Identifying Oomycetes in a Public Garden

By

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Abstract

Oomycetes contain some of the most destructive plant pathogens, namely species from within the genera of Phytophthora and Pythium. *Phytophthora ramorum* is known to cause the mortality of a wide range of tree and woody shrub species, whereas Pythiums although more ubiquitous than Phytophthoras and commonly found in the same soil and waterbodies, are typically known to affect young plants and herbaceous species. The Royal Horticultural Society (RHS) is the UK's largest gardening charity, with five unique gardens in locations throughout England that house important tree and shrub, specimen plants as well as national collections. This research was undertaken in collaboration with the RHS after outbreaks of *Phytophthora ramorum* had been described at the RHS Rosemoor garden in Devon. The aim of this study was to identify Phytophthora and Pythium species within the garden. Twenty sites within the garden were included this study; soil samples were taken from around nineteen *P.ramorum* susceptible plant species and a sample of water was taken from the garden lake. The samples were then analysed to determine the presence of Phytophthora and Pythium species. Cultures were isolated from the soil samples using the apple baiting methods and genus identification was determined using morphological analysis of sporangia. Molecular identification of the samples to species level was confirmed by sequencing the internal transcribed spacer (ITS) of ribosomal DNA. A mixture of Phytophthora and Pythium species was found in seven of the soil samples. *Phytophthora gonapodyides* was identified in the water sample and the following Pythium species were confirmed soil samples taken from across the garden: *P.attrantheridium, P.intermedium, P.macrosporum, P.senticosum, and P.sylvaticum*. No correlation was found between plant species and pathogen species or pathogen species and garden location. It is interesting to know no Phytophthora species were identified in the soil samples analysed in this study, even though an outbreak of *P.ramorum* had been reported prior to sampling. *Pythium senticosum* was associated with a Cornus cultivar displaying disease symptoms. It is not known if this oomycete was the cause of these symptoms. To our knowledge, this is the first time *P.senticosum* has been reported in the UK. Further work is required to examine the pathogenicity of this species and potentially, this could be a novel pathogen in the UK.

Keywords

Chlamydospores, heterothallism, oomycete, oospores, Phytophthora, Pythium, zoospores.

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Contents

CHAPTER 1: INTRODUCTION

1.1 Soil-borne diseases

Soil-borne diseases refer to plant pathogens in the soil, namely mycelium, hyphae, sporangia, and spores. These pathogens infect aerial plant parts by various means, such as wind dispersal and rain splashes. However, infection occurs more often via the roots. Exudates released into the soil from the roots of a susceptible host plant elicit a recognition response from a virulent pathogen; as the plant cannot mount a successful defence response, the pathogen can initiate infection (Rezzonico et al., 2017). Some pathogens have a broad host range, infecting species across multiple genera within various plant families; for example, *Phytophthora ramorum* has over 170 known plant host species (EPPO, 2020) and causes a variety of symptoms, including foliar blight, bleeding cankers, wilt and dieback on a wide range of woodland and ornamental species (Denman et al., 2009). In contrast, others have a narrow host range, affecting only a limited number of species and in some cases, restricted to a single species, such as *Phytophthora lateralis,* whose known host range comprises of just *Chamaecyparis lawsoniana* and *Taxus brevifolia* (T. Jung et al., 2018).

For a disease to occur, environmental conditions must be favourable for both the pathogen and the host plant. The inoculum density in the soil will impact the amount of disease; low density will cause less disease, whilst high density will cause more disease (Martin and Loper, 1999). There are factors within the soil that can impact the development of disease. This includes other microorganisms competing with the pathogen for space, nutrients and temperature, soil pH, and water (Veena et al. 2014). Historically, diseases caused by soil-borne pathogens seldom occurred in natural environments (Shearer, 2000). The agricultural sector has faced a more significant risk due to the monoculture of host species, which led to increased populations of virulent pathogens and reduced competition from other soil organisms (Schippers et al., 1987). In recent years, the movement of plant material throughout the globe has grown increasingly common, facilitating the introduction of new plant pathogens responsible for spreading new diseases in natural environments, farms, nurseries and gardens worldwide (Bandyopadhyay & Frederiksen, 1999).

Soil-borne diseases result from diverse organisms, including bacteria, viruses, fungi, and oomycetes. The latter represents a group of plant pathogens that pose significant threats to natural ecosystems, agriculture, horticulture, and forestry (Thines, 2018).

1.2 Oomycetes

Oomycetes are water moulds that frequent cool, damp environments. In addition to damp soils and water bodies, they are commonly found in runoff and irrigation water (C. Brasier, 1967; Redekar & Parke, 2018; Yang & Hong, 2016). Oomycetes are a phylum of organisms that contains numerous pathogenic species that cause disease in humans, animals, plants, and fungi. They are a complex group of organisms that has prompted considerable taxonomic debate. *Pythium* and *Phytophthora* are two of the most important groups of plant pathogens, and recent findings based on the morphological distinction between reproduction structures have led to their reclassification. During a recent *Phytophthora* diversity survey, six novel species were isolated and assigned to a novel sister group of *Phytophthora* under a new genus called *Nothophytophtora*. (T. Jung et al., 2017). Furthermore, species formerly classified as *Pythium* have now been divided into Pythium and a further four novel genera: *Pythopythium, Globusporangium, Elongiosporangium, and Pilasporangium* (Ho, 2018). For the purpose of this study, pathogens within these groups will be referred to as *Pythium.*

Oomycetes are frequently considered alongside fungal pathogens owing to various shared characteristics, including occupying similar ecological niches and producing hyphae and spores (Fry and Grunwald 2010). However, these organisms are distinguished by several characteristics. Most notably, oomycetes produce motile zoospores with two flagella (Thines, 2018), can be diploid or polyploidy, have non-septate hyphae with uniformly dispersed multiple nuclei, and contain cellulose in their cell walls. True fungi may be haploid or dikaryotic, possessing septate hyphae and chitin cell walls (Hardham 2007; Hayden et al. 2013).

The oomycetes encompass diverse species responsible for soil-borne and aerial diseases in plants. The dissemination of oomycete propagules is facilitated by various mechanisms, including wind, rain, splashing, and animal movement. The actions carried out by humans can inadvertently increase the dissemination of propagules, leading to the introduction of novel species (Hayden et al., 2013). *Pythium* and

Phytophthora are two of the most important genera of oomycetes, responsible for some of the most destructive plant diseases (Green et al., 2021).

1.3 Pythium

The genus Pythium belongs to the Pythiaceae family within the Pythiales order (H Ho, 2018). There are more than 130 described Pythium species, some of which cause human, animal and plant diseases (Dubey et al., 2020). *Pythiums* typically have a propensity for affecting young plants and seeds; they are cause for concern in the agriculture sectors as they are known to affect grain crops, notably *Zea mays,* with annual losses in maise production in the USA totalling $25 million (Bickel & Koehler, 2021). Damping-off (a disease affecting germinating seeds and young seedlings) is the most significant disease caused by *Pythium* species. It is responsible for the mortality of seeds and seedlings, resulting in reduced yields and economic losses in the agriculture and horticulture sectors (Bickel & Koehler, 2021; Dubey et al., 2020; Lamichhane et al., 2017). In pre-emergence damping-off, germination is inhibited as infection causes the seeds to decay. If the hypocotyl and epicotyl regions are affected, they are rapidly engulfed by rot and die (Figure 1.1). In post-emergence damping-off, symptoms include water-soaked roots and lesions and lesions on the basal shoots, which ultimately result in the complete collapse of the seedling (Figure 1.1) (Lamichhane et al., 2017). Damping-off disease spreads quickly and is common in nurseries. The warm, humid growing conditions required for developing young plants in nurseries support the germination temperature (10-30°C) and damp environment required for Pythium species responsible for damping-off disease. (Lamichhane et al., 2017). *Pythium debaryanum, P. ultimum, P. aphanidermatum,* and *P. graminicola* are the most frequently associated species of damping-off disease (Veena et al., 2014).

The damping-off lifecycle commences with the production of a rapidly growing white mycelium that gives rise to sporangia. (Figure 1.1). Pythium has both an asexual and a sexual reproduction stage.

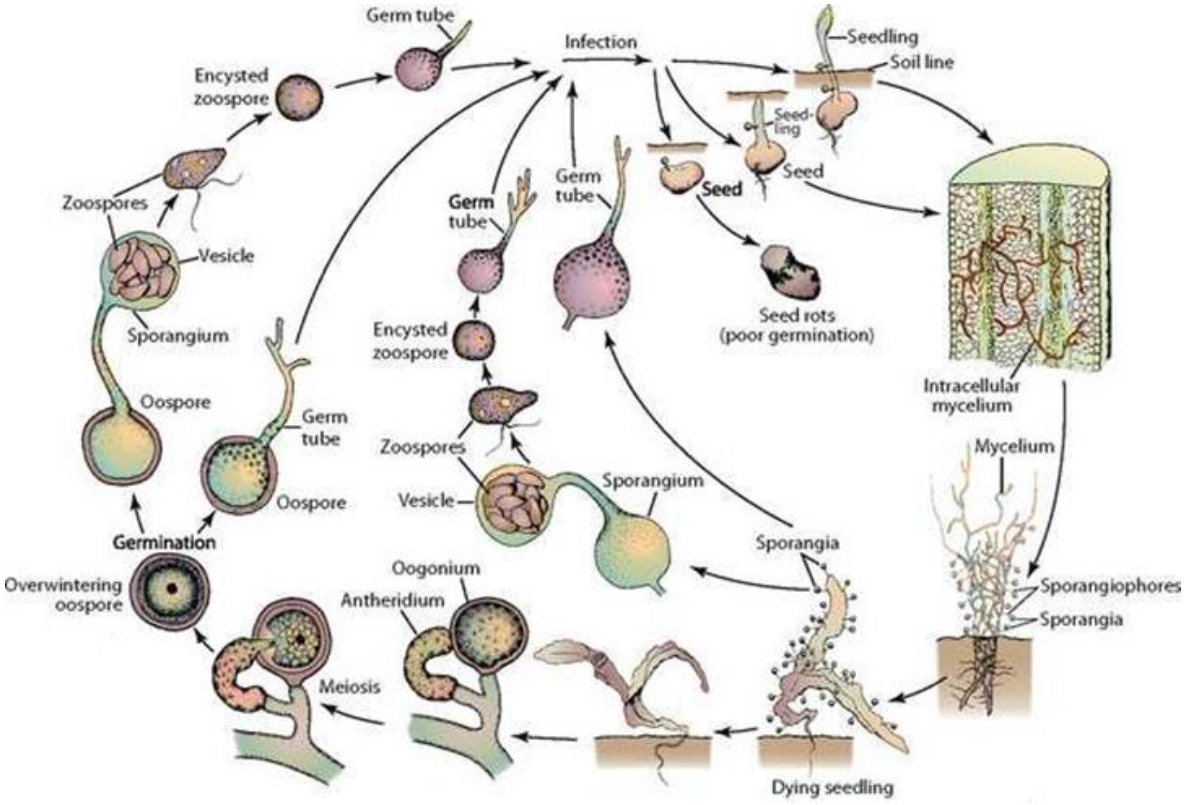

Figure 1.1: Lifecycle of Damping-off disease. Illustration of the asexual and sexual (production of oospore via haploid male antheridium and female oogonium) stages of Pythium in the context of Damping-off disease. Pre-emergence damping-off is shown via direct and indirect germination in seeds and seedlings below the soil. Post-emergence damping-off is shown via direct and indirect seedling germination above the soil. Image taken from (Agrios, 2005).

Pythium demonstrates diploidy throughout its asexual lifecycle (Hardham 2007). During this stage, direct germination takes place by the development of sporangia, which subsequently generate germ tubes that enter the host tissue. (Figure 1.1). Direct germination favours temperatures above 18°C, however, it is inhibited when this exceeds 30°C (Agrios 2005). Asexual reproduction also gives rise to chlamydospores (not shown in Figure 1.1), which are thick-walled resting spores that enable the pathogen to overwinter or survive long periods in unfavourable conditions (Van West et al., 2003). Indirect germination involves the development of a secondary vesicle originating from sporangia from which motile zoospores are produced that swim to the host and, upon contact, encyst and develop a germ tube that penetrates the host tissue (Figure 1.1). Indirect germination favours lower temperatures between 10-18°C (Agrios, 2005).

Sexual reproduction is initiated following the formation of haploid male (antheridium) and female (oogonium) gametangium (Figure 1.1). The male antheridium forms a fertilisation tube that penetrates the female oogonium. This is followed by meiosis, which results in the formation of a diploid zygote (Hardham, 2007). The fertilised oogonium develops a thick membrane, resulting in the formation of the oospore (Figure 1.1). Oospores are sexually produced resting spores and, like the asexual chlamydospores, enable the pathogen to overwinter and withstand hostile environments until germination conditions are favourable (Van West et al., 2003). Germ tubes developed from the oospore or vesicles producing zoospores facilitate direct and indirect germination (Figure 1.1) (Agrios, 2005).

1.4 Phytophthora

Phytophthora belongs to the Peronosporaceae family in the order of Peronsporales (EPPO, 2020). The lifecycle of *Phytophthora* is very similar to that of Pythium, shown in Figure 1.1. Species of *Phytophthora* are among the most destructive plant pathogens in the world (Kroon et al., 2012). The name derives from the Greek words 'phyto' denoting plant and 'phthora' meaning destruction, which aptly reflects the damaging nature of these pathogens (Agrios, 2005). *Phytophthora* affects a diverse range of trees, shrubs, and annual and perennial ornamental plants, in addition to important crops. These organisms pose a substantial risk to forest ecology, ornamental nurseries and gardens and food security (Beal et al., 2021; Cooke et al., 2012; T. Jung et al., 2016). Currently, 200 species have been described, although some estimates place the likely number of species between 200-600 (C. Brasier et al., 2022). *Phytophthora infestens* is a significant plant pathogen that is responsible for causing blight in tomatoes and potatoes (Fry et al., 2015). This particular species of *Phytophthora* has garnered significant attention in academic research due to its central involvement in the catastrophic potato famine that occurred during the mid-19th century. (Cooke et al., 2012). Such is the prevalence and risk to important crops; blight warnings are published in the UK when the risk of an outbreak is high. This information is based on the Hutton Criteria (Dancey et al., 2017) that monitor temperature and relative humidity (RH). If two consecutive days sustain a minimum temperature of 10°C for six hours and the RH exceeds 90%, the risk is deemed high. Details of affected areas are published on the BlightCast (Sygenta, 2023) and Blightspy (AHDB, 2023) websites.

Phytophthora root rot (PRR) is one of the most damaging soil-borne diseases (Giachero et al. 2022). PRR is typically associated with waterlogged soils and affects plants in natural environments and in cultivation (T. Jung et al., 2016). Disease occurrences are commonly found following flood events, heavy rainfall and irrigation practices, resulting in plants exposed to extended periods of standing in water that present favourable *Phytophthora* sporulation conditions. However, like *Pythium*, *Phytophthora* produces oospores and chlamydospores (Figure 2.1), enabling them to survive unfavourable conditions (Katan, 2017). PRR infects via the primary or feeder roots, extending to aerial parts (Frederickson-Matika, 2022). Typically, the first sign of PRR is the presence of disease symptoms in the plant's aerial parts, such as poor growth, yellow leaves, wilting, or the rapid collapse of the plant. Subsequent examination of the root system reveals soft, brown/black, water-soaked roots (O'Neil. T and Ann. D 2016). A recent study conducted by the Royal Horticultural Society (RHS) plant health team revealed *P.cryptogea, P.cactorum* and *P. pachypleura* to be the most common species affecting non-woody hosts and *P.plurivora, P.cinnamomi* and *P.citrophthora* in woody hosts species in UK gardens (Beal et al., 2021).

Phytophthora ramorum and *Phytophthora kernoviae* are pathogens recently and inadvertently introduced to the UK (Brasier et al. 2005; Werres et al. 2001). *P.kernoviae* was initially discovered in Cornwall in 2004 on a *Fagus sylvatica* (beech tree) while surveying for *P.ramorum* was conducted. This pathogen now poses an ecological threat to the area, having spread to hundreds of beech trees (C. M. Brasier et al., 2005). Currently, *P.ramorum* has over 170 known plant host species (EPPO, 2020) and causes a variety of symptoms, including root rot, foliar blight, bleeding cankers, wilt and dieback on a wide range of woodland and ornamental species (Denman et al., 2009). *P.ramorum* was initially identified from isolates obtained from *Rhododendron* and *Viburnum* cultivars exhibiting twig blight in Germany and the Netherlands in 2001 (Werres et al. 2001). Subsequently, it was confirmed as the causal agent of sudden oak death, a disease affecting tanoaks (*Lithocarpus* species) and *Quercus* species, first observed in California in 1994 (Rizzo & Garbelotto, 2003). Sudden oak death has reached epidemic proportions, spreading from mid-California to southern Oregon (Rizzo et al., 2005). In 2009, the first confirmation of *P.ramorum* disease was reported in the UK when it was found to cause dieback and mortality of *Larix kaempferi* (Japanese larch) in southwest England (Harris & Webber, 2016).

Subsequent outbreaks have been confirmed across UK forests, resulting in the culling of thousands of larch trees (Forest Research, 2023). Furthermore, incidents of *P.ramorum* in nurseries and gardens are increasing owing to the movement of affected nursery stock (C. M. Brasier et al., 2005; T. Jung et al., 2018).

P.ramorum is typically a foliar pathogen disseminated via wind, rain and splashes. However, it can be found in soils and riparian habitats and can be spread via the movement of infested soil and plant material (Grünwalde et al., 2008). This species produces caducous sporangia, believed to be a survival adaptation to aid dispersal (T. Jung et al., 2018). The typical lifecycle of *P.ramorum* is shown in Figure 1.2 (Grünwald et al., 2008). Sporangia are produced on the foliage and bark of infected hosts. *Rhododendron pontifucum* is known to be a sporulating host of *P.ramorum*, serving as a source of inoculum on nearby *Larix* trees. Initially, *Larix* was believed to be a terminal host (non-sporulating), however, sporulation has now been observed in these trees (Harris & Webber, 2016). Infection can also occur via the roots (not shown in Figure 1.2) (Parke & Lewis, 2007). Germination is known to occur between 2-30°C (Defra, n.d.), however, direct germination has not been observed *in vitro* (Figure 1.2) (Grünwald et al. 2008). *P. ramorum* exhibits heterothallism and thus requires two distinct mating types: A1 and A2 (Figure 1.2). Currently, the A1 is the predominant mating type in Europe, however an A2 mating type has been isolated from a nursery imported *Viburnum* species in Belgium (Werres & De Merlier, 2003). Thus far, sexual reproduction has exclusively been observed *in vitro* (Figure 1.2) (Werres et al. 2001), however, the danger posed by the potential introduction of more virulent strains in the natural environment cannot be underestimated.

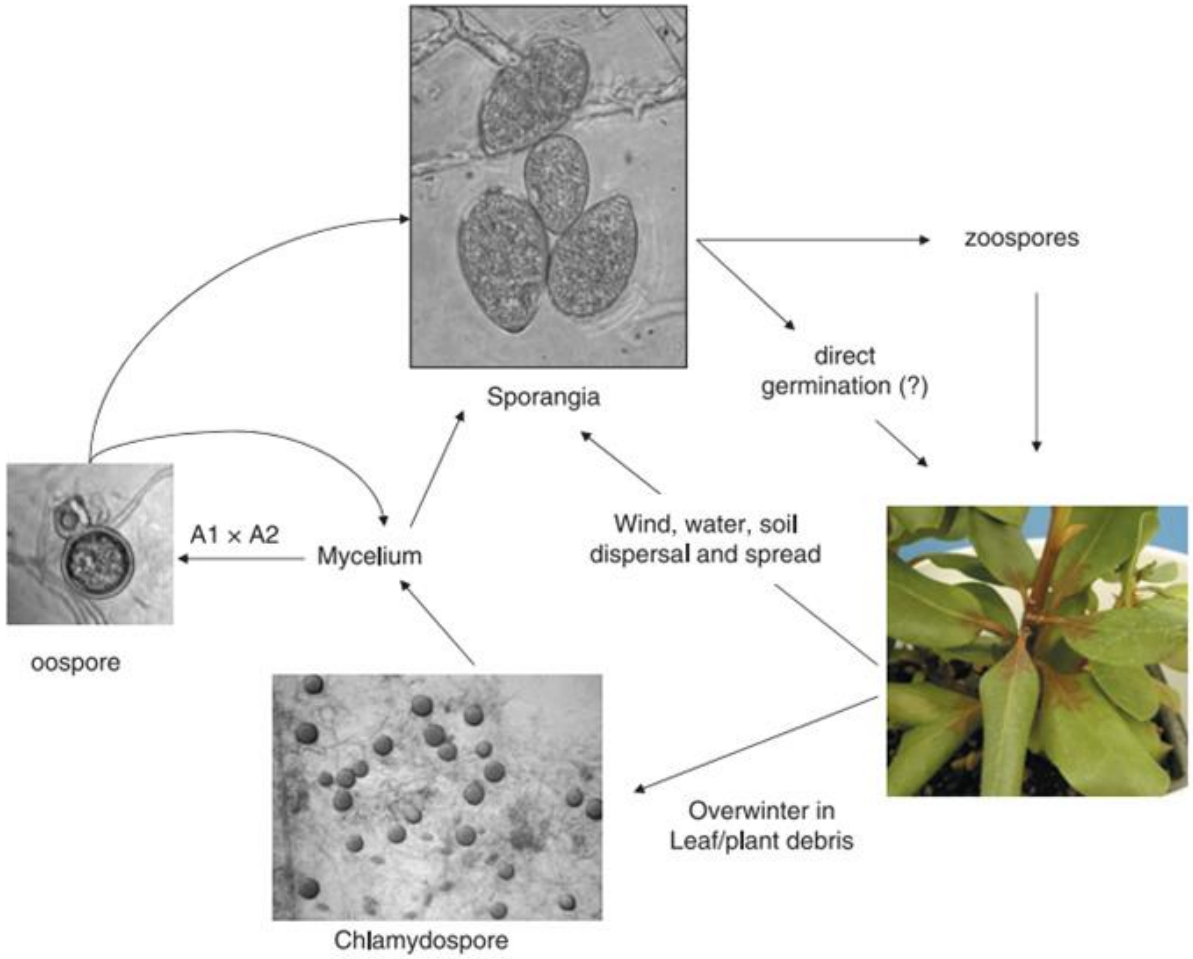

Figure 1.2: Generic Lifecycle of *Phytophthora ramorum*. Sporulation typically occurs on infected foliage or stems. Sporangia and chlamydospores are produced during asexual reproduction. Sexual reproduction requires two mating types: A1 & A2. Germination is direct or indirect; only direct germination via zoospore is observed in vitro. Image from (Grunwald et al 2008).

Due to the risk that *P. ramorum* and *P.kernoviae* pose to the natural environment, both these species have been classified as a regulated quarantine pest (EPPO A2 listed organism). In the UK, the Animal and Plant Health Agency (APHA, 2023) has implemented specific regulations to manage and contain the further spread of outbreaks. In the event of an outbreak, a statutory action notice is issued, which usually requires the destruction of infected plants and other known hosts within a two-metre radius. Planting host species within a four-metre radius is prohibited for three years and an annual visual inspection of the affected site is conducted by an APHA inspector for three years.

The Royal Horticultural Society (RHS) is the biggest horticultural charity in the United Kingdom, with five unique gardens across England. Within each garden, there are important plant specimens and national plant collections. According to the Association of Leading Visitor Attractions (ALVA, 2023), in 2021, the RHS's garden, Rosemoor in North Devon in the Southeast of England received over 233,000 visitors. The garden is situated on a 63-acre adjacent to the river Torridge and features a combination of natural woodland, formal, bog, and edible gardens. The area is known for the cool, damp weather conditions favoured by *Phytophthora* and *Pythium*. In the last five years, cases of *P.ramorum* disease in the garden have resulted in the issue of statutory plant health notices and the subsequent destruction of plants. The garden also hosts the national collections of *Ilex* and *Cornus,* both genera, which are known hosts of *P.ramorum* (Forest Research, 2023).

1.5 Aims and Objectives

The oomycetes contain some of the most destructive plant pathogens in the world. The aim of this study was to determine which species of Phytophthora and Pythium were present in the RHS Rosemoor garden. Sampling sites were chosen based on observations from the Rosemoor gardeners and the RHS plant health team. Soil was sampled from around susceptible plants displaying disease symptoms and from heritage cultivars belonging to the national collections. Oomycete identification from samples was determined by conducting the following processes:

- Oomycete Isolation: baiting soil and water samples
- Obtaining clean oomycete cultures: Using selective and nutrient rich agar media
- Morphological identification: Microscopic analysis of sporangia morphology based on known identification keys
- Molecular identification: Sequencing of the ITS region of the ribosomal DNA of the collected samples

Identified oomycetes were correlated to a locations map of the sampling sites. This would enable us to correlate sampling locations, plant species and oomycete species which will assist the RHS in implementing effective management strategies within affected areas.

CHAPTER 2: METHODS

2.1 Sampling and Mapping

Soil sampling, as described by (Pérez-Sierra et al., 2022), was conducted between the 18th and 20th of October 2022. Soil samples were collected from around known host species of *P.ramorum* (FERA, 2015), exhibiting *Phytophthora* like symptoms. Furthermore, soil samples were obtained from non-symptomatic specimen plants of significant importance. These plants were specifically selected from the RHS's 'Threatened in Cultivation' list and belonged within the two national collections hosted by the Rosemoor Garden, namely, *Cornus* and *Ilex.* Both these genera are known to be host species of *P.ramorum* (FERA, 2015). Additionally, three litres of water were collected from the garden's lake in one litre Duran bottles. The location for each sampling site was recorded using the 'what3words' mobile phone application (Table 1). The batch converter in this application was used to convert the three allocated words to coordinates. The coordinates and a base map of the site provided by the RHS were entered into ArcMAP 10.8.2 software to produce the location map (Figure 2.1). A total of twenty samples were analysed in this study (Table 1), the soil samples were stored at 4°C and the water at room temperature.

Table 2.1: Sample number and reference associated plant and location details from which soil was collected. NS – plants with no symptoms, S – disease symptoms observed, R – disease plant removed prior to sampling, NA – lake water.

Sample No	Reference	Plant Name	Plant status	Bed	Plant Accession	Garden Location	what3words
1	RM1810-S8	*Ilex* 'Shien Nien' *(m)*	NS	RH0803	R907087-H	Lock's Trail Rosemoor House	collides.sifts.mouse
2	RM1810-S9	*Ilex crenata* 'Carolina Upright *(m)*	NS	RH0803	R887987-A	Lock's Trail Rosemoor House	detained.stormy.chairing
3	RM1810-S10	*Ilex* 'Shin Nein'*(m)*	NS	RH0803	R907087-B	Lock's Trail Rosemoor House	represent.copy.bashful
4	RM1810-S14	*Ilex aquifoliulm* 'Crispa' *(m)*	NS	RH0804	R887975-F	Lock's Trail Rosemoor House	enacted.cascaded.boot
5	RM1810-S7	*Ilex crenata* 'Korean Gem'	NS	RH0104	R20040483-A	Rosemoor House Beds	ritual.headrest.occupy
6	RM1810-S16	*Cornus*	NS	RH0805	NA	Lock's Trail Rosemoor House	mugs.friday.announced
7	RM1810-S15	*Cornus mas* 'Schonbrunner Gourmet Dirndi' (F)	NS	RH0805	R20170027-A	Lock's Trail Rosemoor House	crucially.cyber.sleeping
8	RM1910-S2	*Hamamelis x intermediate* 'Pallida'	S	RF1307	R20110639-A	Winter formal garden	glorified.rotations.data
15	RM1910-S7	*Hamamelis x intermediate* 'Arnold Promise'	S	RF1310	R20070019-A	Winter formal garden	bunch.sunblock.adopts
18	RM1910-S4	*Viburnum x bodnatense* 'Dawn'	S	RF1310	R960441-C	Winter formal garden	contour.spruced.milder
22	RM1910-S3	*Hamamelis x intermedia* 'Nina'	S	RF1310	R20180077-A	Winter formal garden	grass.cascaded.hound
23	RM1910-S6	*Hamamelis*	S	RF1310	NA	Winter formal garden	treat.wisely.overgrown
24	RM1910-S5	*Hamamelis*	S	RF1310	NA	Winter formal garden	interacts.usages.coughed
29	RM1910-S23	*Rhododendron* 'Scintillation'	S	RG0202	R20040602-A	Lock's Trail Rosemoor House	group.regime.inspects
32	RM1910-S25	*Rhododendron* 'Blurettia spot'	R	RH0804	R888298	Lock's Trail Rosemoor House	creeps.command.unloads
33	RM1910-S26	*Cornus alba* 'Spaethii'	S	RG0204	R919952-A	Rock Gully lap side	puff.punters.gender
34	RM1910-S27	*Group of Cornus alba* (Kessselringii) *by lake and near Betula utilis* jacquemonii	S	RL0105	R919735-A &R919732-B	Lake and Lower Bog	boat.crossings.unhelpful
35	RM1910-S28	*Cornus controversaq* 'Pagoda'	S	RH0703	R921516-A	Drive beds Rosemoor House	kindest.maps.obviously
36	RM1910-S29	*near Acer rubrum* 'October glory'	S	RL0105	R886915-A	Lake and Lower Bog	dockers.leaned.scrub
37	RM1022-LW	Rosemoor Lake Water	NA	-	-	Rosemoor Lake	evening.walks.roughest

Figure 2.1: Aerial map of RHS Rosemoor, Devon, UK. Location and status of sampling sites collected in October 2022. Numbers correspond to sample numbers in Table 2.1.

2.2 Initiating Infection

The soil samples were thoroughly homogenised by manual mixing, ensuring the removal of any stones, roots, and vegetation debris. The utilisation of Granny Smith apples served as a means of initiating infection by soil-borne pathogens that may have been present. The preparation of the apples as baits was prepared as described by (Pérez-Sierra et al., 2022). The apple surface was cleaned and sterilised using 70% ethanol. Three cores measuring approximately 2x2x2cm were removed from each apple using a 2cm diameter fruit corer. Soil was firmly packed into the holes using a metal spatula. Distilled water was used to moisten the soil to facilitate the movement of any motile spores present. Clear tape secured the soil inside the apples (Figure 2.2a), which were then incubated for 4-7 days in the dark at 18-20°C until the apples showed signs of infection characterised by brown rot (Figure 2.2b). Typically, a firm brown rot indicates *Phytophthora,* whilst soft, lighter brown rot indicates *Pythium (*Pérez-Sierra et al., 2022). A control was prepared following the same method using sterile soil from randomly selected samples decanted into 100ml universal bottles that were autoclaved for 30 minutes at 121°C using a top-loading PS/MID/60 autoclave (Priorclave, UK).

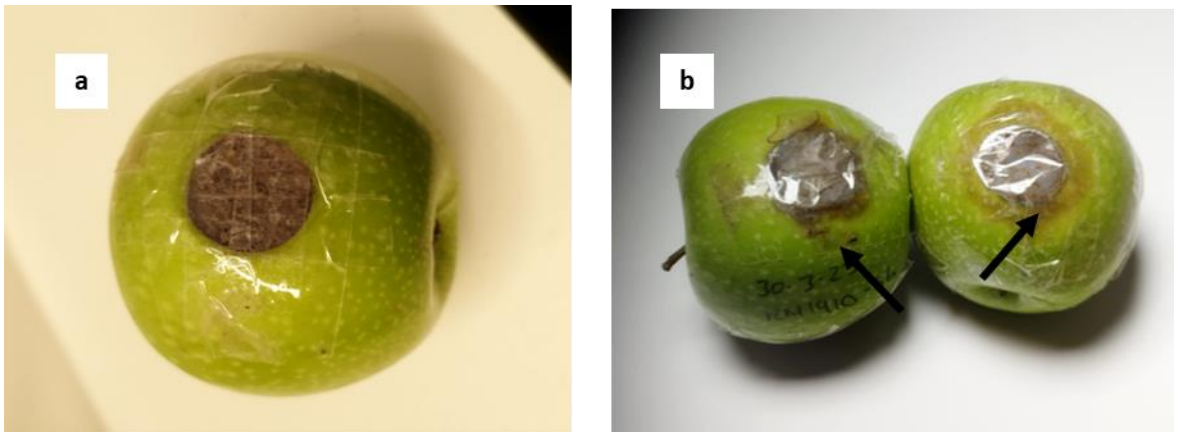

Figure 2.2: Images of baited apples. (a) Newly prepared apple bait pre-incubation. (b) Apple baits post-incubation, arrows highlighting brown rot.

Soil

Pathogen isolation for baiting apples was conducted using PARP selective media (one litre of V8 agar-16g agar, 2g $CaCO_3$, 100ml V8 juice, 900ml distilled water) (T. Jung et al., 2002) with the addition of (10µg/ml pimaricin, 10µg/ml rifampicin, 200µg/ml ampicillin, 50µg/ml nystatin and 25µg/ml pentachloronirtrobenzene) (Jeffers. S.N &

Martin. S.B, 1986), prepared with the omission of hymexazol as described by Pérez-Sierra et al., (2022). Isolates of approximately 10mm x 5mm were removed from the leading edge of the apple rot (Figure 2.3a) and plated onto the PARP media (Figure 2.3b). The plates were subsequently allowed to incubate (SLS Lab Pro Laboratory incubator, UK) for 5-7 days at 20°C until colonies could be detected when holding the plate to the light (Figure 2.3c). Growing hyphae were then transferred to V8 or Carrot agar (CA) (200g carrots, 1L tap water, 15g/L agar) (Pérez-Sierra et al., 2022) for subculture. CA agar was prepared as described by (C. Brasier, 1967). The subcultures were allowed to incubate for 5-7 days at 20°C until extended hyphal growth was observed (Figure 3.1b).

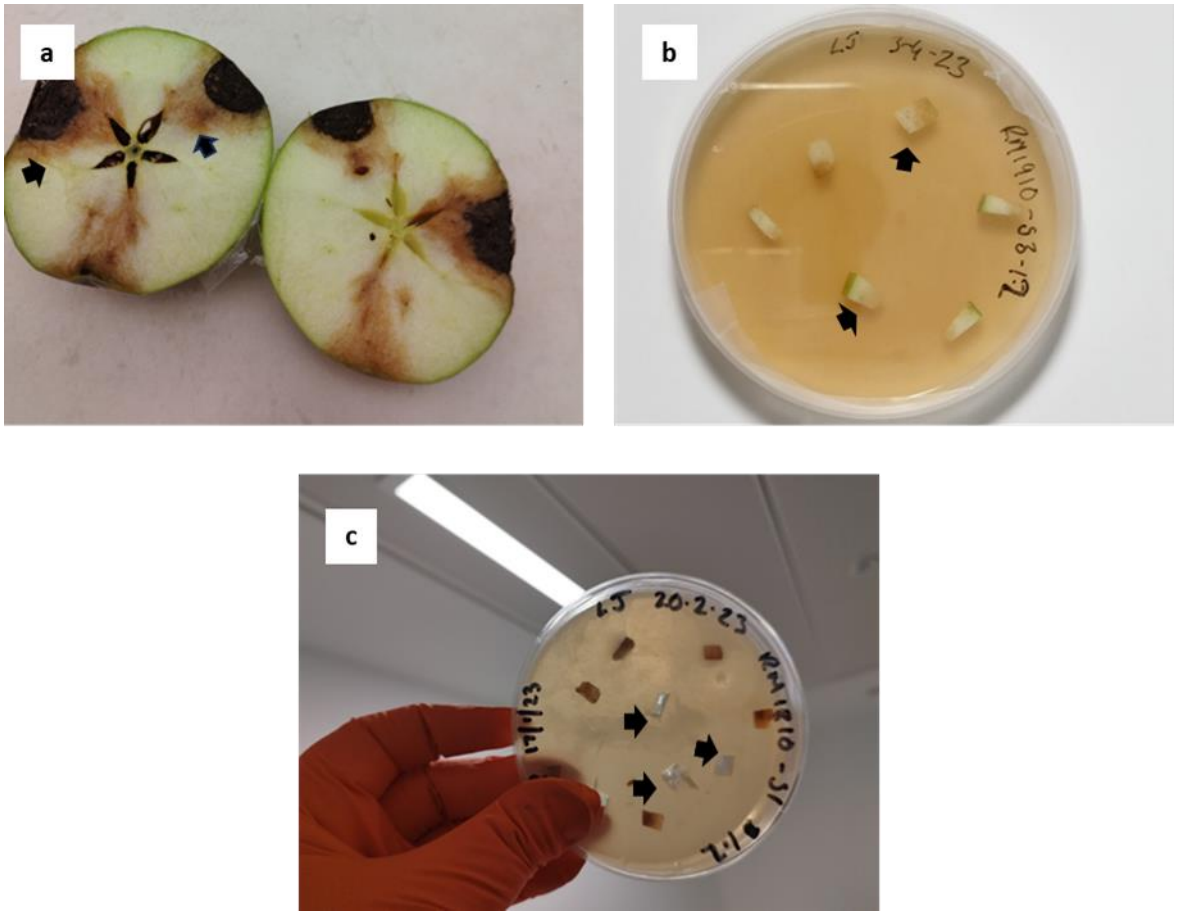

Figure 2.3: Progress of isolating pathogen. (a) Inside of baited apple post-incubation, arrows depict the leading edge of rot. (b) Isolates from the apple transferred to PARP selective media; arrows highlight isolates containing rot and non-rotted apple flesh. (c) Hyphal growth visible in PARP when held to the light post-incubation; arrows depict areas of growth excised for subculture.

Lake Water

The lake water was passed through Whatman filter paper to remove any debris, and the collected water was placed in petri dishes. Washed (70% ethanol) and filter paper dried PARP plugs (0.5cm diameter) were used as baits. The plates were covered with a lid and left on the bench top for 4-7 days until hyphal growth was observed using a dissection microscope (Huvitz, UK). Sections of hyphal growth were removed and transferred to V8/CA agar and incubated as per the cultures obtained from soil.

2.3 Microscopy

To induce sporulation, sections of the agar growing media with hyphal growth were transferred into petri dishes half filled with tap water, these were sealed with a lid and left on the bench top for 3-5 days. From day three, the plates were checked for sporangia using a dissection microscope. When sporangia were observed, hyphae samples were removed to a slide and viewed at magnifications of x200 and x400 using a compound microscope coupled to the Leica Application Suite version 4.12.0 imaging software (Lecia, Switzerland). The shape of observed sporangia (globular indicative of *Pythium* or ovoid indicative of *Phytophthora*) was recorded, and images were captured when possible. Prior to subculture, the same method was used to identify sporangia morphology from hyphal growth on PARP plugs floating in lake water.

2.4 DNA Extraction and PCR (polymerase chain reaction)

The process of DNA extraction was conducted by extracting tufts of hyphal growth from subculture plates and transferring them to a 2ml Eppendorf tube containing 100µl of molecular-grade water. Subsequently, this was heated to a temperature of 95°C to induce cell lysis, following the methodology outlined by (Grünwald et al., 2011). Samples were stored at -20°C until required.

PCR reaction assays with a total volume of 50µl were prepared for each extraction. Each reaction consisted of 2µl of extracted DNA, 30µl BioMix™Red (Meridian Bioscience), 14µl of molecular grade water and 2µl of 0.4µM of both the forward and reverse primers.

Table 2.2: Sequence of ITS6 and ITS4 primers.

Primer	Sense	Length (bp)	Sequence (5'- 3')
ITS6	Forward	21	GAAGGTGAAGTCGTAACAAGG
ITS4	Reverse	20	TCCTCCGCTTATTGATATGC

The amplification of the internal transcriber spacer regions (ITS) of the ribosomal DNA, which consists of the ITS1 spacer, 5.8s (subunit) and ITS2 spacer (Grünwald et al., 2011), was conducted using the ITS6 forward primer (Cooke et al., 2000) and the ITS4 reverse primer (White et al., 1990) (Figure 2.4) (Table 2.2). The PCR reaction consisted of an initial denaturing cycle of 3 minutes at 94°C followed by 35 cycles consisting of 1 minute denaturing at 94°C, 1 minute annealing at 55°C and 1 minute extension at 72°C. This was followed by a final 10-minute extension at 72°C.

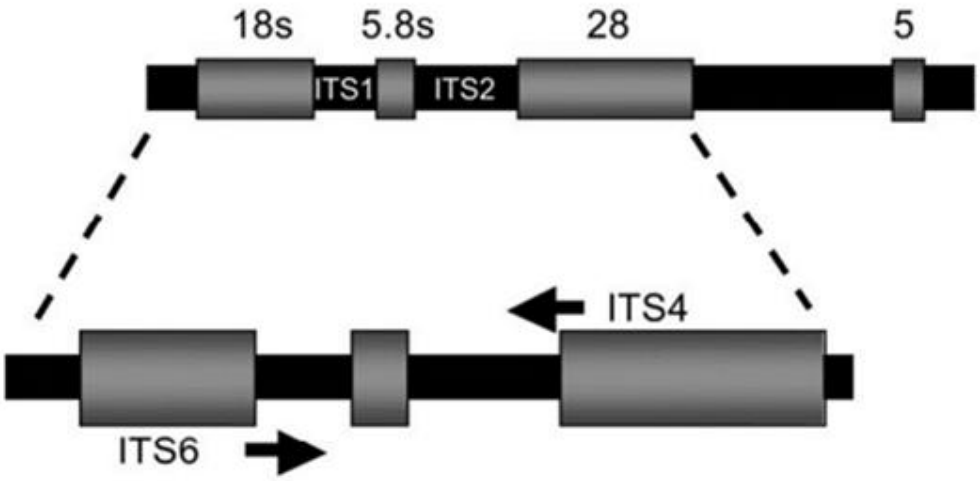

Figure 2.4: Illustration of the internal transcriber spacer regions found in the genome of oomycetes. The arrows depict the position of the ITS6 and ITS4 primers. Image taken from (Grünwald et al., 2011).

Amplicons were separated by electrophoresis in a 1.5% agarose gel (2.25g agarose powder, 150ml TAE buffer, 1.5g SYBR Safe™ DNA dye (Invitrogen). DNA bands were visualised under UV light using Gel Doc™ XR+ with Image Lab software (BIO-RAD) and compared to 1Kb and 100 bp DNA ladders (Invitrogen/DirectLoad™Plus). Gels were transferred to a Vilber Lourmat UV transilluminator (Vilber, Germany) and bands

between 750 and 1050 bp (Lévesque & De Cock, 2004) were cut from the gel using a scalpel and placed into 1.2ml Eppendorf tubes. The DNA was purified using a QIAquick® gel extraction kit (QAGEN) following the manufactures instructions. The DNA concentration was determined using a Qubit 4 fluorometer (Invitrogen) attained from an assay using 2µl of purified DNA added to the Qubit High Sensitivity dsDNA assay prepared as per the manufacturers' instructions. Subsequent sequencing of isolated fragments was undertaken by DBS Genomics, Durham University.

2.5 Sequence Analysis

Sequence files in ab1 trace format were imported into Sequencher 5.4.6(4) software (Gene Codes Corporation, USA) for analysis. An analysis was conducted on the collective sequence data acquired for each sample. The first step of analysis entailed trimming the sequence ends. Subsequently, each chromatogram was carefully examined for any ambiguities, and manual editing was performed whenever feasible. Assembly parameters were set to accommodate 'Dirty Data' with a minimum match percentage of 85 and a minimum overlap of 20 base pairs. The consensus strand for any resulting contigs or individual read was exported to file in FASTA format and then entered in a BLAST search to identify the best sequence match from the GenBank database.

CHAPTER 3: RESULTS

3.1 Obtaining 'Clean' Cultures

Soil sampling was conducted across the garden according to Pérez-Sierra et al. (2022), from important specimen plants and those showing disease-like symptoms. Water from the lake was also collected for sampling. Apple baiting, as described by Pérez-Sierra et al. (2022), was performed to recover potential pathogens from the soil. After 5-7 days of incubation, symptoms of brown rot on the apple exterior were observed (Figure 3.1a). Three distinct observations were made upon dissection: 1) Mild bruising at the site of soil entry in control specimens, indicating no infection (Figure 3.1b). 2) Slow-growing, firm, brown rot indicative of *Phytophthora* infection (Figure 3.1c) (Pérez-Sierra *et al.*, 2022). 3) Extensive, soft, brown rot with little visible intact flesh, indicative of *Pythium* infection (Figure 3.1d) (Pérez-Sierra *et al.*, 2022).

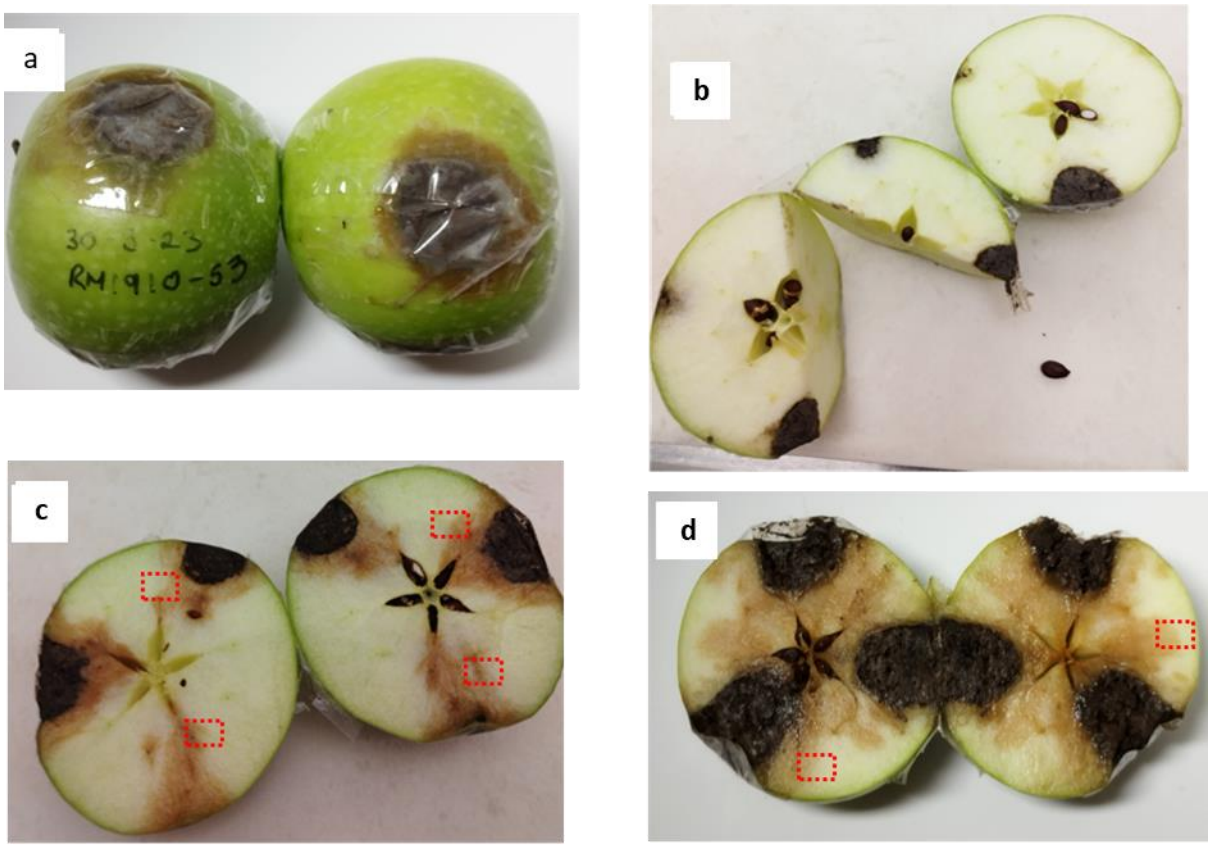

Figure 3.1: Images of rot on baited apples post-incubation. (a) Brown rot on the exterior of an apple indicative of infection. (b) Control showing no rot indicating no infection. (c) Slow growing firm brown rot in an apple post-incubation. (d) Fast growing soft brown rot in an apple post-incubation. Red boxes highlight the leading edge of infection.

Clean cultures were obtained from samples taken from the leading edge of infection (shown by highlighted red boxes in Figures 3.1c and 3.1d) and transferred to PARP which is a growing media that selects for *Pythium* and *Phytophthora* species (Jeffers. S.N & Martin. S.B, 1986; Pérez-Sierra et al., 2022).

To obtain clean cultures (cultures that appear uniform to the eye and show no obvious signs of contamination (Figure 3.2c)), numerous rounds of culturing and subculturing were performed using PARP selective media (Figure 3.2a). Clean cultures were obtained from subsequent outgrowth (Figure 3.1b) on growing on media (V8/CA) (Table 3.1). Clean cultures from PARP plugs floated in the lake water were derived similarly. The 'pancake method' devised by (Pérez-Sierra *et al.*, 2022) was used to obtain clean cultures from plates contaminated with bacteria (Figure 3.2c). This involved removing a fragment of agar containing colony growth ('clean up region') (Figure 3.2c) from a contaminated plate and transferring it to the lower surface of PARP media within a separate plate, as described by (Pérez-Sierra et al., 2022). PARP media contains antibiotics and fungicides, cultures selected on this media should be free from contamination. The clean cultures were used in subsequent studies.

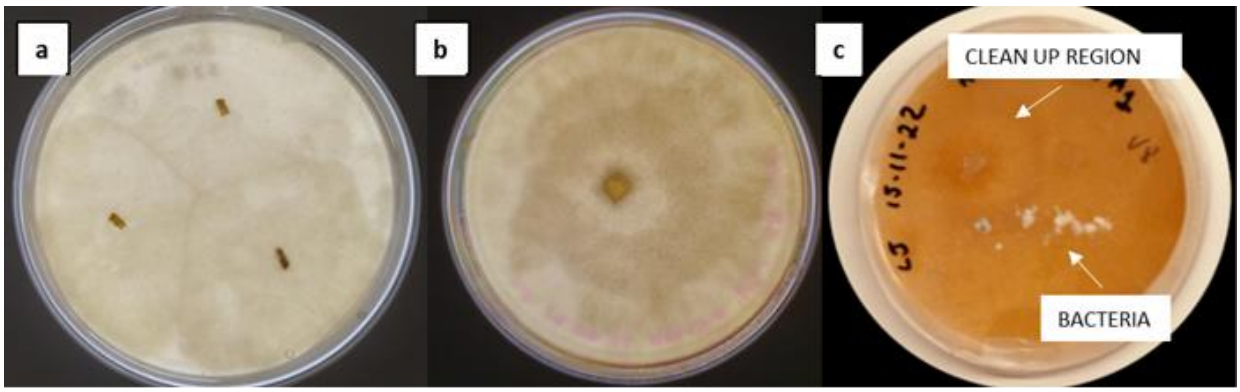

Figure 3.2: Growth on a Plate. (a) Hyphal growth extending from apple cultures on PARP selective media. (b) Subcultured colonies form PARP growing on in V8/CA media. (c) Contaminated plate showing hyphal grow suitable for 'clean up' via the 'Pancake method' (Pérez-Sierra et al., 2022) and bacterial contamination.

Table 3.1: Progress of obtaining a clean culture for biological and molecular analysis. HG – Hyphal Growth.

Sample Reference	Apple Rot	PARP	V8/CA	Clean-Up using 'Pancake method'	Clean Culture taken forward
RM1810-S7	Infection	HG	HG	Y	Yes
RM1810-S8	Infection	HG	HG	-	Yes
RM1810-S9	Infection	HG	HG	-	Yes
RM1810-S10	Infection	HG	HG	-	Yes
RM1810-S14	Infection	HG	HG	-	Yes
RM1810-S15	Infection	HG	HG	-	Yes
RM1810-S16	Infection	HG	HG	Y	No
RM1022-LW	NA	HG	HG	Y*	Yes
RM1910-S2	Infection	HG	HG	-	Yes
RM1910-S3	Infection	HG	HG	Y	Yes
RM1910-S4	Infection	HG	HG	-	Yes
RM1910-S5	Infection	HG	HG	-	Yes
RM1910-S6	Infection	HG	HG	Y	Yes
RM1910-S7	Infection	HG	HG	Y	Yes
RM1910-S23	Infection	HG	HG	-	Yes
RM1910-S25	Infection	HG	HG	Y	Yes
RM1910-S26	Infection	HG	HG	-	Yes
RM1910-S27	Infection	HG	HG	Y	Yes
RM1910-S28	Infection	HG	HG	-	Yes
RM1910-S29	Infection	HG	HG	-	Yes

*Water sample not subjected to apple baiting methods

3.2 Biological Analysis of Cultures

A subset of stab cultures was generated to determine whether the cultures observed on the plates represented the pathogenic agent accountable for the infection in apples. Hyphae was stab inoculated to apples using a sterile tip. Figures 3.3a-c illustrate the sequential punctures, with the number 1 arrows representing the initial puncture, the number 2 arrows indicating the second puncture, and the number 3 arrows denoting the final puncture. As anticipated, a decline in infection can be seen in Figures 3.3a and 3.3b, as the quantity of hyphae introduced decreases with each succeeding puncture. The initial apple depicted in Figure 3.3a exhibits a slight discoloration in both instances and in the apple illustrated in Figure 3.3c, a sterile tip was employed to create these punctures. To determine whether an infection caused the minor discolouration, samples were taken from the area highlighted in red (Figure 3.3c) and plated on PARP media; per previous culturing (Figure 3.3d), no growth was observed. The subset of samples subjected to reinoculation is highlighted in Table 3.2.

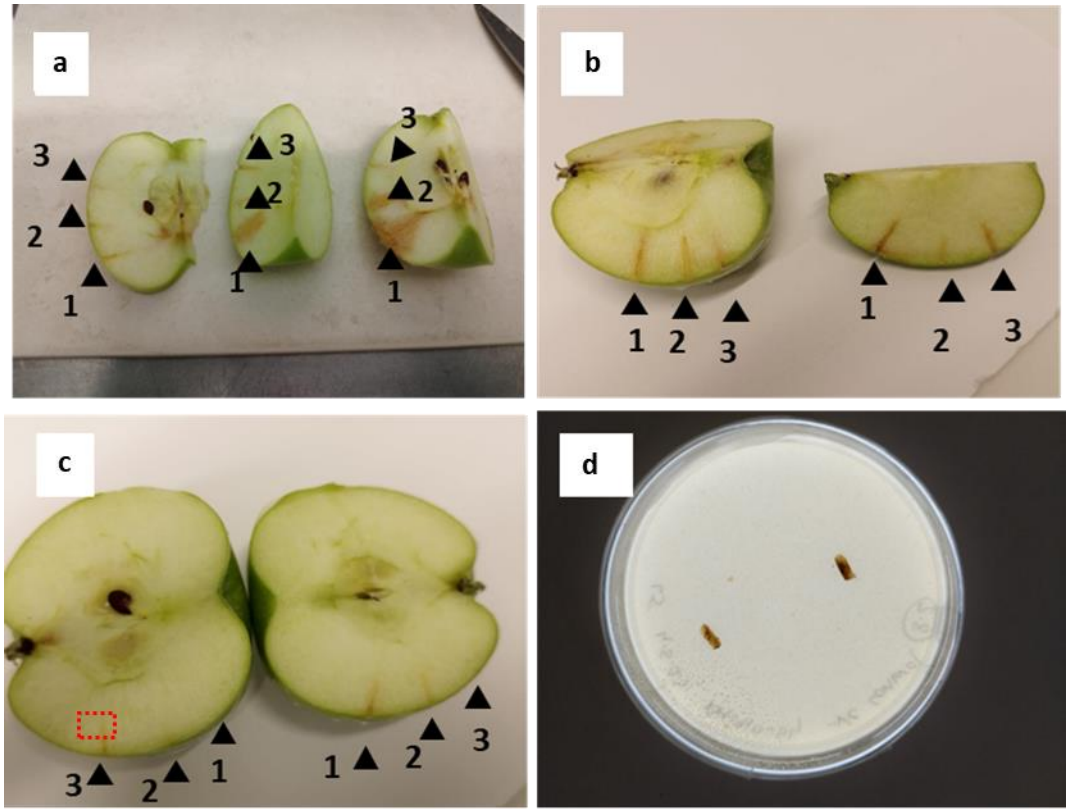

Figure 3.3: Hyphae Stab Inoculation in Apples. (a) Three dissected apple fragments: the left fragment was inoculated with a sterile pipette tip; the middle and right fragments were inoculated with hyphae derived from clean cultures. (b) Two dissected apple fragments inoculated with hyphae derived from clean cultures. (c) A dissected apple inoculated with a sterile pipette tip; red box indicates area transferred to PARP. (d) Post-incubation of culture on PARP removed from apple in image c.

To enable the identification of cultures, sporulation was induced. Spore morphology was identified based on the shape of sporangia visible under a compound microscope, globular for *Pythium* species, ovoid for *Phytophthora* species. Evidence of mixed infection was evident in samples when both sporangia shapes were observed (Figure 3.4a). Morphological identification was attributed if only one sporangia shape was observed in a sample, as in the *Pythium* sporangia seen in Figure 3.3b.

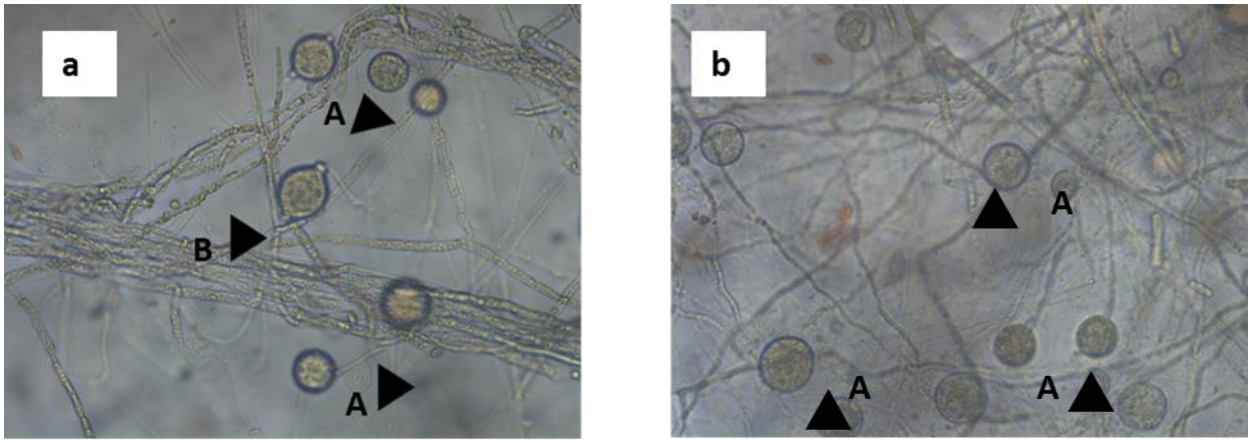

Figure 3.4: Sporangia Identification. (a) Mixed infection: A arrows highlight Pythium globular sporangia, B arrow highlight Phytophthora ovoid sporangia. (b) Single globular sporangia observed in sample indicating Pythium species.

The attribution of a pathogen, as determined by the shape of its sporangia, is shown in Table 3.2. Eight samples were found to contain mixed infection of *Pythium* and *Phytophthora*: containing both globular and ovoid shaped sporangia. No data was available to determine the morphology of cultures obtained from the lake water. The remaining samples were found to be associated with *Pythium species,* with only globular shaped sporangia observed. The remaining eleven samples were found to be associated with Pythium as only globular shaped sporangia were observed.

Table 3.2: Attribution of pathogen based on sporangia shape and results of sample subset subjected to apple reinoculation. NT – Not Tested, Y – Yes, N – No.

Sample	Reinoculation	Circular Sporangia	Oval Sporangia	Conclusion
RM1810-S7	N	Y	N	Pythium
RM1810-S8	N	Y	N	Pythium
RM1810-S9	N	Y	Y	Mixed species
RM1810-S10	N	Y	N	Pythium
RM1810-S14	N	Y	Y	Mixed species
RM1810-S15	N	Y	N	Pythium
RM1810-S16	N	Y	Y	Mixed species
RM1022-LW	N	-	-	ND
RM1910-S2	Infection	Y	N	Pythium
RM1910-S3	N	y	N	Pythium
RM1910-S4	N	Y	Y	Mixed species
RM1910-S5	N	Y	Y	Mixed species
RM1910-S6	Infection	Y	N	Pythium
RM1910-S7	N	Y	Y	Pythium
RM1910-S23	N	Y	N	Pythium
RM1910-S25	N	Y	Y	Mixed species
RM1910-S26	N	Y	N	Pythium
RM1910-S27	Infection	Y	Y	Mixed Species
RM1910-S28	N	Y	N	Pythium
RM1910-S29	N	Y	Y	Mixed infection

3.3 Molecular Analysis of Cultures

Following the procedure described by (Grünwald et al., 2011), a crude DNA extraction was performed followed by PCR reactions the ITS6 and ITS4 primers. These primers typically generate amplicons of between 750 and 1050bps (Lévesque & De Cock, 2004) The majority of amplicons separated by gel electrophoresis were single bands in the 1000bp region, but one sample failed to generate any bands and another sample generated two bands in the 1000bp and 850bp regions, as seen in Figure 3.5.

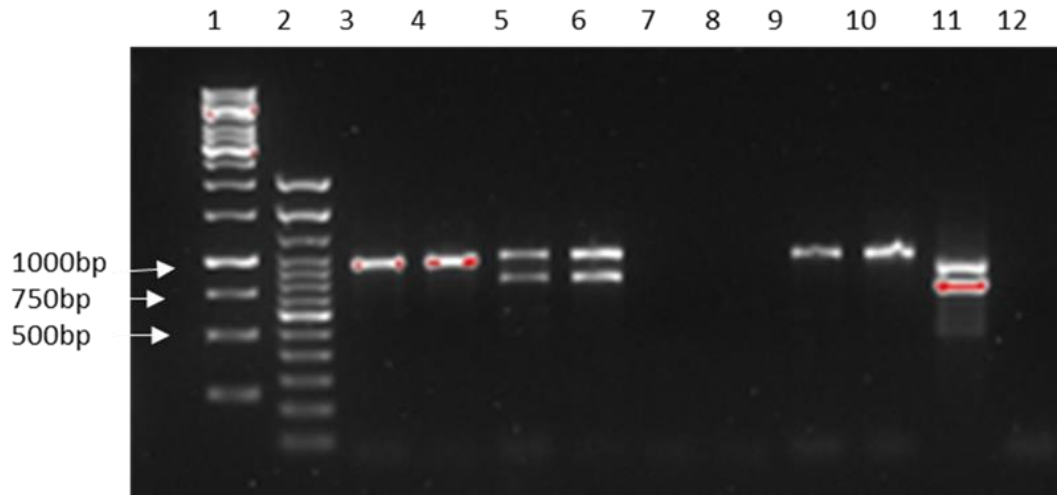

Figure 3.5: Image of Gel Results. Wells 1 - 1kb DNA ladder, well 2 – 100bp DNA ladder, wells 3-4 (Lake water) DNA bands c1000bp, wells 5-6 (RM1910-S14) DNA bands c1000bp and c850bp. Wells 7-8 (RM1810-S8) no DNA bands, wells 9-10 (RM1910-S10) DNA bands c1000bp, well 11 (positive control, gifted by Dr Liz Beal, RHS) DNA bands c900bp and 800bp, well 12 (negative control) no DNA bands.

The gel bands were excised directly from the gel; in the incidences of double bands, these were excised separately. Following subsequent DNA purification and quantification fragments were sequenced directly.

A total of 28 consensus sequences were derived from either contigs or Individual reads. The quality of the consensus sequences was scored based on the length of overlapping contigs assembled, one-directional contigs assembled or single reads. Instances when no data was produced resulted in unusable (U) sequence data. In cases where the sequence read lengths measured below 500bps, it was categorised as poor (P). Sequence read lengths falling between 500-800 bps were classified as

good (G), while lengths ranging from 800-1100 bps were designated as very good (VG). Details of the sequence analysis are presented in Table 3.3.

Each consensus strand generated by the sequence analysis software was subjected to a BLAST search to identify the pathogen at the species level. The best BLAST result was only considered if the query cover was greater than 94%, the e-value was close to 0, and the identity percentage was greater than 96%. Details of the best blast results are displayed in Table 3.3.

Table 3.3: Consensus sequence and quality derived from sequence analysis and best blast results. Consensus quality abbreviations; U – unusable, P – Poor, G – good, VG – very good.

Sample	Consensus length	Consensus quality	Sequencher Assembly status	BLAST Query Cover %	BLAST e-value	BLAST Ident. %	BLAST Hit	Genbank reference
RM1910-S7	893	VG	Overlapping contig of 2	100	0	100	*Pythium sylvaticum*	KF806441
RM1810-S8	na	na	na	na	na	na	na	na
RM1810-S9	909	VG	Overlapping contig of 3	100	0	100	*Pythium sylvaticum*	MN901147
RM1810-S10	0	na	na	na	na	na	na	na
RM1810-S14T	207	P	ITS4 contig of 2	100	9.00e-92	97	*Pythium intermedium*	AB512926
"	241	P	ITS6 contig of 2	100	5.00e-12	99	*Pythium attrantheridium*	MK886855
RM1810-S14B	0	na	na	na	na	na	na	na
RM1810-S15	964	VG	Overlapping contig of 4	99	0	100	*Pythium sylvaticum*	DQ528741
RM1810-S16	na	na	na	na	na	na	na	na
RM1022-LW	807	VG	Overlapping contig of 2	99	0	99	*Phytophthora gonapodyides*	OM984701
RM1910-S2	58	P	Single read ITS4	100	4.00e-17	100	*Pythium attrantheridium*	MN306101
"	84	P	Single read ITS6	100	6.00e-22	100	*Pythium attrantheridium*	MN306101
RM1910-S3	463	G	Contig of 2 ITS4	100	0	100	*Pythium sylvaticum*	MN901147
"	457	G	Contig of 2 ITS6	100	0	100	*Pythium sylvaticum*	MK583652
RM1910-S4	62	P	Single read ITS4	100	6.00e-22	100	*Pythium attrantheridium*	MN306101
"	726	G	Single read ITS6	99	0	100	*Pythium attrantheridium*	MN306101
RM1910-S5	911	VG	Overlapping contig of 3	100	0	93	*Pythium macrosporum*	AY598646
RM1910-S6	na	na	na	na	na	na	na	na
RM1910-S7	310	P	Single read ITS6	100	4.00e17	100	*Pythium intermedium*	MT647270
RM1910-S23	980	VG	Overlapping contig of 4	97	0	100	*Pythium sylvaticum*	MK583652
RM1910-S25	1002	VG	Overlapping contig of 4	99	0	100	*Pythium sylvaticum*	ON146326
RM1910-S26	312	P	Contig of 2 ITS4	100	6.00e-16	100	*Pythium macrosporum*	AY598646
"	264	P	Single read of 1	98	1.00e-17	100	*Pythium macrosporum*	AY598646
RM1910-S27	57	P	Contig of 2 ITS4	100	2.00e-34	100	*Pythium attrantheridium*	MN306101
"	85	P	Contig of 2 ITS6	100	2.00e-34	100	*Pythium attrantheridium*/ *Pythium intermedium*	MN306101/ AB512940
RM1910-S28	857	VG	Overlapping contig of 4	94	0	100	*Pythium senticosum*	HQ643773
RM1910-S29	884	VG	Overlapping contig of 4	98	0	100	*Pythium anandrum*	AY598650

3.4 Isolate Attribution

When morphological analysis revealed both forms of sporangia in a sample, this was attributed to the presence of mixed species. If no molecular results were obtained for a sample, but morphological analysis revealed a single form of sporangia, genus identification was assigned. Identification at the species level was assigned to a sample if the morphological analysis revealed a single form of sporangia, and the best BLAST result returned a species within that genus. An exception to this was the lake water sample, which was attributed to a species, without available morphological data, however, molecular analysis yielded a species result. Interestingly, a single sample (RM1910-S27) was assigned to two species (*Pythium attrantheridium* and *Pythium intermedium*) due to identical BLAST scoring matches (Table 3.3).

This study identified five *Pythium* species in nine soil samples, one *Phytophthora* species in lake water and three non-distinct *Pythium* species in three additional soil samples. The remaining seven samples were shown to contain mixed species consisting of *Pythium* and *Phytophthora.* Details of the final attribution are presented in Table 3.3.

Soil was sampled from *Ilex, Cornus, Hamamelis, Viburnum, Rhododendron, Betula* and *Acer* species. Analysis of the organism/s found in the soil samples showed no correlation with the plant species from which the soil samples were collected. Analysis of oomycete organisms revealed no location affects within the garden. An aerial map detailing the distribution of Oomycetes identified in this study is shown in Figure 3.5.

Table 3.3: Final attribution of Oomycetes identified in this study based on the morphological identification of spore type by shape and the best BLAST results. The sample number relates to the sample locations and the colour of the final attribution font correlates to the colour points assigned to the variables in the final attribution map in Figure 3.5.

Sample Number	Sample Reference	Identification by spore type	Identification by sequence	Final Attribution
5	RM1810-S7	*Pythium*	*Pythium sylvaticum*	***Pythium sylvaticum***
1	RM1810-S8	*Pythium*	NA	***Pythium sp.***
2	RM1810-S9	*Pythium*	*Pythium sylvaticum*	***Pythium sylvaticum***
3	RM1810-S10	*Pythium*	ND	***Pythium sp.***
4	RM1810-S14	*Phytophthora & Pythium*	*Pythium intermedium & Pythium attrantheridium*	**Mixed species**
7	RM1810-S15	*Pythium*	*Pythium sylvaticum*	***Pythium sylvaticum***
6	RM1810-S16	*Phytophthora & Pythium*	ND	**Mix species**
37	RM1022-LW	ND	*Phytophthora gonapodyides*	***Phytophthora gonapodyides***
8	RM1910-S2	*Pythium*	*Pythium attrantheridium*	***Pythium attrantheridium***
22	RM1910-S3	*Pythium*	*Pythium sylvaticum*	***Pythium sylvaticum***
18	RM1910-S4	*Phytophthora & Pythium*	*Pythium attrantheridium*	**Mixed species**
24	RM1910-S5	*Phytophthora & Pythium*	*Pythium macrosporum*	**Mixed species**
33	RM1910-S6	*Pythium*	ND	***Pythium sp.***
15	RM1910-S7	*Pythium*	*Pythium intermedium*	***Pythium intermedium***
29	RM1910-S23	*Pythium*	*Pythium sylvaticum*	***Pythium sylvaticum***
32	RM1910-S25	*Phytophthora & Pythium*	*Pythium sylvaticum*	**Mixed species**
33	RM1910-S26	*Pythium*	*Pythium macrosporum*	***Pythium macrosporum***
34	RM1910-S27	*Phytophthora & Pythium*	*Pythium attrantheridium & Pythium intermedium*	**Mixed species**
35	RM1910-S28	*Pythium*	*Pythium senticosum*	***Pythium senticosum***
36	RM1910-S29	*Phytophthora &Pythium*	*Pythium anandrum*	**Mixed species**

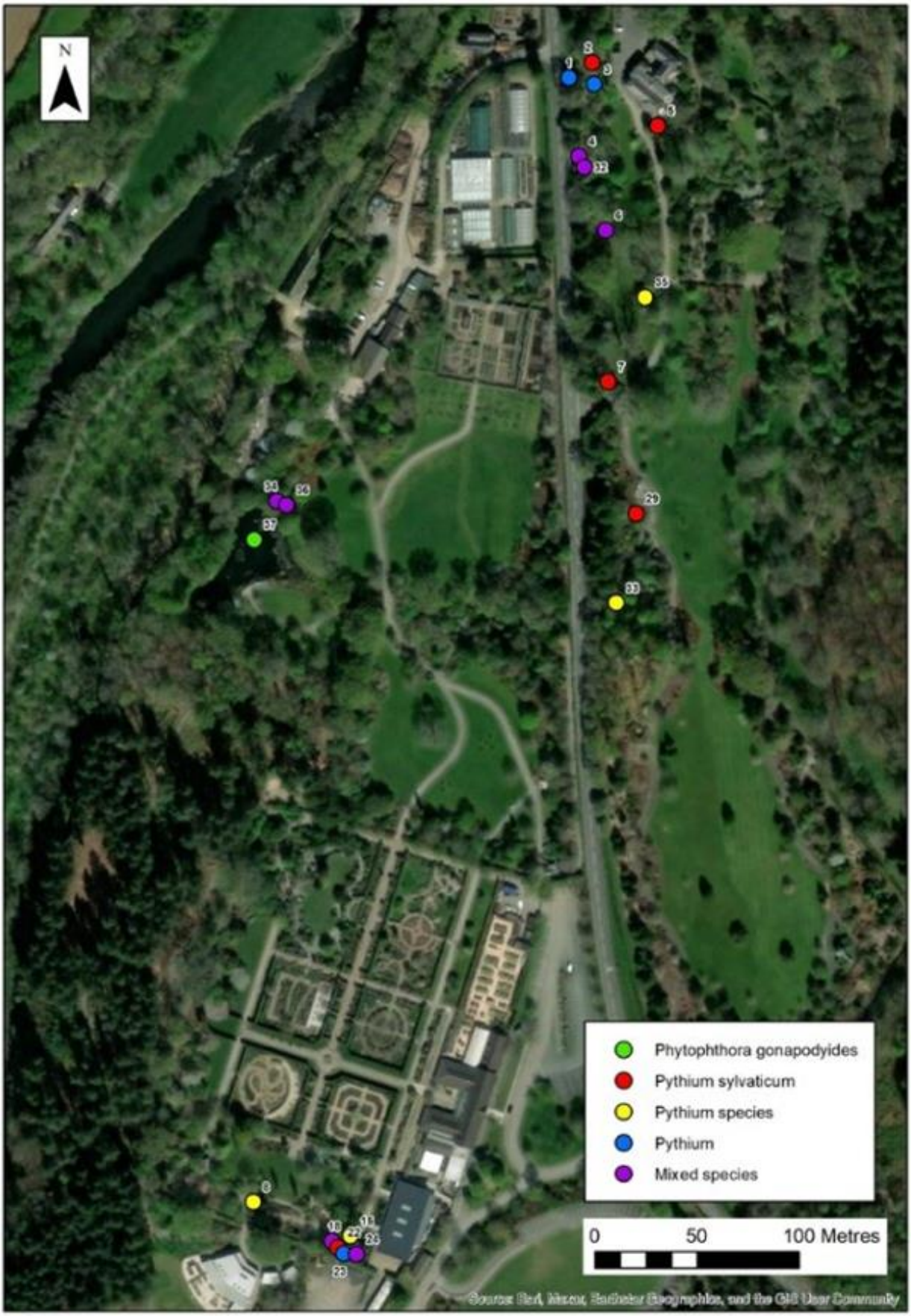

Figure 3.5: Final Attribution of Oomycetes Identified in RHS Rosemoor from samples collected in October 2022.

CHAPTER 4: DISCUSSION

4.1 Overview

In this study, one species of *Phytophthora* and five species of *Pythium* were isolated from locations within the garden at RHS Rosemoor. Each of these species will be discussed in turn. However, for seven of the samples analysed, it was not possible to provide an attribution due to morphological and sequence data suggesting mixed culture. The PARP media used selected against bacterial and fungal species and *Pythium* and *Phytophthora* species have been isolated. The addition of hymexazol to the media would further select against *Pythium* (Pérez-Sierra et al., 2022). A mixed culture on the plate, would result in mixed DNA template for PCR and result in fragments of mixed sequence. When sequenced directly (as performed in this study) such mixed fragments will result in poor, high noise, mixed sequence data, from which no clear attribution could be obtained. Cloning PCR products as described by Finneyet al. (2001) from mixed infection samples and sequencing several clones, would enable clean, high quality, sequence data to be obtained and species attribution assigned. Morphological analysis of the culture obtained from sample 36 (RM1910-S29), suggested a mixed culture (Table 3.2) as both *Pythium* (globular) and *Phytophthora* (ovoid) spores were identified. However, sequence analysis obtained from this sample enabled a species attribution of *Pythium anandrum* (Table 3.3). Morphological characteristics such as hyphal swellings can often be misidentified as *Phytophthora* sporangia (PennState University, 2023). It is possible such misidentification may have been made in this case, however, due to the morphological analysis this sample was still given a final attribution of mixed species.

4.2 *Phytophthora gonapodyides*

The attribution of *Phytophthora gonapodyides* to the lake water sample was consistent with previous reports as this species is recognised for its extensive distribution across various aquatic ecosystems (Brasier et al., 1993; Ristaino, 2023). *P.gonapodyides* was initially discovered, albeit classified under an unidentified genus, during the early 20th century after the decay of apples submerged in a freshwater pond was observed (Brasier et al., 1993). In addition to being associated with root and collar rot in ornamental species and the occurrence of root rot, twig blight, and stem lesions in both coniferous (Brasier et al., 1993) and broad-leaf (Jung et al.,1996) woodland

species, this pathogen has now been identified as a causative agent of fruit rot on a variety of edible species, with raspberries being the most recent species affected (Burlakoti et al., 2023; Ristaino, 2023). *P.gonapodyides* is typically considered a minor pathogen associated with wet areas (Ristaino, 2023). It was recently reported that it was responsible for the death and deterioration of numerous commercially cultivated *Juglans regia* (walnut) trees in Italy. The trees were located next to an irrigation canal and had been standing in water for some time due to severe rains, the authors suggested this was contributing factor for the pathogen's unusual aggressiveness, as these conditions were optimal for sporulation (Belisario et al., 2016).

4.3 *Pythium sylvaticum*

Studies have shown *P.sylvaticum* is associated with root rot and damping-off disease in herbaceous annuals and perennials, fruit crops, and woodland species (Mcpherson & Wakeham, 2015; Millner, 2006). *Pseudotsuga menziesii* (Douglas fir) is an important coniferous species with 200 million seedlings commercially produced each year in North American forest nurseries. A study by (Weiland et al., 2013) reported 44% mortality in seeds inoculated with *P.sylvaticum*, thus deeming it a highly virulent pathogen to this species. Other pathogenicity tests conducted on *Chrysanthemum* species supported the designation of *P.sylvaticum* as a virulent pathogen (Mcpherson & Wakeham, 2015; Pettitt et al., 2011). However, analysis in the aforementioned studies has been conducted using seeds and young plants, no analysis could be found to support *P.sylvaticum* being a virulent pathogen on mature ornamental plants. Soil samples 22 (RM1910-S22) and 29 (RM1910-S23) (Table 3.5) from which this pathogen was isolated from were associated plants showing disease symptoms. Therefore, it is recommended a root inspection be conducted on these plants to determine if root rot is a contributing factor.

4.4 *Pythium senticosum*

This study attributed a single identification of *Pythium senticosum* to soil from around a *Cornus* species (sample 35) reported to be showing disease symptoms (Table 2.1) (Figure 2.1). *P.senticosum* was first isolated from soil samples collected in a cool temperate forest in Japan as part of a study examining the role of *Pythium* species in the decomposition process within natural ecosystems (Senda et al., 2009) and again in Vietnam during a study investigating *Phytophthora* diversity in forest ecosystems (T.

Jung et al., 2020). In 2013, it was isolated from watercourses in Tennessee while surveys for *P.ramorum* were being conducted (Shresth et al., 2013). That same year, it was implicated in the demise of *Quercus mongolica* species from within forests in the central Sikhote-Alin mountain range in Russia (Malysheva et al., 2013abstract only). No known pathogenicity testing has been conducted to date on this species. This study could be the first occasion *P.senticosum* has been isolated in the UK. Interestingly, Senda et al., (2009) described the sporangia as ovoid shaped, this was not observed in this study, therefore it is soil sample 35 (RM1910-S28) contained mixed species.

4.5 *Pythium macrosporum*

A single attribution of *Pythium macrosporum* was made in this current study. *P.macrosporum* is a globally distributed pathogen that has been identified as the causal agent of root diseases in flower bulbs (Van Os et al., 1999) and carrots (Allain-Boulé, Lévesque, et al., 2004). Pathogenicity testing conducted on cucumber and cabbage seeds demonstrated that *P.macrosporum* exhibited pathogenic properties towards these plants, leading to pre-emergence damping-off and subsequent seed mortality. However, the severity of the disease caused by *P.macrosporum* was significantly lower compared to the effects observed from *P.ultimum* investigated within the same study (Uzuhashi et al., 2008). Currently, there is no available data to support *P.macrosporom* affecting mature trees and woody shrubs.

4.6 *Pythium attrantheridium*

One attribution of *Pythium attrantheridium* was made in this current study. *P.attrantheridium* was first described in 2004 when isolates were obtained from cavity spot lesions observed in commercially grown carrots and from wild cherry seeds in North America (Allain-Boulé et al., 2004). Further studies have associated *P.attrantheridium* with damping-off disease in soya bean seeds (Broders et al., 2007). Published data on the effects this pathogen has on other species is limited. The isolation of this pathogen in this study was attributed to a soil sample 8 (RM1910-S2) (Table 3.5) Information received from the RHS Rosemoor plant records team (27.06.23) confirmed the death of the plant in this location (Table 2.1). Symptoms of "root rot and butt rot" and a negative test for *Phytophthora ramorum* were recorded by the RHS plant health team, no further details were available. The plant (sample 8)

had been located in a separate bed to four other plants associated with this current study of the same genus (samples 15,22,23 and 24) (Table 2.1). These four plants were planted adjacent to each other, in a bed located near the dead plant (Figure 1.1). On a visit to the garden in June 2023, it was noted these plants had been heavily pruned and appeared to be in good health.

4.7 *Pythium intermedium*

This current work, attributed *Pythium intermedium* to soil sample 15 (RM1910-S7) (Table 3.5). Previous literature has associated this pathogen with damping-off disease in *Capsicum* species grown for the production of dried chilli (Arora et al., 2021). Interestingly, *P.intermedium* and *P.attrantheridum* are two of the most genetically similar *Pythium* species (Allain-Boulé et al., 2004). The sequence data generated from RM1910-S2 and RM1910-S7 (Table 3.3) was of poor quality. RM1910-S2 produced single reads of 58bp (ITS4) and 84bp (ITS6) and RM1910-S7 produced a single read of 310bp (ITS6). Therefore, it is possible these sequences did not include enough genetic variation to truly distinguish between them.

4.8 Conclusion

Following morphological and molecular analysis of samples collected from twenty sampling sites across the RHS Rosemoor garden, *Phytophthora gonapodyides* was attributed to the lake water sample, and one of five *Pythium species* were attributed to distinct soil samples when a positive correlation was found between the shape of sporangia observed in the sample and the sequence data result. There is no observation of any subsequent correlations, as is clearly shown in figure 3.5 where attributions are mapped across the garden.

It was not possible to compare the data generated in this study with pre-existing observations within the garden as records have been kept in various formats and do not permit direct comparison. The development of a unified record for each disease observation, incorporating historical data to which new data could be added would be a valuable tool in the monitoring of disease over time. The ability to assess and monitor diseases over time periods will be invaluable in assessing the effects of climate change on outbreaks. According to the Met Office (2023), projections associated with a 2°C and a 4°C global warming increase would increase the average maximum daily temperature to 15.67°C and the average minimum daily temperature

to 8.49°C. The average annual rainfall would decrease; however, the intensity would be greater, with a higher potential for severe flooding. Germination temperatures for *Pythium* and *Phytophthora* would still be within these ranges (Agrios, 2005; Martin & Loper, 1999b). Coupling favourable temperatures with standing water would significantly increase the pathogens' presence in the soil, thus increasing the potential of disease outbreaks. Testing a larger number of soil samples at regular intervals from host plants across the garden could provide significant information on the status of oomycetes within the soil at RHS Rosemoor and monitor the impact of climate change within the garden. No consensus could be found in current literature as to how frequently samples should be taken across an area, as in most studies, sampling is taken from plants presenting disease symptoms.

The genomic identification of *Pythium senticosum* from sample 35 in this study, presents an opportunity to potentially identify a novel disease occurrence in the UK. Repeat analysis of the soil to verify the identity of *P.senticosum,* followed by morphological and molecular analysis of root and plant material to establish if this material is infected, should be conducted. Further work should include a Koch's postulates (Rivers, 1937) to confirm if *P.senticosum* is a pathogen of Cornus species.

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