assess the spread of each isolate i.e., how far it had travelled through the root. The roots were assessed every day for 14 days and necrotic zones were recorded.

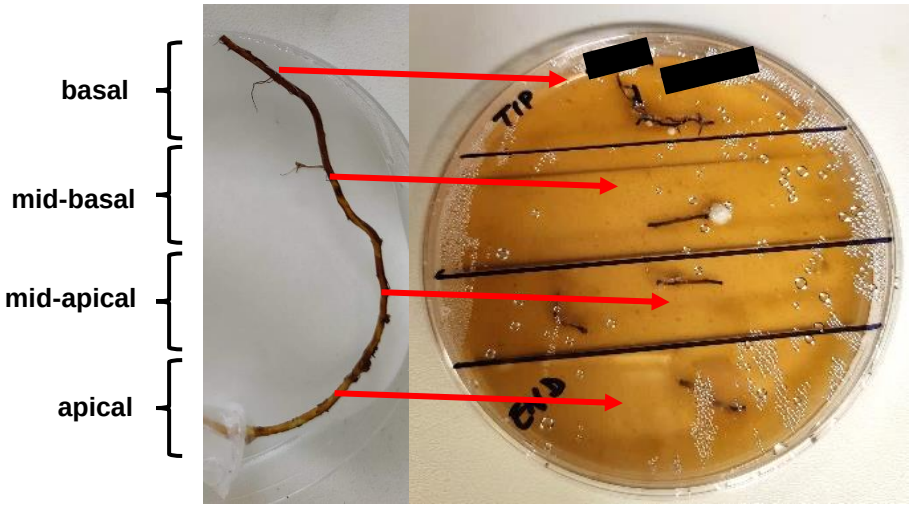


Figure 4.3: Zonation of roots which had been inoculated with the zoospores of isolates of *Phytophthora* and *Phytopythium* isolates and incubated for 14 days. The areas noted on the left; basal, mid-basal, mid-apical and apical indicates where the root was subsectioned for re-isolation.

After 14 days, five root sections per sample were aseptically transferred to 9 cm Petri dishes containing V8-PARP. The diseased root tissue was cut into 10 mm sections, soaked in 70% ethanol for one minute, rinsed twice in distilled water and dried on a sterile filter paper for 30 seconds. Four pieces of root per variety-isolate pairing were carefully submerged in Petri dishes containing V8-PARP. The plates were incubated in the dark at 20°C until mycelial growth was observed. The hyphal tips of growing colonies were transferred onto fresh V8-PARP and their zone of origin was noted. Cultures were transferred to V8 media to ensure no contaminating fungi were present. A rapid DNA extraction, PCR with ITS4 and ITS 5 primers and Sanger sequencing to confirm isolate identity was performed according to the method outlined in Chapter 2.

4.2.3 Whole plant assay

The pathogenicity of four *Phytophthora* isolates; *P. rubi*, *P. pini*, *P. citrophthora* and *P. cryptogea* and two *Phytopythium* isolates; *Pp. vexans and Pp. litorale* were assessed on the five raspberry varieties Tulameen, Valentina, Latham and two proprietary varieties Variety 4 and Variety 5.

Inoculum was prepared by cutting five, 7 mm plugs from the growing edge of the cultures using a cork borer, and aseptically placing them in sterile Petri dishes with the mycelia facing upwards. The dishes were flooded with 20 mL of V8 broth (20 mL V8 juice, 800 mL distilled water, pH adjusted to 7.0 using 9 mL of 1M KOH) which had been sterilised in an autoclave at 120°C for 15 minutes and cooled. The dishes were incubated at room temp (~20°C) in the dark for 4-5 days to produce a mycelial mat. The broth was discarded, and the mycelial mats were washed twice with sterile distilled water to get rid of all presence of broth. The mycelial mats were then flooded with 20 mL sterile soil extract solution and incubated under constant fluorescent light for three days to induce the production of sporangia. After three days, the suspension was aspirated off through a pre-wetted sterile filter paper using a Buchner funnel and vacuum pump and replaced with fresh soil extract. The Petri dishes were chilled at 4°C for 1 h and then transferred into an incubator set at 23°C in constant light for 1 h to release the zoospores. The suspension containing the zoospores was collected by filtering it through folded 113v filter paper into a sterilised conical flask. The concentration of zoospores was determined by taking 500 µL of the zoospore suspension and mixing 500 µL 0.25% (v/v) Victoria blue dye solution. The solution was vortexed for 2 min to remove flagellae. The number of zoospores were counted using a hemacytometer. The concentration of zoospores was adjusted to a final concentration of $1x10^4$ zoospores per mL. A 40 mL aliquot of the zoospore solution was pipetted around the base of each plant. Four non-inoculated plants of each variety treated with 40 mL of sterile distilled water were included as negative controls. The plants were re-inoculated four weeks after the initial inoculation with the same method outlined above.

A total of 160 plants were assessed, four plants per variety:isolate treatment combination. One plant treated with one isolate formed an experimental unit with four replicates. Four un-inoculated plants of each genotype acted as negative controls. Plants were eight months old at the time of inoculation. Plants were potted in 3 litres of a 1:1 sand:compost mix. No fertilizer was added to the compost. Plants were maintained in a 16°C glasshouse with a 12 h day/night cycle. Overhead watering was performed as needed. Plants were arranged in a randomised split-plot design. Foliage of each plant was scored weekly according to disease percentage from 0-100%. The experiment was run for 8 weeks or until all inoculated plants exhibited symptoms of disease. At the end of the experiment,

each root system was scored according to disease percentage from 0-100%. Analysis of variance (ANOVA) of the overall root disease scores was performed in R Studio v1.4.05.5. Re-isolation, rapid DNA extraction, PCR with ITS4 and ITS 5 primers and Sanger sequencing to confirm isolate identity was performed according to the method outlined in Chapter 2.

4.3 Results

4.3.1 Detached Leaf Assay

4.3.1.1 Pilot trial

Infection of detached leaves of the variety used in the pilot trial with isolates of *P. pini*, *P. cryptogea* and *Pp. vexans* resulted in olive green/brown water-soaked lesions which covered the entire surface of the leaf after 7 days incubation at 20°C. Isolates of *P. citrophthora*, *P. erythroseptica* and *Pp. litorale* produced dry brown/black lesions on the leaves (Figure 4.4). Leaves floated in soil extract infected with a *P. rubi* isolate exhibited chlorosis and thinning of the leaves, however no lesions were noted across all replicates.

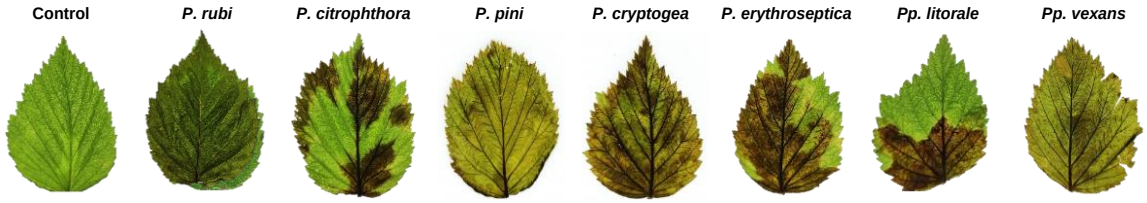


Figure 4.4: Representative leaves from pilot trial of raspberry leaves from a proprietary variety floated in soil extract infected with *Phytophthora* and *Phytopythium* isolates after seven days isolation at 20°C.

The *Phytophthora* species *P. citrophthora*, *P. cryptogea* and *P. erythroseptica* and the *Phytopythium* species *Pp. vexans* exhibited high virulence against the proprietary variety used in the pilot trials, with significantly higher disease percentages on raspberry leaves compared to the control (Figure 4.5).

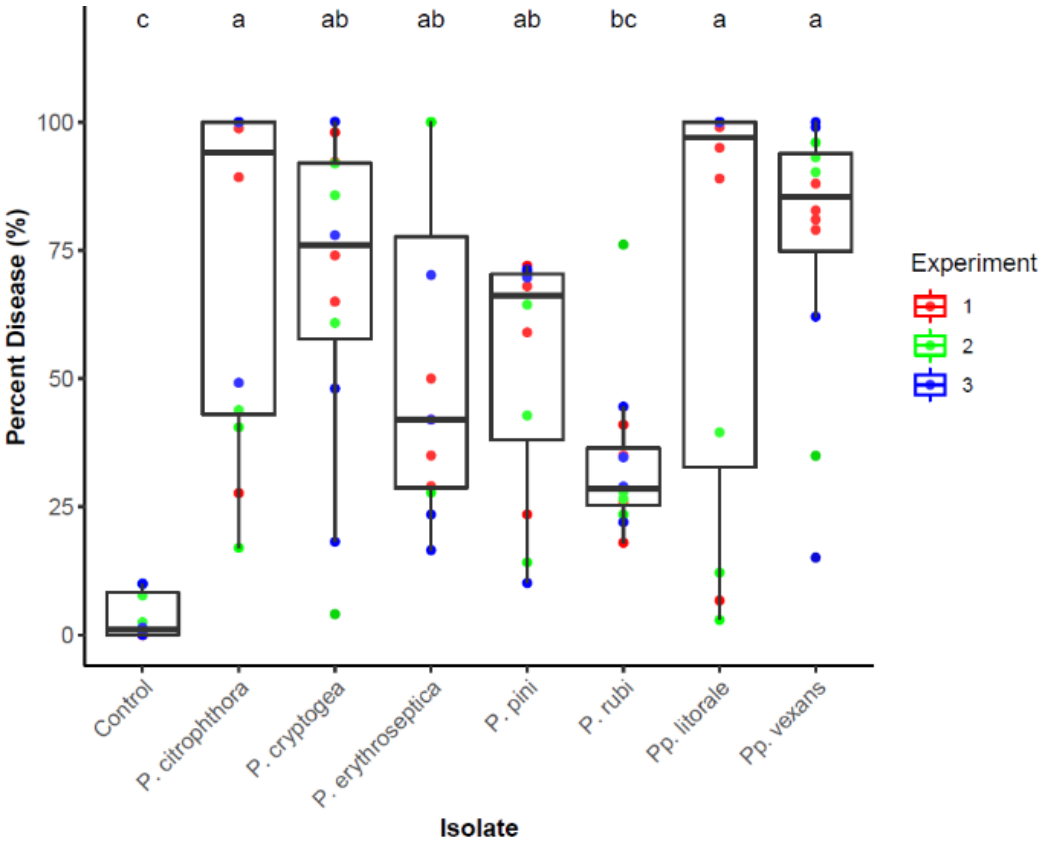


Figure 4.5: All *Phytophthora* and *Phytopythium* isolates were able to cause disease on detached leaves of a proprietary variety. Disease percentage of raspberry leaves floated in soil extract infested with *Phytophthora* and *Phytopythium* isolates over three experimental replicated experiments. ANOVA and Tukey's HSD test were performed in R Studio v1.4.05.5 and statistical differences are given as labelled letters.

4.3.1.2 Genotype resistance trial

The *Phytophthora* species *P. citrophthora*, *P. pini*, and *P. rubi* and the *Phytopythium* species *Pp. litorale* and *Pp. vexans* were chosen for pathogenicity testing on a wider panel of isolates as they displayed reliably pathogenicity across three replicates of the pilot trial.

Visual analysis of raspberry leaves after seven days incubation indicated *P. citrophthora*, *P. pini*, *Pp. litorale* and *Pp. vexans* had the highest lesion areas compared to the control treatment. Leaves of Valentina and Variety 4 floated in soil extract infected with *P. rubi* had large lesion areas, whereas the lesion areas of the other varieties were low, and some leaves had no lesions and appeared healthy (Figure 4.6).

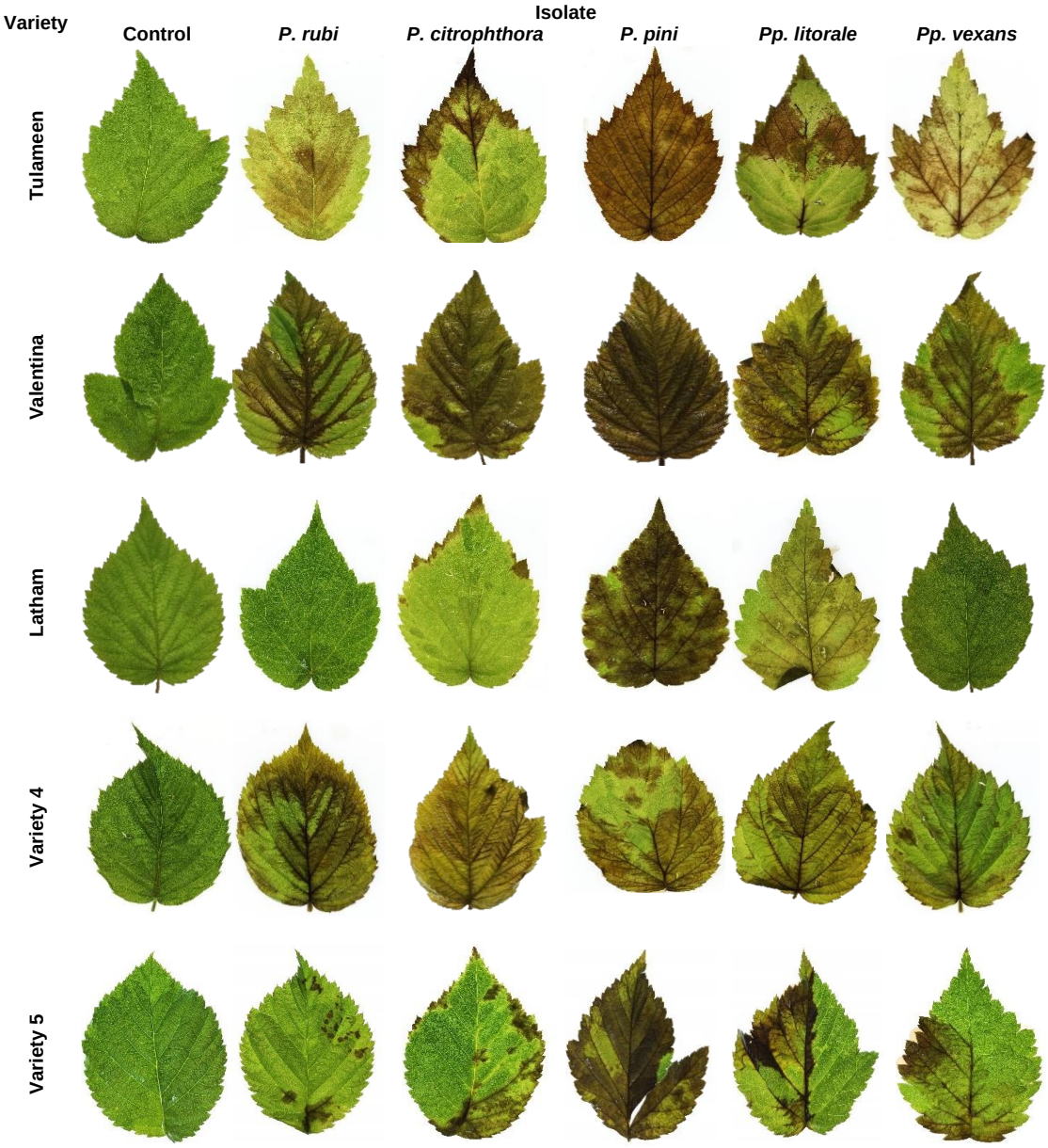


Figure 4.6: Representative raspberry leaves of five varieties floated in soil extract infested with *Phytophthora* and *Phytopythium* isolates after seven days incubation at 20°C.

ANOVA and Tukey's HSD test on the percentage disease on each leaf confirmed this observation on some of the species tested. Leaves infected with *P. citrophthora* and *P. pini* showed significantly higher disease percentage than the control leaves across all varieties (Figure 4.7). *Pp. litorale* showed significantly higher disease percentage than the control leaves in Tulameen, Latham, Variety 4 and Variety 5. No significant difference in the disease percentage of leaves infected with *P. rubi* and *Pp. vexans* compared to the control was observed on all varieties tested (Table A5). An analysis of the effect of plant

variety and isolate revealed no significant effect of the interaction of these two factors on leaf disease percentage (See Table A8 in the Appendix).

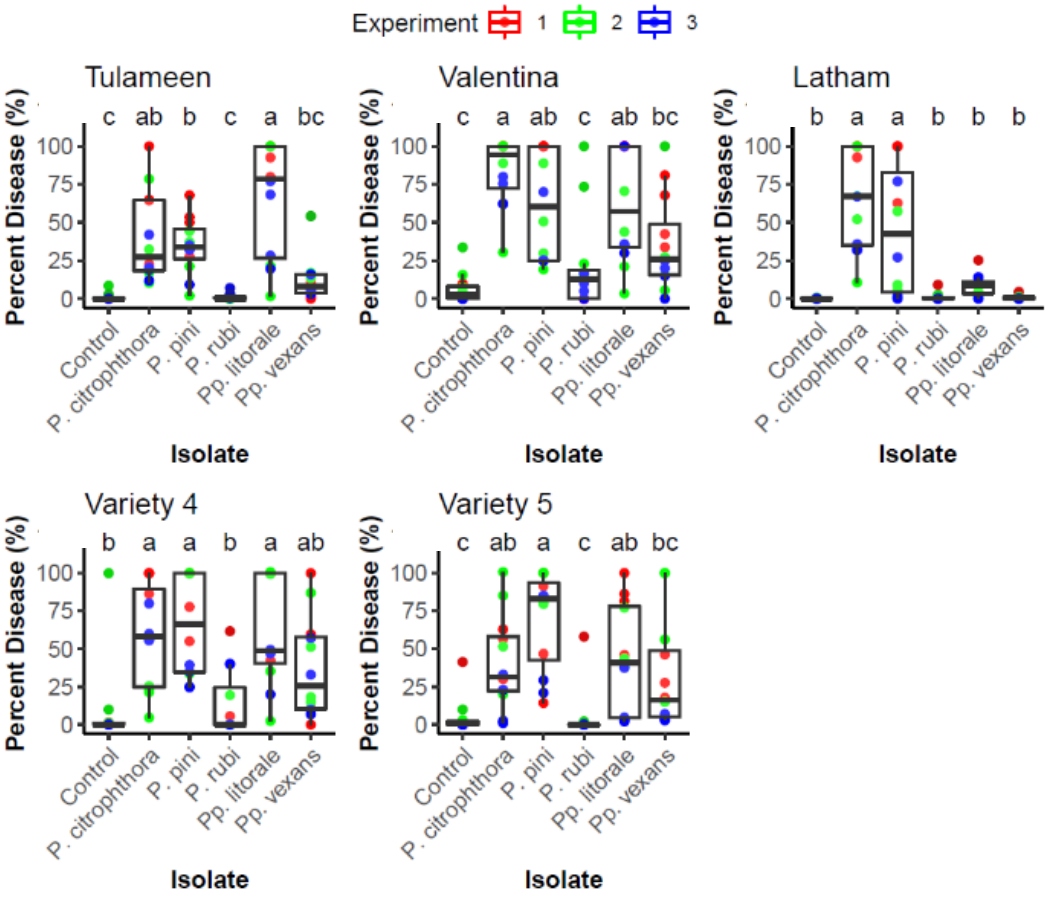


Figure 4.7: Disease percentage of raspberry leaves of five varieties floated in soil extract infested with *Phytophthora* and *Phytopythium* isolates over three experimental replicated experiments. ANOVA and Tukey's HSD test were performed in R Studio v1.4.05.5 and statistical differences are given as labelled letters.

All the isolates tested in the detached leaf assay were successfully reisolated from the disease leaf tissue onto V8-PARP and the resultant isolates' identity was re-confirmed via PCR and Sanger sequencing.

4.3.2 Detached Root Assay

The high humidity in the Petri dish caused some opportunistic root fungi such as *Botrytis cinerea* and yeast to grow on some of the roots during the incubation step, including the control roots. Re-isolation from many of the treatment plates was not possible due to excessive contamination of PARP plates with yeasts and other fungi. The species that were successfully re-isolated and their identities confirmed via PCR and Sanger sequencing are presented in Table 4.4.

Table 4.4: *Phytophthora* and *Phytopythium* species re-isolated from detached raspberry roots.

Isolate	Variety	Area of root
P. citrophthora	Tulameen, Latham	Basal end
P. pini	Tulameen, Latham, Variety 5	Apical end and basal end
Pp. litorale	Tulameen, Variety 5	Apical end and basal end
Pp. vexans	Tulameen, Latham	Apical end and basal end

4.3.3 Whole Plant Assay

The glasshouse housing the plants was heavily infested with two-spotted spider mite, a destructive pest of raspberry leaves, in the sixth week of the trial. All of the above-ground portion of the plants were affected; therefore, the above-ground assessments could not be completed, and the symptoms noted cannot be reliably related to oomycete infection. As two-spotted spider mites do not affect plant roots, the trial was continued for two weeks to allow the oomycetes to infect the roots. After eight weeks, the above ground sections of the plants were removed, and the root balls were assessed and given an overall disease score (Figure 4.8).

The four replicate control plants of Tulameen, Valentina and Latham all showed some disease. No oomycete species were re-isolated from the roots of Latham plants, indicating the root rot disease symptoms noted may come from another pathogen. No statistically significant differences between the overall disease percentage of treated vs control plants were noted (Figure 4.9).

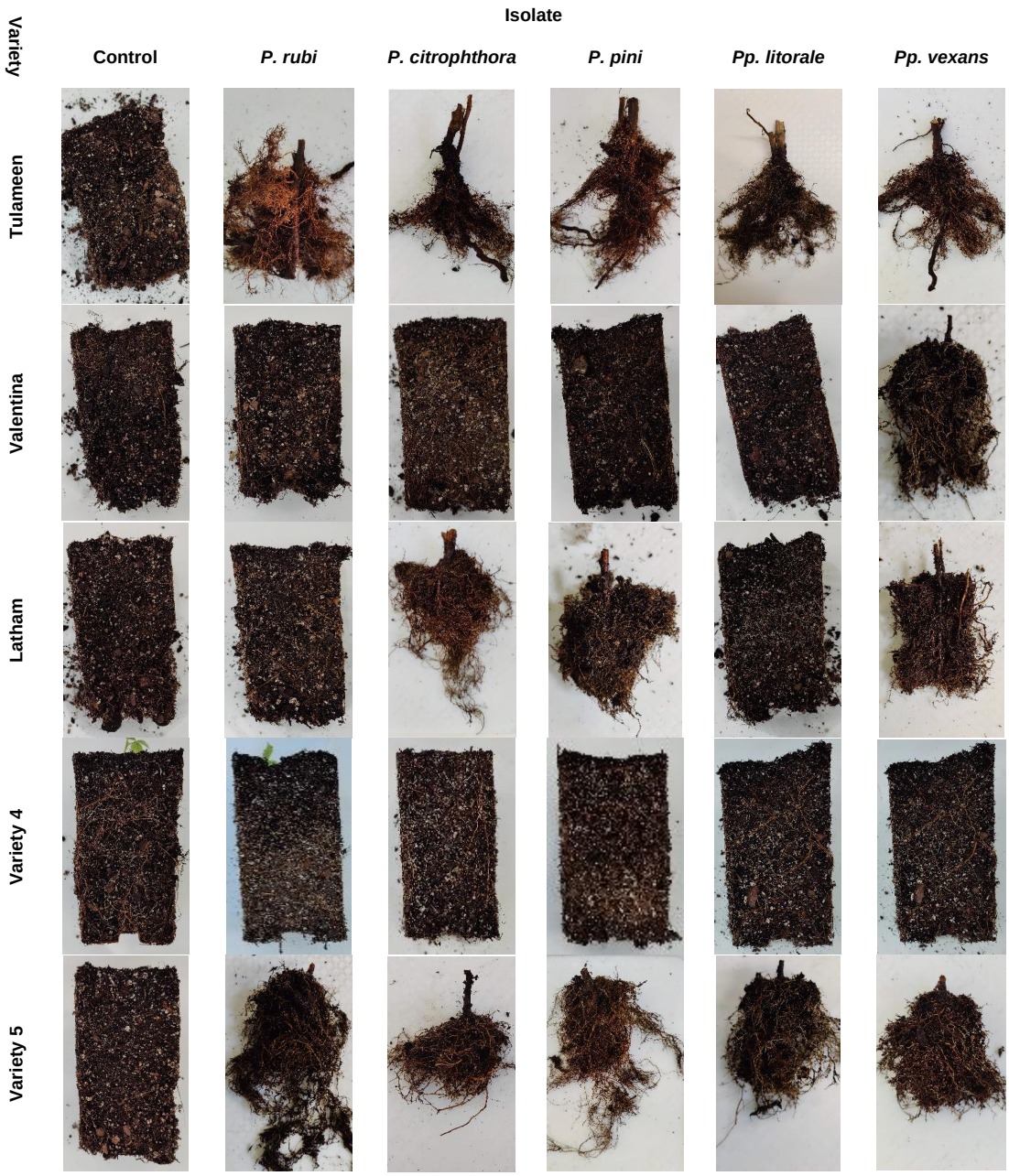


Figure 4.8: The root balls of raspberry plants of five varieties eight weeks post inoculation with zoospores of *Phytophthora* and *Phytopythium*.

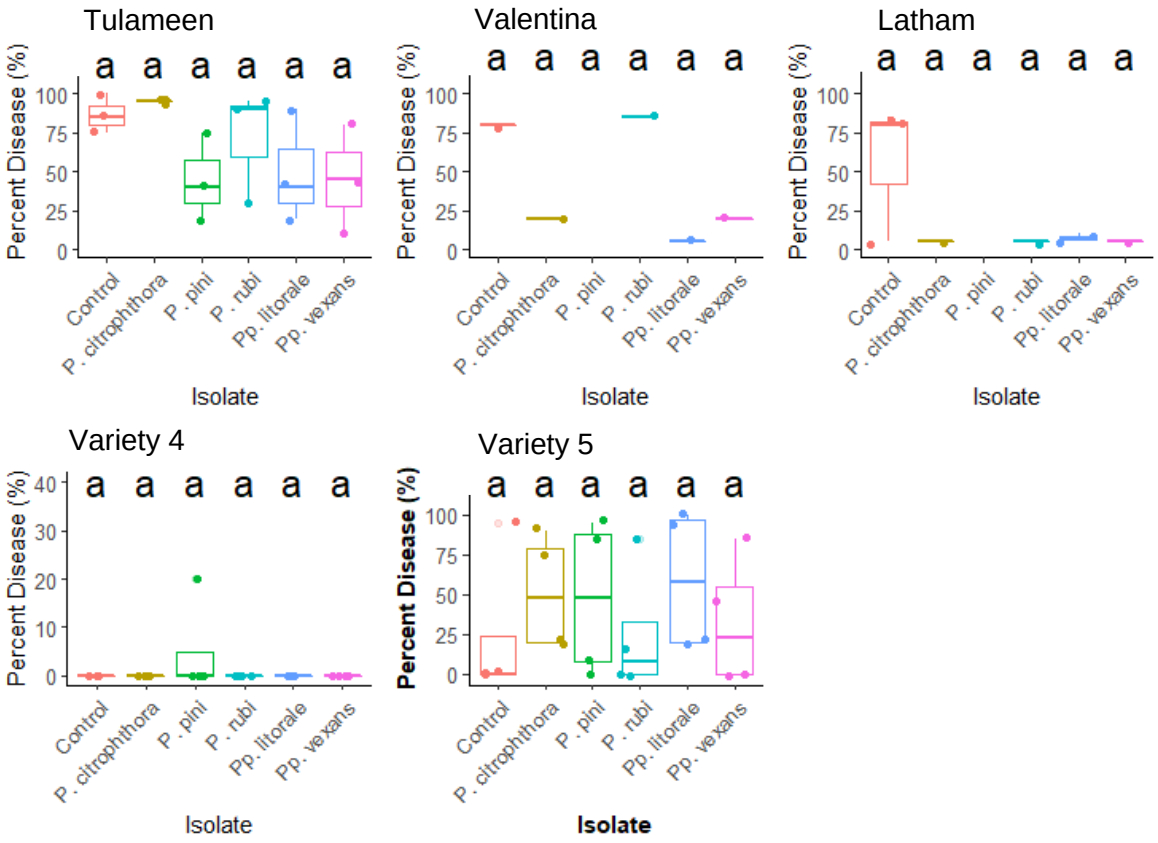


Figure 4.9: Disease percentage of one year old raspberry root balls inoculated with zoospores of *Phytophthora* and *Phytopythium* isolates. ANOVA and Tukey's HSD test were performed in R Studio v1.4.05.5 and no statistical differences were identified (denoted by letter a).

Root and cane dissections of the plants showing root rot symptoms revealed black/brown lesions consistent with *Phytophthora* infection. *Pp. vexans*, *P.pini*, *P. citrophthora*, *Pp. litorale* were successfully re-isolated from these root and cane lesions (Figure 4.10). No oomycetes were isolated from control plants. The ANOVA and Tukey results for this analysis can be found in Table A7 in the Appendix.

Variety	Isolate	Lesions	Number of reps
1	*P. rubi*		1
1	*Pp. vexans*		2
1	*Pp. litorale*		1
1	*P. pini*		1
3	*P. citrophthora*		2
2	*Pp. vexans*		1
5	*P. citrophthora*		1
5	*Pp. vexans*		2

Figure 4.10: *Phytophthora* and *Phytopythium* species re-isolated from inoculated raspberry plants of five varieties. The lesions which were isolated from are shown on longitudinal and lateral sections of the root base and cane. The number of plants from which each species could be re-isolated from out of a total of four replicates per variety are given.

4.4 Discussion

This study adds to the extant knowledge on the pathogenicity of the *Phytophthora* species *P. rubi*, *P. citrophthora*, *P. pini* and *P. cryptogea* on red raspberry. Furthermore, findings are presented on the pathogenicity of the *Phytopythium* species *Pp. litorale* and *Pp. vexans* on raspberry which are yet undescribed on this host species. This study also presents the potential of a detached leaf assay as a reliable method in the preliminary assessment of host resistance to *Phytophthora* and *Phytopythium* and pathogenicity analysis of these species.

Screening bioassays in this study were conducted using detached leaf, detached root, and whole plant pathogenicity assays. Leaf assays provide preliminary information on the etiological relationship between a pathogen and its host. Leaf assays are non-destructive and thus more cost-effective method to screen large breeding populations for host susceptibility and the pathogenicity of numerous isolates, although not a major route of natural infection. The work presented in this study represents a preliminary step in assessing the virulence of *Phytopythium* species in raspberry. Chapter 3 of this thesis presented a much more diverse oomycete community in raspberry than previously thought, indeed, *Phytopythium* may be a much more important genus in the raspberry root rot complex than some *Phytophthora* species.

Koch's postulates were satisfied and thus the pathogenicity of *P. citrophthora*, *P. pini*, *Pp. litorale* and *Pp. vexans* were confirmed on red raspberry using a detached root assay. *P. citrophthora* and *P. pini* appear to be able to travel through the root tissue quicker than the other isolates assessed in this study, however, without a replicated experiment this cannot be confirmed.

Phytophthora species can infect many plant tissues, however, the primary target in raspberry are the root tissues. While leaf assays provide a rapid, cost effective and non-destructive method of pathogenicity and host resistance assessment, investigating the effects of *Phytophthora* and *Phytopythium* on the roots themselves allows for a more well-rounded understanding of the virulence of a putative pathogen. This is typically achieved through whole-plant pathogenicity assays, however, the glasshouse and labour costs associated with full plant trials are high. In this study, a detached root assay is presented as an alternative and complementary method to whole plant assays in determining *Phytophthora* and *Phytopythium* isolate pathogenicity. The detached root technique has only been previously reported for the investigation of *Phytophthora* pathogenicity in avocado roots (Kellam & Coffey, 1985; Zilberstein, 1987; Maas & Kotzé, 1990). This method is not widely used in *Phytophthora* research but presents a potentially more cost-

effective and rapid alternative to the current gold standard of root pathogen pathogenicity testing – the whole plant assay.

The contamination of the root trial with opportunistic fungi and yeasts reduced the reliability of the assay. *Cladosporium*, *Penicillium* and yeast-like growth appeared on the filter paper three days post inoculation and colonised many of the re-isolation plates in two days. These microbes proliferate in damp and humid conditions therefore removing the damp filter paper may mitigate their growth in future experiments. Furthermore, increasing the concentration of PCNB, a fungicide with activity against Ascomycetes and yeasts, in the PARP media may reduce their co-occurrence at the re-isolation stage. The root assay was a valuable method to verify the ability of *Pp. litorale* and *Pp. vexans* to infect and be subsequently isolated from raspberry roots.

The pest infestation in the first month of the whole plant pathogenicity was an unfortunate but all too real element of biological research. The time restraints associated with this project meant it was not possible to repeat this experiment. Although it was not possible to assess the above-ground symptoms associated with *Phytophthora* and *Phytopythium* species infection, the ability of *P. citrophthora*, *P. rubi*, *P. pini* and the *Phytopythium* species *Pp. litorale* and *Pp. vexans* to infect whole raspberry plants was confirmed. Additionally, the resilience of Variety 4, a pre-breeding variety, through a mite infestation and oomycete inoculation is valuable information for the breeding team it came from.

This study identified a significant effect of raspberry cultivar on the pathogenicity of some *Phytophthora* and *Phytopythium* species. Tulameen was reliably susceptible to all species tested in both the detached leaf and the whole plant assay. Latham, with known resistance to *Phytophthora*, showed strong resistance to *Phytopythium* species in the detached leaf assay and low root ball disease percentage in the whole plant assay. This variety is used as a resistant parent in many contemporary breeding programmes and thus this potential resistance to *Phytopythium* infection may be a positive outcome of this work.

As a result of this study, dominant *Phytophthora* pathogens reported in plants with RRR can potentially be included in host resistance and genotype susceptibility studies which at present may focus on *P. rubi* due to its reported dominance in the RRR complex. The focus on *P. rubi*-specific screening may not be as effective with changing patterns in RRR species diversity as reported in this study. The oomycete diversity on UK red raspberry outlined in Chapter 3 of this thesis may ensure more targeted and effective breeding programmes can be maintained.

The potential of *Phytopythium* species to be a major contributor to RRR has yet to be determined, and to our knowledge there have been no reports on the pathogenicity of this

genus on red raspberry. The high pathogenicity associated with *Pp. litorale* is of note. Future studies into the species' pathogenicity should be conducted to further elucidate the potential risk it may pose in the future.

Phytopythium species have previously been reported to be associated with warmer climates, thus the high pathogenicity of *Pp. litorale* observed in this study conducted at 18°C is of note (Jabiri et al., 2022). *Pp. litorale* and *Pp. vexans* have an optimum growth temperature of 30°C and a maximum growth temperature of 35°C (Chenari Bouket et al., 2016; Hosseini et al., 2018; Jabiri et al., 2022). This optimal growth temperature is considerably higher than the optimum temperature of *P. rubi*, at 19-22°C (Graham et al., 2021). The optimum temperature for *P. citrophthora* and *P. pini* are 28 and 25°C, respectively (Ribeiro & Erwin, 1996; Jung & Burgess, 2009). The steadily rising temperatures associated with climate change may lead to more virulent oomycete species which can survive warmer temperatures being even more present in red raspberry in the UK.

These findings highlight the importance of regular surveying and sampling of cultivated raspberry crops as a method of disease management. Additionally, obtaining isolates is critical for pathogenicity testing and assessing the potential risk of new species to UK production. Awareness of the changing patterns of oomycete diversity in raspberry allows researchers to identify changes in life cycles, disease symptoms and pathogenicity of species of interest, which serves to inform growers and the wider scientific community. Due to this studies' limitations with regards the whole plant pathogenicity assay, information concerning the importance of *Phytopythium* as a raspberry pathogen is incomplete, therefore further field testing of these isolates is critical to assess the potential threat of this genus to UK raspberry production.

5 Discussion

As sessile organisms, plants are anchored to their substrates by roots. The root is a complex organ mediating nutrient and gas exchange, longevity and fitness of the plant (De Coninck et al., 2015). Surrounding and infiltrating the roots exists a highly complex community of bacteria, fungi, oomycetes and archaea noted to be one of the richest and most diverse ecosystems on Earth (Pascale et al., 2020). The carefully balanced ecosystem of the rhizosphere; the area of soil surrounding the root directly affected by root exudates, can be easily disrupted by the infiltration of root pathogens such as *Phytophthora*. *Phytophthora* root rot is the most destructive disease of raspberry in the UK, and the majority of previous reports indicate *Phytophthora rubi* as the primary causal agent. This work aimed to assess if colloquial reports of a more diverse *Phytophthora* species composition in raspberry were correct.

To this end, extensive surveying and root sampling on commercial UK raspberry farms was conducted to collect samples in which *Phytophthora* species could be detected through traditional isolation and molecular diagnostics. Furthermore, the effect of plant cultivar, as a widely used cultural management technique, and farm location on the *Phytophthora* community structure was assessed. Together the results of these studies aim to improve our knowledge of raspberry root rot, the species involved in the disease, and factors which can affect its occurrence and severity.

The grower questionnaire responses indicate a complete reliance on resistant phenotypes as a prophylactic measure in root rot control by the growers surveyed in 2020. Additionally, all growers integrated a chemical control method with preventative and curative modes of action against *Phytophthora*. This thesis presents a potential challenge to these management approaches through identifying several species of *Phytophthora* and *Phytopythium* which can infect resistant genotypes. Furthermore, the sensitivity of these species to common fungicides is yet unknown.

Isolation of species from symptomatic root tissue onto *Phytophthora*-specific media revealed the presence of *P. citrophthora*, *P. pini*, *P. cryptogea*, *P. pseudocryptogea* and *P. erythroseptica* in the raspberry samples. The apparent absence of *P. rubi* in isolations was notable, but not unexpected. *P. rubi* is notoriously difficult to isolate from diseased tissue, potentially owing to its slow growth in comparison to the other species isolated. A notable find of this study was the isolation of *Phytopythium* species *Pp. litorale* and *Pp. vexans* from diseased root tissue, a genus which is undescribed both in raspberry and in the UK. The *Phytopythium* species isolated in this study exhibited rapid growth and produced sporangia and zoospores in two to three days in comparison to the *Phytophthora* isolates

which took six to seven days to produce these reproductive structures. The rapid growth and reproduction rate may constitute a major factor in the success of these pathogens. Methods are currently in production to better detect *Phytopythium* in crops, which may prevent its spread through propagation networks and ultimately mitigate its effects on growers. The results outlined in Chapter 4 of this thesis show *Pp. litorale* can be detected using the *Phytophthora* genus-specific LFD. While *Pp. vexans* was not detected using this method, a rapid Loop-Mediated Isothermal Amplification Method (LAMP) has been developed for detection of this species by Wang et al. (2021) which can be used for screening plant material from propagation stock and by plant pathologists surveying for this species.

Molecular analysis of the environmental DNA of the samples by PCR and Sanger sequencing detected further *Phytophthora* species such as *P. plurivora, P. meadii*, *P. hedraiandra* and *P. ilicis. P. rubi* was detected in just one sample, from Farm 1 in Hereford. Sanger sequencing is used by plant pathologists worldwide to test grower samples and breeding populations for the presence of *Phytophthora*. It is a standard method used by governmental plant health clinics around the world which sequences a single barcode in a sample, as such, just one species can be identified per sample. Genus-specific primers, rather than species-specific, were used in this analysis due to the diversity of species isolated from the diseased roots.

While species-specific primers are available for the *Phytophthora* species detected in this work, many plant pathologists may opt to use *P. rubi*-specific primers to test their raspberry crops as this is reported as the main, and at times only, *Phytophthora* species causing root rot in the UK. In contrast with the two other methods of detection, *P. rubi* was identified in 100% of samples through metabarcoding. This work indicates that while *P. rubi* may be present, some isolates do not exhibit high pathogenicity on commercial raspberry varieties. Furthermore, other *Phytophthora* species isolates such as *P. citrophthora* and *P. pini* and isolates of the *Phytopythium litorale* are significantly more virulent than the *P. rubi* isolate used in this study. Variation in isolate pathogenicity is a well-reported concept; some isolates of the same species can exhibit high virulence while others have a low virulence on the same host (Pánek & Střížková, 2021). Bezanger (2021) reported intra-species variation in highly conserved effector genes produced by *P. rubi* isolates through targeted enrichment of pathogen sequences. The study notes the lack of polymorphism and mutation in these genes, suggesting they are critical for isolate pathogenicity (Bezanger, 2021).

Similarities and some stark differences were observed in the pathogen populations detected via isolation and PCR, and those detected through metabarcoding. *Phytophthora*

citrophthora, *P. pini*, *P. cryptogea*, *P. pseudocryptogea* and *Pp. litorale* and *Pp. vexans* were detected across all three methods. *P. erythroseptica* was only detected through direct isolation, and *P. ilicis* and *P. plurivora* were only detected through PCR and Sanger sequencing with *Ypt1* primers. Metabarcoding revealed a much more diverse *Phytophthora* and *Phytopythium* community than predicted. Notably, *P. cactorum* and *P. bishii* were detected in all samples analysed. *P. cactorum* is not reported as a pathogen of raspberry, however, all the farms sampled also grew strawberry, a well-reported host of *P. cactorum* (Pánek et al., 2022). Cross-contamination may have occurred through poorly sanitized irrigation lines or run-off of excess water from strawberry growth tunnels to raspberry tunnels. A slow-growing isolate from raspberry plants in Australia in 1996 was formally designated as *P. bishii* in 2008 by Abad et al. (2008). The species was also detected by Stewart et al. (2014) in Washington in 2014. There are no reports of this species as a pathogen of raspberry, however pathogenicity was confirmed in strawberry (Z. G. Abad et al., 2008). This analysis identified an additional *Phytopythium* species, *Pp. citrinium* in 44% of samples. Both *P. bishii* and *Pp. citrinium* may be potential future targets for pathogenicity and host resistance studies.

Metabarcoding has contributed significantly to our knowledge of the pathogen communities associated with root rot-affected plants, and can be a useful tool in optimizing the soil-borne disease mitigation strategies such as biological amendments through rhizosphere community monitoring (Mazzola et al., 2015). Traditional isolation provides a physical isolate which is critical for virulence analysis and HTS provides an insight into the fascinating ecosystem of the pathosphere and how biotic and abiotic factors can affect its diversity. This work highlights the benefits of combining traditional and rapidly developing novel diagnostic methods such as HTS in plant pathology. This integrated approach to plant pathogen diagnostics was explored by Spada et al. (2022) who detected 22 *Phytophthora* species by isolation and metabarcoding of soil samples from natural and managed ecosystems. Of these 22 species, five were detected solely through baiting, while 12 species were detected by metabarcoding alone (Spada et al., 2022). Thus, these techniques do not compete with, but rather complement each other. Through combining traditional isolation methods with HTS, research into these root rot pathogen populations can provide critical information for growers and researchers, improving our understanding of the threat they pose to UK raspberry production.

The presence of multiple *Phytophthora* species in each sample is of note as this may play a role in the ability to isolate certain species from diseased roots with higher frequency than others. The interactions between *Phytophthora* species are poorly understood. Kozanitas et al., (2017) investigated the factors affecting the co-occurrence of *P. ramorum* with *P. nemorosa* and *P. pseudosyringae* in areas affected by Sudden Oak Death. They

noted that even in sites where *P. nemorosa* is previously dominant, the ability of *P. ramorum* to sporulate in wetter conditions led to its greater competitive ability. The study suggests a competitive exclusion of *P. nemorosa* and *P. pseudosyringae* by *P. ramorum* (Kozanitas et al., 2017). A similar exclusion may occur in root rot-affected raspberry, wherein a species with dominance, e.g., *Phytophthora cactorum* (optimum temperature 25°C) is out-competed by a species which can grow at higher temperatures such as *Phytopythium vexans* (optimum temperature 30°C)(Pánek & Střížková, 2021).

The isolates behaved reliably across the detached leaf and detached root assays, with *P. pini*, *P. citrophthora* and *Pp. litorale* having the highest pathogenicity in all detached tissue assays. The isolate of *P. rubi* used in this work had low virulence across all assays. *Pp. vexans* had high pathogenicity in the preliminary detached leaf assay which used a separate variety not included in the second leaf trail, however, it had low virulence on varieties tested in the second leaf trial.

This indicated these varieties had resistance against *Pp. vexans* and indicates a detached leaf assay is a reliable method of preliminary assessing host resistance of raspberry to Peronosporales species. Tulameen showed the lowest resistance to all isolates across all three assays. Tulameen is a known susceptible to *Phytophthora* and performed predictably. Varieties 4 and 5 are experimental varieties thus their susceptibility to *Phytophthora* is unknown. Variety 4 showed resilience to *P. rubi* in the detached leaf and root assay and all plants of this variety remained healthy during the whole plant trial. Variety 5 showed high sensitivity to all the isolates tested. This work further informs the breeding team on a new method of germplasm screening.

The resistant variety used in this trial was Latham, which is frequently used by breeding programmes to produce new varieties with resistance to *P. rubi* (J. Graham et al., 2011). Latham's parentage was also noted to be the source of resistance in the varieties Prelude, Anne, Caroline, Nova and Josephine to *P. rubi* isolates (Pattison et al., 2004). Latham showed resistance to *P. rubi*, *Pp. vexans* and *Pp. litorale* in the detached leaf assay. No root lesions were noted, and *Phytophthora* and *Phytopythium* were not re-isolated from the roots in the whole plant assays in this study. *P. citrophthora* and *P. pini* were highly virulent the detached leaf and roots of Latham but the zoospores of these species did not cause lesions on the whole plants. As the whole plant assay is the most efficient method of predicting the pathogenicity of an isolate in the field, the results from the detached tissues assays cannot definitively note the threat of these species to red raspberry. As such, the results outlined in this study give preliminary insights into the pathogenicity of two novel raspberry pathogens and the host susceptibility of a panel of commercially relevant raspberry varieties to UK *Phytophthora* isolates.

This work highlights the importance of combining a traditional and molecular approach in investigating the pathogenicity of oomycete species and host resistance. The outputs from this thesis provide a baseline for future research into management systems for raspberry *Phytophthora* and *Phytopythium* species other than *P. rubi*.

Reports of the deleterious effects of oomycete-controlling fungicides on soil, aquatic animals and invertebrates have prompted more investigation into alternative and sustainable agents to effectively control root rot (Teather et al., 2001; Oliveira et al., 2013; Zhang et al., 2020; Fan et al., 2021;). Thus, biocontrols; microbes with either antagonistic or competitive activity towards pathogens, are increasingly being investigated as a potentially sustainable management practice to control *Phytophthora*. Bacteria and fungi have both shown to illicit inhibitory activity on *Phytophthora* present in Rosaceae plants (Norman et al., 1996; Agustí et al., 2011; Iqbal et al., 2021; Trzciński et al., 2021). Just one study, published by Valois et al. (1996), investigates the efficiency of biocontrol agents on raspberry root rot. This report noted the production of enzymes β-1,3-, β-1,4-, and β-1,6-glucanases by fungal actinomycete strains which inhibited *P. rubi* mycelial growth. The enzymes' inhibitory effect was via hydrolysis of the glucans integral for cell wall structure in oomycetes (Valois et al., 1996)**.** Panth et al. (2021) noted the *Bacillus amyloliquefaciens* biofungicide Stargus® reduced the disease severity on ginkgo and maple plants infected with *P. vexans*. At the time of writing there are no reports of *Pp. litorale* sensitivity to fungicides or biofungicide. Testing the sensitivity of *Phytopythium* to the fungicides and biofungicide used in raspberry production in the UK is important due to their ability to grow and reproduce quicker than *Phytophthora* species.

6 Conclusion and suggestions for future work

This work presents an insight into the previously unexplored community of *Peronosporales* species associated with raspberry root rot, and the effect of key factors such as farm location and variety on their diversity and abundance. This analysis presents new potential target species for resistance testing and adds to our knowledge of oomycete pathogens of soft fruit. Furthermore, this work highlights the potential risk of these species to UK raspberry production through pathogenicity testing on commercial raspberry varieties grown by UK producers. The limitations of this work include the use of just one isolate of each species in the pathogenicity assessments as just one isolate of *P. pini* and *Pp. vexans* were available. A larger panel of multiple isolates of each species may aid in addressing the intra-species pathogenicity variation observed in previous studies of *Phytophthora* and *Phytopythium* isolates. Due to time and financial limitations of the

project, it was only possible to sample the farms in one season (Autumn), however sampling the plants on the grower sites prior to planting and exposure to the potential oomycete community in the planting substrate/irrigation system of the grower may shed some light on the source of these diverse *Phytophthora* species. Future study is needed in order to gain a more comprehensive understanding of the raspberry root rot complex:

- Re-sampling the farms in multiple times of year to assess if season affects the raspberry *Peronosporales* species composition.
- Field pathogenicity trials of *P. citrophthora*, *P. pini*, *P. cryptogea*, *Pp. litorale* and *Pp. vexans*
- Assessing the effect of biocontrols on *P. citrophthora*, *P. pini*, *P. cryptogea*, *Pp. litorale* and *Pp. vexans*
- Fungicide and biofungicide sensitivity testing of all species testing in this study.
- Testing the pathogenicity of *P. bishii* and *Pp. citrinium* on UK plants to assess their risk to UK raspberry production.

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Appendix

All raw data is stored in NIAB East Malling EMQA system within the Genetics, Genomics and Breeding files. The Project code is 300070.

Data is filed in: Y:\Nellist\Projects\PhD Eithne Browne C-300070\Science

Figure A1 - raspberry root rot questionnaire distributed to UK growers to assess their fruit production, mitigation strategies and incidence of root rot. The survey was distributed in September 2020.

Respondent's name	
Farm	
No. of hectares of raspberry production	
Contact number	
GPS Reading	
Date	

Raspberry *Phytophthora* Survey 2021

1. Which raspberry varieties do you have in production?
2. What is the cropping history of the ground you have raspberry plants in?
3. Are your plants grown in pots or in the ground?
    i. For pots – what growing media do you use?
    ii. For soil – what is the soil type you grow in?
4. Where do you source your raspberry plants i.e. which propagator/co-operative?
5. How long do you keep each plant in production for; do you buy in floricanes/primocanes?
6. What incidence of root rot have you observed on your farm in 2019 (%)? (Please circle)

0% 5% 10% 15% 25% 50% 75% 100%

Fig. 1: *Phytophthora* symptoms on roots

7. Have you noticed root rot symptoms increasing with crop age?
8. When in the season do you see most root rot incidence?
9. What control methods are you currently using (please tick relevant boxes)?

Paraat/ if other fungicide, please note product name	Biocontrol (if possible, please note product name)	Growing resistant cultivar	Gypsum application	Other

Fig. 2*: Phytophthora* symptoms above-ground

10. What other root rot control methods have you used in the past?
11. How would you rate raspberry root rot impact on your business? (Please circle which are relevant)

Large impact Moderate Impact Negligible Impact No Impact

12. Any other comments:

Table A1: Summary of the 41 operational taxonomic units (OTUs) generated using a 97% sequence similarity. OTUs sequences were compared to the GenBank nt database using the BLASTN+ for species identification. Species in bold are those with the highest reads per OTU.

Species	Similarity (%)	Clade (*Phytophthora* only)	Number of reads/OTU	% of total reads	Fragment size (bp)
Globisporangium intermedium	99.56	–	214	0.0	225
Globisporangium perplexum	100	–	84	0.0	220
Globisporangium rostratum	100	–	1085	0.0	295
Globisporangium ultimum	100	–	176	0.0	217
Hyaloperonospora parasitica	99.54	–	3195	0.1	219
Phytophthora bishii	**100**	**Subclade 2d**	**293,780**	**4.3**	**221**
Phytophthora cactorum	**99.54**	**Subclade 1a**	**775,951**	**19.9**	**216**
Phytophthora cactorum	**100**	**Subclade 1a**	**441,299**	**5.8**	**216**
Phytophthora citricola/pini	**100**	**Subclade 2c**	**2,888,842**	**10.2**	**182**
Phytophthora. citrophthora	**100**	**Subclade 2a**	**510,950**	**28.8**	**195**
Phytophthora. citrophthora	**100**	**Subclade 2a**	**59,089**	**3.3**	**193**
Phytophthora cryptogea	**100**	**Subclade 8a**	**14,657**	**0.3**	**205**
Uncultured Phytophthora clone sp1	95.58	Clade 1	323	0.0	212
Phytophthora crassamura	100	Subclade 6b	6,140	0.1	225
Phytophthora pseudocryptogea	100	Subclade 8a	13,540	0.2	206
Phytophthora pseudocryptogea	100	Subclade 8a	5,603	0.1	206
Phytophthora rubi	**100**	**Subclade 7**	**1,389,673**	**32.0**	**230**
Peronospora sparsa	**100**	**–**	**1,235,657**	**28.9**	**214**
Peronospora medicaginis-minimae	100	–	48,296	0.9	219
Peronospora cf. fagopyri	100	–	22	0.0	218
Phytopythium citrinium	97.6	–	13,003	0.2	250
Phytopythium citrinium	98.4	–	11,054	0.2	251
Phytopythium citrinium	98.8	–	486	0.0	249
Phytopythium citrinium	98.8	–	263	0.0	249
Phytopythium citrinium	**98.4**	**–**	**61,856**	**1.8**	**250**
Phytopythium citrinium	98.4	–	32,233	0.6	250
Phytopythium citrinium	100	–	65	0.0	249
Phytopythium citrinium	100	–	127	0.0	251
Phytopythium citrinium	100	–	85	0.0	251

Phytopythium citrinium	100	–	29	0.0	251
Phytopythium citrinium	100	–	30	0.0	251
Phytopythium litorale	**100**	**–**	**166260**	**12.9**	**235**
Phytopythium litorale	100	–	2626	0.0	235
Phytopythium vexans	100	–	30364	0.6	237
Phytopythium vexans	99.16	–	3211	0.1	237
Phytopythium vexans	99.58	–	1250	0.0	237
Phytopythium vexans	100	–	202	0.0	237
Pseudoperonospora urticae	100	–	487	0.0	216
Pythium heterothallicum	97.56	–	8911	0.2	205
Pythium sylvaticum	100	–	363	0.0	288
Pythium anandrum	100	–	523	0.0	162

Table A2: Percentage of total reads of the *Peronospora*, *Phytophthora* and *Phytopythium* species present on nine commercial raspberry farms in 2021.

Farm Number	***Peronospora***		***Phytophthora***		***Phytopythium***	
	% reads	# reads	% reads	# reads	% reads	# reads
III	69	88,636	31	7,185	0	0
IV	10	9,476	90	954,594	0	12
1	57	906,647	43	134,985	1	1,354
2	66	240,126	34	58,567	0	267
3	42	93,140	48	107,477	10	21,982
4	11	128,375	79	2,133,563	10	167,350
5	6	36,521	71	40,342	23	385,389
6	15	82,555	83	67,672	5	22,830
7	12	9,056	72	388,789	15	304,382
8	30	8,424	70	103,11	0	1
9	10	21,647	90	746,634	0	1

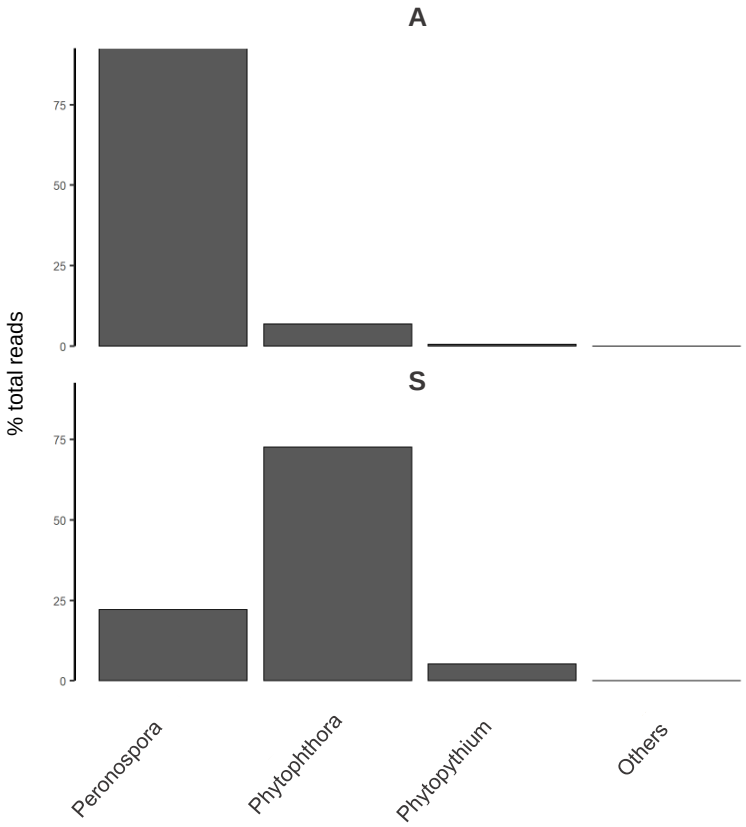

Figure A1: Percentage of total reads of *Peronospora*, *Phytophthora* and *Phytopythium* present in raspberry plants asymptomatic (**A**) and symptomatic (**S**) of root rot.

Table A3: Read distribution of the *Phytophthora* species in samples from the 11 farms sequenced. Number of reads (#) per OTU and percentage of total farm reads (%) are displayed.

Farm	# of samples	*P. rubi*		*P. cactorum*		*P. citrophthora*		*P. pini*		*P. bishii*		*P. cryptogea*		*P. pseudocryptogea*		*P. crassamura*		*Phytophthora clone sp1*	
		#	%	#	%	#	%	#	%	#	%	#	%	#	%	#	%	#	%
III	2	1,428	0.6	2,308	0.0	81	0.0	0	0.0	485	0.2	0	0.0	2	0.0	0	0.0	0	0.0
IV	4	211,565	50.5	7,258	0.0	3	0.0	0	0.0	9,989	2.3	1	0.0	0	0.0	0	0.0	0	0.0
1	10	56,961	4.3	5,073	0.0	12,502	0.9	3,877	0.3	17,072	1.3	425	0.0	419	0.0	5,740	0.4	286	0.0
2	4	2,762	1.0	1,674	1.0	6,976	2.5	2,516	0.9	21,586	7.7	325	0.1	1,889	0.7	183	0.1	19	0.0
3	1	50	0.1	1,566	2.8	5,184	5.3	4,523	4.6	14,130	14.4	397	0.4	331	0.3	0	0.0	0	0.0
4	9	20,062	1.4	8,384	3.8	434,639	30.7	267,757	18.9	58,555	4.1	10,700	0.8	8,896	0.6	2	0.0	0	0.0
5	7	28,820	4.6	16,836	23.6	41,808	6.7	2,796	0.4	2,602	0.4	41	0.0	85	0.0	213	0.0	18	0.0
6	13	1,056,022	61.8	162,086	1.1	9,728	0.6	7,373	0.4	12,205	0.7	1,162	0.1	417	0.0	2	0.0	0	0.0
7	5	6,093	1.1	200,837	8.5	3	0.0	0	0.0	6,979	1.2	1,606	0.3	1,501	0.3	0	0.0	0	0.0
8	2	5,632	2.9	2,500	0.0	25	0.0	0	0.0	515	0.3	0	0.0	0	0.0	0	0.0	0	0.0
9	4	278	0.1	338,878	0.1	1	0.0	0	0.0	46524	9.9	0	0.0	0	0.0	0	0.0	0	0.0

Table A4: Read distribution of the *Phytopythium* species in samples from the 11 farms sequenced. Number of reads (#) per OTU and percentage of total farm reads (%) are displayed.

Farm	# of samples	*Pp. litorale*		*Pp. vexans*		*Pp. citrinium*	
		#	%	#	%	#	%
III	2	0	0.0	0	0.0	0	0.0
IV	4	2	0.0	0	0.0	0	0.0
1	9	906	0.1	773	0.1	2,363	0.2
2	4	79	0.0	0	0.0	181	0.1
3	1	3	0.0	3	0.0	1,239	1.3
4	9	1,996	0.1	7	0.0	50,284	3.6
5	7	33,331	5.3	188	0.0	268	0.0
6	12	36,203	2.1	29,393	1.7	7,521	0.4
7	5	93,736	16.7	0	0.0	0	0.0
8	2	2	0.0	0	0.0	0	0.0
9	4	2	0.0	0	0.0	0	0.0

Table A5: ANOVA and Tukey's HSD analysis of the disease scores of raspberry leaves floated in soil extract infected with *Phytophthora* and *Phytopythium* isolates and incubated at 20°C for seven days. Values in bold indicate significant *p*-values <0.05.

Group1	Group2	*p*-value
Control	*P. citrophthora*	**0.0439**
Control	*P. cryptogea*	**0.0432**
Control	*P. erythroseptica*	**0.0236**
Control	*P. pini*	0.513
Control	*P. rubi*	0.387
Control	*Pp. litorale*	0.145
Control	*Pp. vexans*	**0.016**
P. citrophthora	*P. cryptogea*	1
P. citrophthora	*P. erythroseptica*	1
P. citrophthora	*P. pini*	0.945
P. citrophthora	*P. rubi*	1
P. citrophthora	*Pp. litorale*	1
P. citrophthora	*Pp. vexans*	1
P. cryptogea	*P. erythroseptica*	1
P. cryptogea	*P. pini*	0.944
P. cryptogea	*P. rubi*	1
P. cryptogea	*Pp. litorale*	1
P. cryptogea	*Pp. vexans*	1
P. erythroseptica	*P. pini*	0.842
P. erythroseptica	*P. rubi*	0.999
P. erythroseptica	*Pp. litorale*	0.996
P. erythroseptica	*Pp. vexans*	1
P. pini	*P. rubi*	0.999
P. pini	*Pp. litorale*	0.996
P. pini	*Pp. vexans*	0.772
P. rubi	*Pp. litorale*	1
P. rubi	*Pp. vexans*	0.996
Pp. litorale	*Pp. vexans*	0.989

Table A6: ANOVA and Tukey's HSD analysis of the disease scores of raspberry leaves of five commercial varieties floated in soil extract infected with *Phytophthora* and *Phytopythium* isolates incubated at 20°C for seven days. Values in bold indicate significant *p*-values <0.05.

Group1	Group2	*p*-value				
		Variety 1	**Variety 2**	**Variety 3**	**Variety 4**	**Variety 5**
Control	*P. citrophthora*	**0.0393**	**2.78 x10^{-07}**	**7.11x10^{-09}**	**0.00316**	**0.000791**
Control	*P. pini*	**1.15 x10^{-05}**	**0.000323**	**6.51 x10^{-05}**	**0.000586**	**0.00656**
Control	*P. rubi*	1	0.792	1	0.999	1
Control	*Pp. litorale*	**0.0204**	**0.000277**	0.942	**0.0016**	**1.68 x10^{-08}**
Control	*Pp. vexans*	0.21	0.146	1	0.263	0.628
P. citrophthora	*P. pini*	0.162	0.447	0.208	0.996	0.987
P. citrophthora	*P. rubi*	**0.0394**	**4.21 x10^{-05}**	**1.18 x10^{-08}**	**0.00945**	**0.000791**
P. citrophthora	*Pp. litorale*	1	0.474	**2.94 x10^{-07}**	1	**0.072**
P. citrophthora	*Pp. vexans*	0.977	**0.00281**	**9.53 x10^{-09}**	0.531	**0.0808**
P. pini	*P. rubi*	**1.15 x10^{-05}**	**0.0202**	**0.000103**	**0.00193**	**0.00656**
P. pini	*Pp. litorale*	0.258	1	**0.00165**	1	**0.0126**
P. pini	*Pp. vexans*	**0.0279**	0.311	**8.47 x10^{-05}**	0.247	0.304
P. rubi	*Pp. litorale*	**0.0205**	**0.0179**	0.968	**0.005**	**1.68 x10^{-08}**
P. rubi	*Pp. vexans*	0.21	0.839	1	0.458	0.628
Pp. litorale	*Pp. vexans*	0.926	0.289	0.958	0.402	**8.63 x10^{-06}**

Table A7: ANOVA and Tukey's HSD analysis of the disease scores of raspberry plants of five commercial varieties infected with zoospores *Phytophthora* and *Phytopythium* isolates 8 weeks post inoculation. Values in bold indicate significant *p*-values <0.05.

Group1	**Group2**	***p*-value**				
		Variety 1	**Variety 2**	**Variety 3**	**Variety 4**	**Variety 5**
Pp. litorale	*P. rubi*	0.53	0.819	1	1	0.975
Pp. litorale	Control	0.53	0.853	0.876	0.0528	0.969
Pp. litorale	*P. pini*	0.53	1	0.953	1	0.999
Pp. litorale	*P. citrophthora*	0.53	1	1	1	1
Pp. litorale	*Pp. vexans*	0.53	1	0.86	1	0.996
P. rubi	Control	1	1	0.943	0.0632	1
P. rubi	*P. pini*	1	0.853	0.975	1	0.876
P. rubi	*P. citrophthora*	1	0.932	0.969	1	0.953
P. rubi	*Pp. vexans*	1	0.932	0.999	1	1
Control	*P. pini*	1	0.883	1	0.0901	0.86
Control	*P. citrophthora*	1	0.95	0.996	0.0632	0.943
Control	*Pp. vexans*	1	0.95	1	0.0632	1
P. pini	*P. citrophthora*	1	1	1	1	1
P. pini	*Pp. vexans*	1	1	1	1	0.953
P. citrophthora	*Pp. vexans*	1	0.1	1	1	0.989

Table A8: Two-way analysis of variance (ANOVA) of the effect of plant variety and isolate and their interaction on the disease percentage of detached raspberry leaves floated in soil extract infected with *Phytophthora* and *Phytopythium* isolates incubated at 20°C for seven days. Values in bold indicate significant *p*-values <0.05.

	Df	Sum Sq	Mean Sq	F-value	P-value
Isolate	6	183209	30535	33.207	**$<2 \times 10^{-16}$**
Variety	4	88271	22068	23.999	**$<2 \times 10^{-16}$**
Isolate*Variety	19	18732	986	1.072	0.378
Residuals	354	325515	920		