Peronospora sparsa biology and drivers of disease epidemics in boysenberry

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Downy mildew, caused by the biotrophic oomycetes, *Peronospora sparsa,* is a major disease of boysenberries, with crop loss from disease for conventional growers being up to 45% and for organic growers up to 100% in some years. Most boysenberry plant material (including tissue culture propagated plants) are systemically infected with the pathogen. Disease expression by sporulation of naturally infected leaves, stems/canes and calyxes at 15ºC under high humidity in the field was identified as the source of inoculum in the wider Nelson area in 2010 and 2011 with peak spore dispersal in mid-November. A strong relationship between rainfall pattern, humidity and temperature and *P. sparsa* spore dispersal was observed. Spore dispersal was triggered by the frequency (%) of rainy days, RH and warm temperatures (16-23ºC) in early spring where early wet periods with high moisture levels promoting sporulation and a subsequent dry period allowed spore release.

In vitro, the optimum temperature for spore germination, infection, sporulation and lesion expansion were 20ºC (24 h darkness), 15 or 20ºC (12 h/12 h light/ dark), 15 or 20ºC (12 h/12 h light/ dark) under high humidity, respectively. The optimum spore numbers for infection was 200. Similarly *in vivo* evaluations showed that disease expression on young foliage, stems/canes, calyxes/sepals, petals and stamen by symptoms and sporulation was favoured at temperatures ranging from 5-15ºC, under high relative humidity (90-100%) in potted systemically infected boysenberry plants.

An existing nested PCR for detection of *P. sparsa* was optimised and limits of detection determined in different plant tissues. The optimised Plant & Food Research (P&FR) protocol (using modified Aegerter buffer) extracted more *P. sparsa* DNA with a higher purity than the commercial PowerPlant® DNA isolation kit. The optimised nested PCR could detect as little as 0.4 pg of *P. sparsa* genomic spore DNA from a range of asymptomatic boysenberry tissues including primocane tips, leaves, leaf buds, canes/ stems, roots, flower buds, flowers and berries. This was approximately equivalent to 40 spores. The method was improved to a more rapid and robust one step nested PCR method with an equivalent sensitivity for detecting latent

infection of *P. sparsa.* The results showed that the most robust tissues for reliable detection of latent infection were root or crown.

In vitro and *in vivo* evaluations indicated that dryberry is caused by spore infection of flowers or berries by the spores produced on systemically infected canes/ stems, calyx, and petals under favourable environmental conditions. Accordingly two infection pathways resulting in dryberry were identified: 1) spores produced on petals and calyx may infect pollen followed by fertilisation of infected pollen then systemic infection of the drupelets of the developing berry initials, 2) spore infection of drupelets at the red partially ripe stage. Movement of the pathogen from the systemically infected cane to berries across the pedicel and calyx was not observed.

Of the ten fungicides investigated *in vitro*, chlorothalonil, mandipropamid, fluazinam, azoxystrobin and dimethomorph were the most effective to inhibit *P. sparsa* spore germination and infection. *In vivo* evaluations on young disease-free boysenberry plants showed that dimethomorph, azoxystrobin and metalaxyl-M+mancozeb were the most effective at protecting leaves from infection. Three applications of phosphorous acid (PA) reduced both incidence and severity of dryberry whereas, acibenzolar-s-methyl reduced only incidence in systemically infected plants.

A tissue culture protocol was developed for the production of *P. sparsa* pathogen free clean boysenberry propagation material by heat alone. As this method does not rely on treatment of infected plants with fungicides, there is no subsequent risk of fungicide resistant strains developing. Distribution of clean boysenberry planting material in new gardens or replanting areas in existing gardens in New Zealand is an important initial step to avoid disease epidemics in the field.

Keywords: *Peronospora*, spores, fungicides, rain, temperature, humidity, dispersal, PCR, CTAB and dryberry.

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CHAPTER 1

Introduction

1.1 The New Zealand boysenberry industry

Boysenberries originated in California, USA in 1925 (Langford and Mavromatis, 1981) and were first introduced into New Zealand in the early 1940s. Lack of supply of boysenberries to the USA in 1978/79 created an opportunity for New Zealand boysenberry growers, resulting in an increase in production in the early 1980s and the establishment of the New Zealand Boysenberry Development Council (NZBDC). Currently, there are approximately 21 boysenberry growers in New Zealand across the North and South islands (FreshFacts, 2013) and boysenberries are the main bramble species grown in New Zealand (G. Langford, pers. comm., 2011). The main areas of cultivation are Nelson (Figure 1.1) and Marlborough due to the suitable climate of cool winters, temperate springs and autumns, and dry summers. Boysenberries are also grown in the Hawke's Bay/Poverty Bay/Wairarapa and Waikato/Bay of Plenty (Langford and Mavromatis, 1981).

Figure 1.1 Two boysenberry orchards in the Nelson region in (A) Motueka in the spring and (B) Richmond in the summer, 2011.

New Zealand is the world's major producer and international marketer of boysenberries. The annual production is estimated at 2,400 t based on the 2009/2010 harvest, with approxiamately 900 t sold locally (G. Langford, pers. comm., 2011). Of the total exports, 1,267 t are exported as frozen (block frozen and individually quick frozen) forms and 233 t as puree and juice concentrate. However, in the 2012/2013 season, boysenberry fresh fruit production further increased to 3,100 t earning NZ$4.8 M through domestic sales: however, the sales value from the export market was not reported. For processed fruit, sales values from the domestic and export market were NZ$2.7 M and $3.5 M in 2012 and 2013, respectively (FreshFacts, 2013).

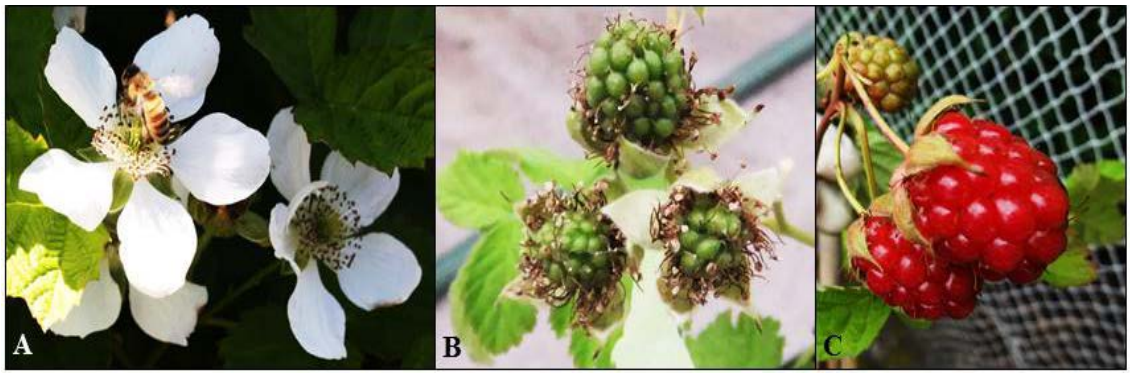
Boysenberries are exported worldwide, with the majority going to Australia, Denmark, Finland, UK, Germany, the Netherlands and Norway. New Zealand also exports to countries such as Fiji,

India, Japan, Malaysia and Tonga (Berryfruit Export New Zealand Ltd, 2004-2011; G. Langford, pers. comm., 2011).

1.2 Boysenberry production in New Zealand

1.2.1 Varieties

There are two types of 'true' boysenberry grown in New Zealand: thorny types, which have dark red berries, are 3.5-4.0 cm long with vigorous plant growth, and semi-thornless types, which have slightly smaller fruit and less vigorous plant growth (Toleman and Storay, 1985a). "Youngberry" is also grown, and in New Zealand both these and the 'true' boysenberry are referred to as "boysenberry". The exact lineage of the boysenberry is unknown (Wood *et al.*, 1999). The boysenberry (*Rubus ursinus*) is a hybrid of 'Himalayan Giant' blackberry (*R. procerus*) and either youngberry or 'Cuthbert' raspberry (a hybrid between *R. idaeus* and *R. strigosus)* (Langford and Mavromatis, 1981; Castillo *et al*., 2006). Youngberry is a crossbreed of brambles 'Phenomenal berry' (a type of Loganberry) and 'Austin Mayes' (a trailing blackberry cultivar) (Wood *et al.*, 1999). The canes of both boysenberry and youngberry have long thorns and are strong, pliable and can be extended over a framework of wire for commercial cropping. Boysenberries are self-fertile and pollination by other berry cultivars can decrease fruit set (Hall and Langford, 2008). As boysenberry pollen cannot be disseminated by wind, pollination is mostly by honey, bumble and wild bees (Figure 1.2A). A large active bee population, with up to five hives per hectare is required to ensure efficient pollination (KingGrapes 2012). The fruit which develop are composed of a cluster of loosely packed drupelets (Figure 1.2B and C) attached to a mid-column of receptacle tissue (Vicente *et al*., 2007).



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Boysenberry fruit are similar to those of the youngberry but are larger in size. Although boysenberries are deep wine-red in colour, youngberries ripen to black. Additionally, ripe youngberry fruit are sweeter (Wood *et al*., 1999). Youngberries are also known as 'early boysenberries' as they ripen slightly earlier (7-10 days) than boysenberries and are sufficiently similar to boysenberries (Langford and Mavromatis, 1981; Hall and Langford, 2008; G. Langford, pers. comm., 2011). Similar to other members of the *Rosaceae* family, such as

raspberries and blackberries, fruit is only produced on second year canes known as 'floricanes'. The new first year canes, which do not produce flowers, are known as 'primocanes' (Toleman and Storay, 1985a).

1.2.2 Site selection

Prior to planting boysenberry, site selection must be considered based on 1) suitable soil type, 2) labour cost, 3) elevation off the ground, 4) exposure to weather conditions, 5) level of infestations of perennial weeds, 6) proximity to transport services, and 7) adequacy of shelter (Toleman and Storay, 1985a). Soil type does not seem to affect boysenberry cultivation since they can be grown on a range of soil types, including heavy clay and stony silt loam (Langford and Mavromatis, 1981; Hall and Langford, 2008). Waterlogged soils or those with fluctuating water tables need to be maintained at optimum moisture by tile drainage. Access to a clean water supply for irrigation is required in drier climates, especially in summer. Hand picking is costly, thus fairly flat land of sufficient length to allow long rows is preferred to facilitate mechanical harvesting. Cold pockets should also be prevented by avoiding low lying land prone to heavy frosts. Heavy infestations of perennial weeds require herbicide application before planting and increases establishment costs. Ideally, the site should also be established close to transport services, cannery or freezing facilities. Shelter is important, especially in New Zealand, during the cropping period. An outer shelter of at least 10 m height and an internal shelter of 5 m are recommended to protect against strong winds (Toleman and Storay, 1985a).

1.2.3 Climate

Boysenberries are winter cold sensitive. Therefore, high sunshine hours, low wind speeds and protection from severe frosts are ideal conditions for optimum growth of boysenberries. Cold temperatures during the blooming period result in poor fruit set and reduced harvest yields. High temperatures during flowering result in low grade fruit set while high temperatures during fruiting cause white drupelet disorder. Low atmospheric humidity at this time can reduce fruit size (Hall and Langford, 2008; Langford and Mavromatis, 1981). In New Zealand, the boysenberry growth cycle begins with bud break in early September, flowering mostly during October (for approximately one month) and fruit harvest from mid-December to mid-January. It is estimated that flowering to fruiting takes approximately 239 GDD (growing Celcius degree days), which is approximately 55 days in Nelson and approximately 2 days longer in Canterbury (G. Langford, pers. comm., 2011).

1.2.4 Propagation

Propagation of boysenberries is mainly via various vegetative propagation methods. Seeds are generally not used, as seed grown plants rarely grow true to type. Methods commonly used include tip layering, softwood tip and semi-ripened wood cuttings, sucker propagation, various cuttings (Toleman and Storay, 1985a) and tissue culture (Wu *et al*., 2009).

Tip layering produces strong plants with strong rooting systems quickly, however a limited numbers of plants are produced. This method is also prone to nematode and dryberry infection (Toleman and Storay, 1985a) due to the ease of transmission to adjacent daughter plants (Jennings, 1988, Wood and Hall, 2001 cited in Wu *et al*., 2009). Cane tips are buried in the soil (10-15 cm depth, in late summer if quick growth is needed) or in soil in polythene planter bags buried in the soil (if later planting is desired). The rooted plants can be moved to permanent positions for planting (Toleman and Storay, 1985a).

Softwood tips and semi-ripened wood from canes from the current season's growth can be rooted using mist units with bottom heat of 20ºC. A rooting medium containing 50% pumice or sand and 50% peat is generally recommended. Softwood tips about 10 cm in length are buried in the rooting medium to a depth of 7 cm. For semi-ripened wood cuttings, cane sections are cut to include at least two buds and treated with a rooting hormone enhance the rooting process. Rooting is rapid under mist propagation (Toleman and Storay, 1985a). Softwood tip and semi-ripened wood cutting can also be propagated by placing in polythene tunnel propagating beds filled with a 50:50 peat and sand mix. These tunnels are generally 80 cm wide of any desired length and lined with polythene. The tunnel provides high humidity by acting as a mist propagation unit, which enables cuttings with leaves to be planted without wilting. Cuttings are placed in these beds in summer.

Sucker growth can be used as propagating material and placed in polythene tunnel beds. Rather than using cuttings from fruiting canes, softwood tips and stem cuttings 1-2 nodes long are cut from sucker growth (Figure 1.3). These are cut at the base directly beneath a node, the base dipped in rooting hormone powder, followed by inserting the cuttings in the tunnel bed and watering well. The cuttings are placed on the beds in summer. Rooting with this method is reported to be approximately 75% for boysenberries (Toleman and Storay, 1985a).

Root leaf buds, tips and semi-ripened wood cuttings treated with hormone rooting powder and placed in cold frames can also be used for propagation. However, rooting is slower and often results in a reduced numbers of well-established plants (Toleman and Storay, 1985a).

Figure 1.3 Well rooted softwood stem cutting of boysenberry (cv. Mapua) of 1-2 nodes long, propagated in a plastic tunnel bed by mist propagation at the Lincoln University nursery.

Traditional propagation by cutting, layering or suckering is still used commercially and successful propagation depends on adequate plant growth and favourable seasonal conditions. It can be difficult to induce rooting using traditional methods (Wu *et al*., 2009). Tissue culture provides a more rapid method for *in vitro* propagation and enables the production of large numbers of genetically identical and virus-free *Rubus* plants using limited space and without being affected by seasonal variation (Zimmerman, 1991, Sobczykiewicz, 1992 cited in Wu *et al*., 2009). Thus, tissue culture is increasingly being used to commercially propagate boysenberry plants in New Zealand using a protocol adapted from Wu *et al*. (2009). Single-bud stem cuttings (1-2 cm) taken from the young cane tips from mother plants are used as the explant materials. These are pre-rinsed in warm water with antimicrobial soap before being sterilised in 0.6% sodium hypochlorite solution. They are then rinsed with sterile water under sterile culture conditions and kept submerged in sterile water prior to being used to initiate tissue culture. They are first placed in an initiation culture medium comprising 1/3 strength MT (Murashige and Tucker; Wu *et al*., 2009) basal medium with supplements then transferred into multiplication medium, comprising 1/3 strength MT basal medium with supplements and charcoal. All cultures are maintained in a growth room at 22ºC under cool-white fluorescent light of reduced light intensity. Rooting medium is not required for boysenberry as they can be rooted in the multiplication medium (B. Shunfenthal, pers. comm., 2011).

1.2.5 Irrigation

The irrigation requirements for boysenberries depend largely on the water holding capacity of the soil and local rainfall totals. Sandy soils should be watered little and often, clay soils less frequently.

Sprinkler and trickle systems are the major irrigation methods used in New Zealand for boysenberries as flood irrigation is rarely used for brambles. Sprinkler irrigation is more suitable for very light and stony soils which, for permanently installed sprinklers, can also provide frost protection. Trickle irrigation is ideal for semi-permanent crops. For most boysenberry blocks, irrigation rates for trickle systems provide up to 15-20 L/plant/day depending on plant spacing, soil type and season (Toleman and Storay, 1985a).

1.2.6 Pruning

In New Zealand boysenberries are pruned after fruiting. Pruning should not be delayed as the new canes can become tangled with the old canes, making removal of the old canes difficult. There are three main methods of pruning as follows:

1. Pruning and removal of old canes immediately, followed by burning of the old canes. Using this method new canes can be maintained free from pests and disease as potentially infected old canes are removed; however, soft new canes can be damaged by the removal of old canes.
2. Cutting the old canes but leaving them on the wires with removal after they have dried up. This minimises the possibility of damaging the new canes at removal; however, pest and diseases are able to spread from the old canes to new canes.
3. Pruning the entire plant to the ground post-harvest (end January). This method is suitable only if the plants are vigorous and have sufficient growth (Toleman and Storay, 1985b).

1.2.7 Desuckering

New canes that emerge on fruiting boysenberry plants interfere with harvesting and increase the lushness of growth, contributing to high humidity (less sun penetration) which increases disease problems. Thus, desuckering by hand or spraying with a mixture of dinoseb and oil is recommended in New Zealand. Spraying is conducted when suckers are 10-15 cm long. In addition, the base of the trained cane (up to 40 cm) can be sprayed to remove the spurs which will hang on the ground. Desuckering is recommended only on plants with vigorous growth in well-established blocks (Toleman and Storay, 1985b).

1.2.8 Training

Training involves lifting of the new canes (primocanes) off the ground onto a trellis, which facilitates both the interception of light by the plant and harvesting. Also, the physical separation of the primocanes and floricanes (fruiting canes) allows effective management of

both cane types. Boysenberry canes are trained using two common methods in New Zealand: fan training (Langford and Mavromatis, 1981; Toleman and Storay, 1985b) and weave training (tumble systems) (Toleman and Storay, 1985b). Both provide support for weak and relatively supple boysenberry canes. The main differences between the two methods are the cane lengths and amount used, which will also affect the amount of light intercepted early in the season. In early spring in most of the USA and New Zealand plantings new canes and lower laterals are burnt off with herbicide to enable more efficient machine harvesting (Langford and Mavromatis, 1981). For fan training all cane material 10-15 cm above the top wire is removed, then canes are spaced 10-15 cm space along the wire and held tight by alternating the cane between the two top wires (Figure 1.4A, C, and D). The end of the cane is then wound around the top wire or tucked into the neighbouring cane to hold it in place (Toleman and Storay, 1985b). This facilitates even distribution of the fruiting canes, increasing fruiting potential due to improved light interception. In addition, fan training improves spray access and makes harvesting easier. With weave training, single or loose groups of 2-3 canes are trained by placing on the top wire and training down to an intermediate or bottom wire and sometimes (depending on growth) up again (Figure 1.4B). The canes are tied tightly into a single layer on the trellis. Canes need to be spaced on the trellis as evenly as possible. This is generally suitable for first year orchards since there are often not enough canes to cover the trellis using the fan method (Toleman and Storay, 1985b).

1.2.9 Harvesting

There are two methods of harvesting: hand harvesting and machine harvesting (Toleman and Storay, 1985b). Hand harvesting is the traditional method and is labour intensive, requiring 10-15 pickers/ha for the whole season. Even experienced pickers can only harvest 200 kg/day, with selective picking of slightly smaller berries necessary for canning resulting in lower volumes being picked. The main advantage of machine harvesting is the lower harvesting cost due to the lower labour requirements. However, for this to be economical the area should be large enough for machines. Mechanical harvesting can cause damage to next year's fruiting canes resulting in a reduction in subsequent harvests. The estimated pick volume for a machine harvester is 10 t/ha and 0.5 t/h (Toleman and Storay, 1985b).

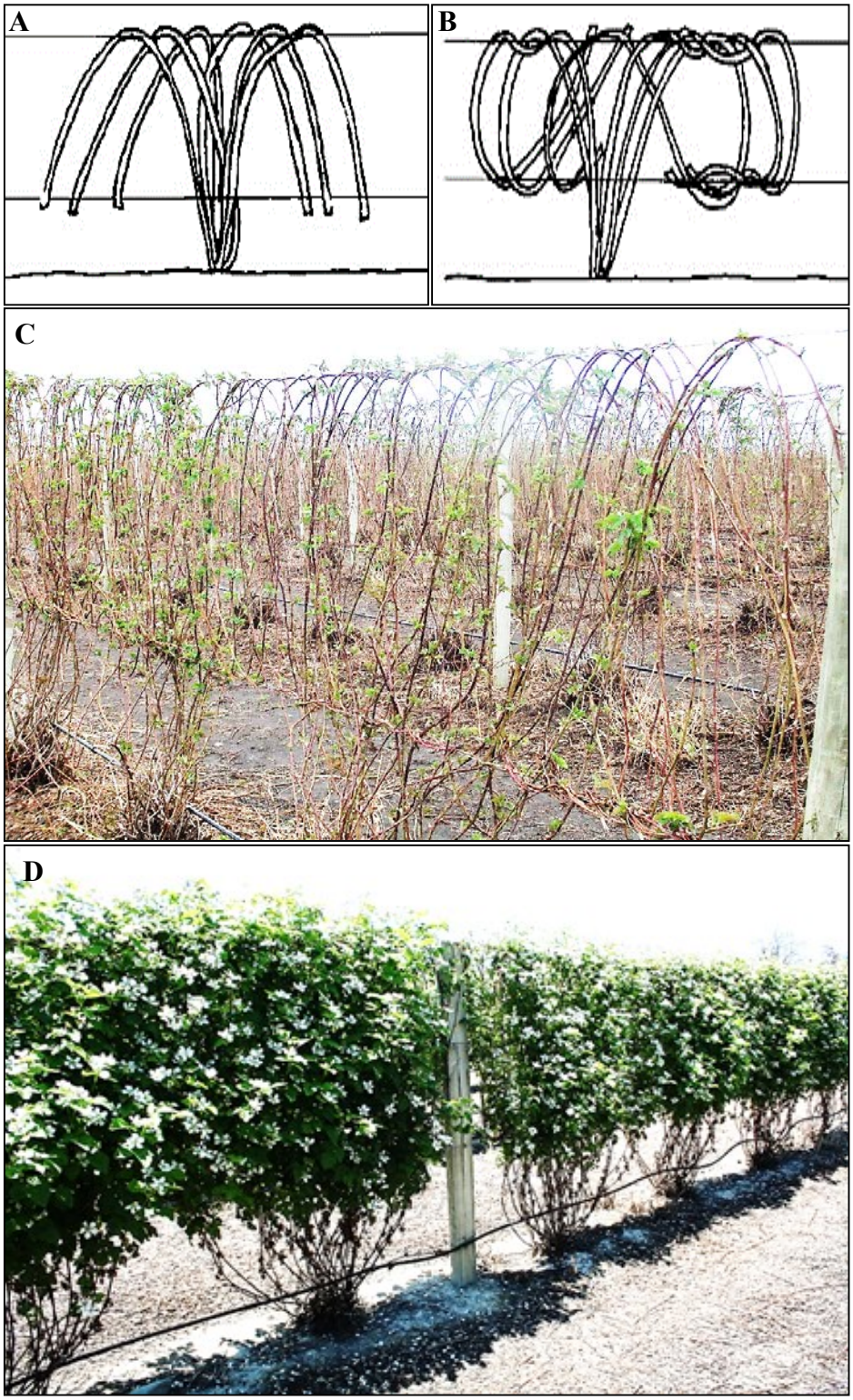

Figure 1.4 Boysenberry cane training methods used in New Zealand. (A) fan training and (B) weave training. Fan trained boysenberry canes in the (C) spring and (D) summer. (Source of Figures A and B: http://www.depi.vic.gov.au/agriculture-and-food/horticulture/fruit-and-nuts/berries/cultivated-blackberries-pruning-and-training).

1.3 Health benefits of boysenberries

Consumption of boysenberries is reported to have a range of health benefits (Bushman *et al*., 2004). Boysenberries have high levels of vitamin C (6.06 mg/100 g of fruit), dietary fibre (1.75 g/100 g of fruit) and ellagic acid (5.98 mg/g of dry weight) that have been shown to prevent or reduce cancers. Ellagic acid is a phenolic compound that possesses powerful anti-viral and anti-bacterial properties in addition to anti-carcinogenic properties. The ratio of free ellagic acid to total ellagitannins in boysenberries is particularly high, which has been suggested to increase the availability of ellagic acid for absorption into the body (Oregon Raspberry and Blackberry Commission, 2008). The ORAC value (Oxygen Radical Absorption Capacity), used to measure antioxidant content of foods, of boysenberries is 42 μmoles/g, double that of blueberries. Anthocyanins, found at relatively high concentrations (120-160 mg/ 100 g fruit) act as antioxidants and contribute to the deep, dark colour of boysenberries (Oregon Raspberry and Blackberry Commission, 2008). Boysenberries are flavour enriched fruits that contain a large concentration of sugar, approximately 12^{o} Brix (Hall and Langford, 2008).

1.4 Downy mildew disease (dryberry disease)

Boysenberries are prone to disease outbreaks. The three main diseases detrimental to boysenberry cultivation in New Zealand are witches' broom caused by *Cercosporella rubi*, grey mould caused by *Botrytis cinerea*, and downy mildew caused by *Peronospora sparsa* (Hall and Langford, 2008). Of these, downy mildew is considered the most economically important (Ellis *et al*., 1991) and will be the focus of this study.

Downy mildew disease, caused by *Peronospora sparsa* Berk (synonym *P. rubi*) (Tate and Van Der Mespel, 1983) is a serious issue for boysenberry growers in New Zealand. In 2001/02 in the Nelson region, downy mildew fruit infection reduced boysenberry yield by approximately 50% for crops grown under conventional management (Richards, 2002), with total losses in 2002 valued at NZ$1.8 M (Dodd *et al*., 2007). For organic growers the situation was worse, with a total loss of fruit for that season. Severe fruit infections were also reported in the Nelson region in 2005-2006 and 2006-2007, incidence of fruit infection being 86% and 90%, respectively (Kim *et al*., 2014). More recently in Whakatane downy mildew was estimated to reduce yields by up to 25% in 2009-2010 (C. Julian, pers. comm., 2011). The threshold of downy mildew fruit infection at which serious economic losses occurs in the New Zealand boysenberry industry has been reported to be >10% (Kim *et al*., 2014). Walter *et al*. (2004) reported that high disease pressure (with up to 100% of the fruit infected) could occur in wet climates and that even in relatively dry climates optimum disease conditions could cause extremely rapid increases from < 5% to >75% incidence within 6 days.

Peronospora sparsa also infects other crops such as rose (*Rosa* spp.), blackberry (*Rubus fruticosus*), arctic raspberry (*Rubus arcticus*) (Lindqvist *et al*., 1998) tummelberry (*Rubus loganobaccus* L. H. Bailey), strawberry (*Fragaria ananassa*) and caneberry (*Rubus* spp. L.)

(Aegerter *et al*., 2002; Bolda, 2009; O'Neill *et al*., 2002; Kim *et al*., 2014). In Finland between 1994 and 1996, 50-100% yield losses were reported in arctic raspberry due to dryberry (Lindqvist *et al*., 1998), with the causal agent reported as *P. sparsa* (Lindqvist-Kreuze *et al*., 2002). Since the early 1990s, bare-root nursery cultivation of roses in California has also been affected by downy mildew caused by *P. sparsa* (Aegerter *et al*., 2003). In Colombia, the leading exporter of flowers to the USA (Asocoloflores, 2005 cited in Castillo *et al*., 2010) including roses which make up 48% of total exports, downy mildew caused by *P. sparsa* is the biggest disease problem (Filgueira and Zambrano, 2014). Losses due to *P. sparsa* infection are commonly reported to be 8% to 10% of production (Castillo *et al*., 2010; Gómez and Filgueira-Duarte, 2012). Similarly, *P. sparsa* is a major issue for rose production in Mexico, with up to 100% of flowering rose stems being lost due to the disease (Álvarez-Romero *et al*., 2013). The disease is common in both field and greenhouse grown plants (Debener and Byrne, 2014) and affects productivity and quality as well as increasing production costs, mainly due to the need for additional fungicide applications (Castillo *et al*., 2010; Álvarez-Romero *et al*., 2013).

The pathogen has a worldwide distribution, being identified from Africa (Egypt, Morocco, S. Africa, Zimbabwe), Asia (Brunei, Iran, Israel, Japan, Mauritius, Philippines), Australasia (Australia, New Zealand), Europe (Austria, Britain (incl. Jersey), Bulgaria, Czechoslovakia, Estonia, France, Germany, Greece, Iceland, Italy, Latvia, Netherlands, Portugal, Poland, Romania, Sweden, Switzerland, Yugoslavia, USSR, N. America (Canada, USA), S. America (Argentina, Brazil, Colombia, Venezuela) (Francis 1981; Gómez and Filgueira-Duarte 2012); and C. America (Mexico) (López-Guisa *et al*., 2013) (Figure 1.5).

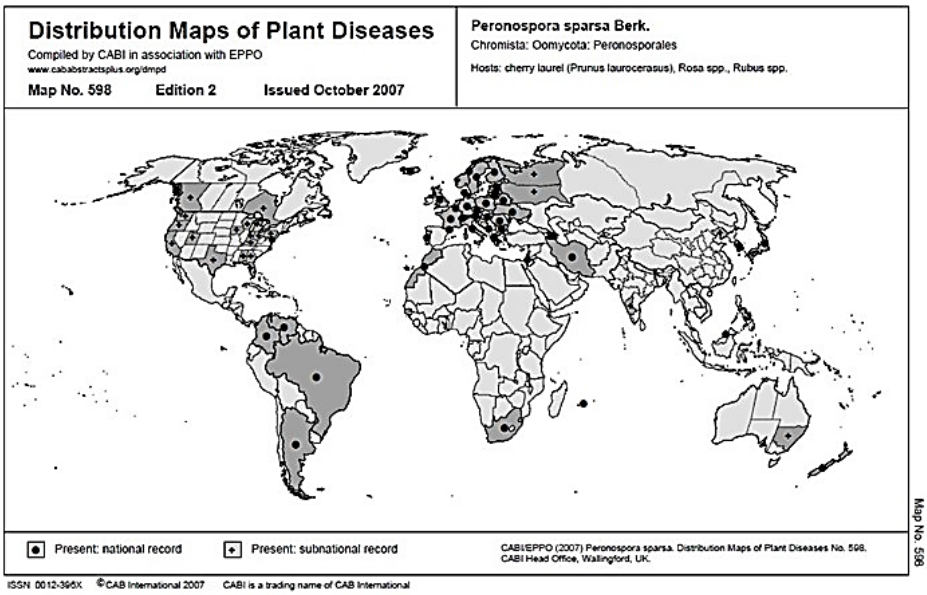

Figure 1.5 Distribution map for *Peronospora sparsa (CABI/EPPO (2007). Distribution Maps of Plant Disease No. 598, CABI Head Office, Wellingford, UK)*

1.5 Downy mildew symptoms on boysenberry

In New Zealand, leaf infections first appear as purple, angular lesions in early spring (from mid-September onwards). These lesions are visible as blotches between the veins or along the midrib and major veins. Over time, infections can be seen to expand along the veins. Distortion of the leaves and severe shortening of shoots may result due to severe infections of the primocane. On the undersides of the leaves, blotches appear as pink to grey areas of sporulating tissue which are short-lived (Richards, 2002). The spores infect expanding leaves and developing flowers and fruit. Tate (1981) suggested that leaf lesions expanding along the veins are due to systemic infections (Figure 1.6A) whilst discrete spots on the leaves result from spore-initiated infections (Figure 1.6B). The spores, which are disseminated by air are produced during wet nights in spring and early summer. The spores land on the foliage and developing flowers, and later the fruit (Richards, 2002). In raspberry, blackberry and hybrids, such as boysenberry, early infection of green fruit causes premature reddening, shrivelling and rapid hardening of the fruit, with the fruit also becoming dull in appearance compared to the healthy fruit (Figure 1.6C and D); hence the disease is also known as dryberry. Infection at later stages of berry development often results in only a part of the berry being infected. In addition, distinct reddening of the pedicels can be observed (Ellis *et al*., 1991).

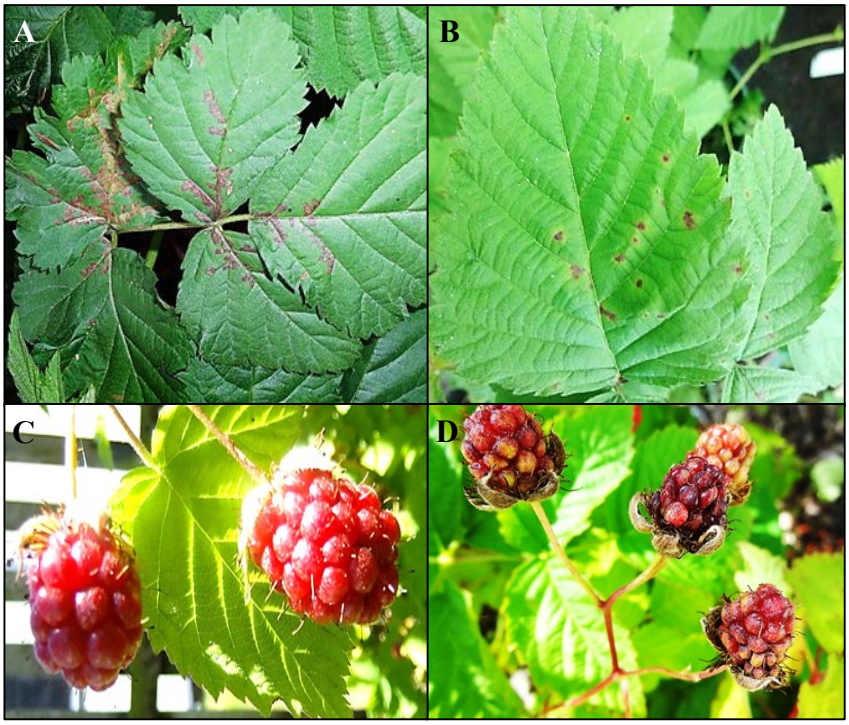

Figure 1.6 *Peronospora sparsa* infected tissues. (A) Leaf lesion expansion following veins due to systemic infection and (B) discrete leaf spots caused by spore infection. (C) Healthy fruit and (D) infected fruit showing typical dryberry symptoms.

1.6 Causative agent - *Peronospora sparsa*

Downy mildew diseases are caused by a number of species from the oomycetes class *Bremia*, *Peronospora*, *Plasmopora* and *Sclerospora* (Hukkanen *et al*., 2006). Though the higher level classification of oomycetes has not been fully resolved, they belong to the kingdom Stramenopiles, phylum Oomycota and class Oomycetes. Oomycetes are more closely related to diatoms and seaweeds than the filamentous forms of the kingdom Mycota (Thines, 2014). The Oomycota contain two main orders: the Saprolegniales and the Peronosporales, with many serious plant pathogens in the latter, such as *Phytophthora*, *Pythium* and *Peronospora*, which include the downy mildew pathogens (Thines, 2014).

In New Zealand and California, the causal agent of downy mildew of *Rubus* spp. was identified as *P. sparsa* due to the presence of sporangia (sporangiospores) and the size of its oospores (Ellis *et al*., 1991). In Europe, the pathogen causing downy mildew of *Rubus* species was initially identifed as *P. rubi* due to the lack of oospores (Ellis *et al*., 1991). Breese *et al*. (1994) showed in their *in vitro* experiments that isolates from either *Rubus* or *Rosa* spp. can infect the other host and suggested that *P. sparsa* and *P. rubi* are conspecific or variants of the same species. Further, Williamson *et al*. (1998) reported that the sequences of the internally transcribed spacer (ITS) of the rRNA gene region of isolates of *P. sparsa* were completely identical to sequences from *P. rubi*. The pathogen is now referred to as *P. sparsa* due to nomenclature priority.

Peronospora species are obligate specialised biotrophs. They parasitise host cells by the production of haustoria and the intracellular spaces within the plant tissue is often completely colonised by the mycelium. They produce asexual spores (sporangia/conidia) (Agrios, 1997) that are borne on the tips of dichotomously branched sporangiophores (Figure 1.7), but unlike many other downy mildew causing genera, they do not produce motile asexual spores (zoospores) (Hukkanen, 2008). Since the sporangia of *Peronospora* spp. germinate directly to produce a germ tube, sporangia are also referred to as conidia (Agrios, 1997). The sporangia are disseminated by wind or free water, initiating secondary infections on new leaves, flowers and fruits (Hall, 1989; Ellis *et al*., 1991; Hukkanen, 2008). *Peronospora sparsa* sporangiophores measure 490-600 x 4-6 µm and are branched 3-4 times (Figures 1.7A, C and D). Branch ends are 12-16 µm long, thin and diminished. The sporangia, 18–24 × 16–20 µm in size (Figure1.7 B), are ellipsoidal to near spherical and pale yellow in colour. Short stalks can be seen on some detached sporangia. Oospores are 22-30 µm in diameter (Figure 1.7E and F). The outer oospore wall is hyaline, 2 µm thick and often folded (Francis, 1981).

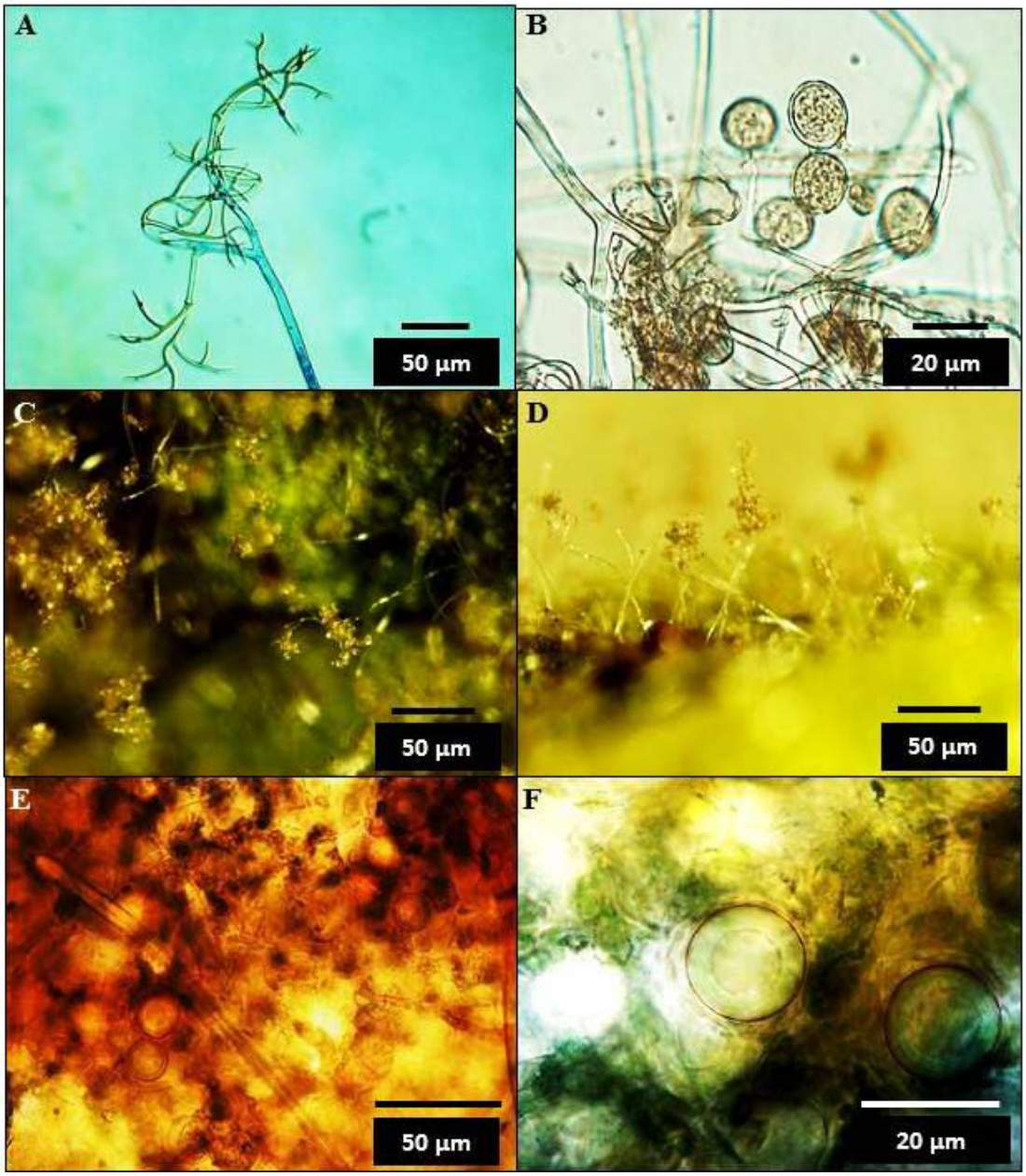

Figure 1.7 *Peronospora sparsa* (A) sporangiophore and (B) sporangiophores with sporangia (stained with lactoglycerol cotton blue). (C) and (D) stereomicroscopic views of sporangia on sporangiophores on the abaxial surface of infected boysenberry (cv. Mapua) leaves, oospores in boysenberry leaf mesophyll tissues (E) and (F).

1.7 Epidemiology of downy mildew

Although the epidemiology of downy mildew of boysenberry has not been widely studied, some research has been conducted on *P. sparsa* on rose species (Fox, 1996; Aegerter *et al*., 2002; O'Neill *et al*., 2002).

Beckerman (2009) stated that wet conditions with high relative humidity (≥85%) allowed development of the downy mildew pathogen on a suitable host. The general disease cycle of *P. sparsa* on raspberry, blackberry and hybrids such as boysenberry has been outlined by Ellis *et al*. (1991). The disease is favoured by warm temperatures between 18 and 22°C and wet

weather conditions generally found during mid to late spring and autumn in both New Zealand and North America (Ellis *et al*., 1991). Similar temperatures of 15 to 20ºC (Aergerter *et al*., 2003) or 15 to 18°C (Filgueira and Zambrano, 2014) were reported to be the optimum for infection of rose leaves by *P. sparsa.* Further, recent studies on downy mildew disease of roses conducted in Colombia showed that *P. sparsa* displayed adaptation to extreme temperatures, being able to initiate new infections over a wide range of temperatures (4 to 33°C) (Filgueira and Zambrano, 2014).

Peronospora sparsa is reported to overwinter as mycelium within the roots, crowns, buds and canes (Tate, 1981). As the primocanes resume growth in the spring, the growth of the pathogen is suggested to closely follow the apical shoot growth, infecting the unfolding leaves. The pathogen is restricted to the outer cortex parenchyma and is not found in the vascular tissue. Wet or humid conditions when leaves are unfolding are required for systemic leaf infections (Tate, 1981). Sporangia are produced on these infected leaves during cool wet nights, with most produced on the lower side (abaxial surface) of the leaves. Symptoms usually appear within 11 days of infection, with sporulation occurring 5-11 days later (Aegerter *et al*., 2003; Walter et *al*., 2004). In rose, the optimal temperature for *P. sparsa* infection of detached leaves was demonstrated to be between 15 and 20ºC, whereas for colonisation it was 20 to 25ºC (Aergerter *et al*., 2003). However, recent studies on roses in Colombia (Filgueira and Zambrano, 2014) reported that *P. sparsa* can be infective over a broader range of temperatures, with sporulation occurring from 4 to 33°C. However, Filgueira and Zambrano (2014) also reported that for spore production the optimum temperature range was between 15 and 18°C. Only 2 h of leaf wetness was required for infection at the optimal temperatures when the RH levels were maintained between 90 and 100% (Aegerter *et al*., 2003). However, confirmation of these results by inoculation of whole plants has not been reported.

Restrepo and Lee (2007) observed that incidence of *P. sparsa* infection on inoculated glasshouse roses decreased as the length of time (6-18 h) increased when exposed to low RH (30-44% RH) during the day time, whereas the incidence increased when exposed to increasing time (6-18 h) at higher RH (72-86% RH). However, the interaction between RH and temperature for *P. sparsa* infection of boysenberry has not been well studied. Whether environmental factors have a similar effect on infection of boysenberry by *P. sparsa* is not known. Kim *et al*. (2014) have developed a model to predict disease risk by identifying weather patterns associated with high incidence of downy mildew infection on boysenberry in the field. However, the model is based on disease incidence in the field and not on detailed studies on the effect of environmental conditions on *P. sparsa* infection of boysenberry. Photoperiod and light intensity have also been shown to affect *P. sparsa* sporulation on rose (Soto and Filgueira, 2009). Continuous exposure to light inhibited sporulation and resulted in the production of atypical small sporangia, with a photoperiod of 8-16 h at 560 lux resulting in maximum sporulation, and 12 h at 790 lux also resulting in moderate sporulation levels.

The significance of oospores in transmission of *P. sparsa* is unclear (Francis, 1981). Tate (1981) reported that oospores were only found in the outer dead cortex of systemically infected roots, with no oospores observed in any aboveground tissue. However, Tate (1981) only examined these tissues in spring. Based on these observations, he suggested that in boysenberry oospores may be involved in soil transmission, causing infection of healthy plants planted into sites where infected boysenberries had previously been grown. However, in the annual disease cycle under New Zealand conditions, he did not consider oospores to have a major role. Annual disease was suggested to initiate from internally infected shoots produced from the canes, crowns, roots and buds where the pathogen primarily overwinters. However, Hall and Shaw (1982) observed oospores in infected leaves and sepals collected in November and early December in New Zealand, although none were seen in plant material collected in summer. Oospores were also induced to develop on artificially inoculated leaves incubated under *in vitro* conditions (Breese *et al*., 1994; Williamson *et al*., 1998). A study by Xu and Pettitt (2004) to determine the possible modes of overwintering of *P. sparsa* on rose in the UK suggested that systemic infection is unlikely, with oospores likely to be the primary overwintering inoculum rather than mycelium or any other pathogen structures. However, further research is required to confirm these results as well as the factors affecting the germination, viability and pathogenicity of oospores (Wallis *et al*., 1989).

Plants propagated by either cuttings or tissue culture can be systemically infected and potentially introduce the disease into new growing regions as the symptoms are not visible until after the plants have been released for growing (Wallis *et al*., 1989; Williamson *et al*., 1998). Therefore, development of a robust and sensitive method for detection of systemic infections by *P. sparsa* is required.

1.8 Identification of *Peronospora sparsa*

The biotrophic nature of *P. sparsa* does not provide a means of cultivation on artificial media. Also, overgrowth by other fungi on symptomatic plant samples may mask the presence of the target pathogen (Lindqvist *et al*., 1998). In the absence of symptoms, detection of the pathogen by general microscopy is difficult, with the mycelial structure of different fungi being similar (Aegerter *et al*., 2002). Sporulation by *P. sparsa* on infected tissues is often not seen, with the pathogen often being systemic and not expressing symptoms. To overcome these problems, diagnostic techniques for detection and quantification of the pathogen's DNA have been developed (Lindqvist *et al*., 1998).

1.8.1 Polymerase Chain Reaction (PCR) for detection of *Peronospora sparsa*

Polymerase chain reaction (PCR) based methods have been widely used to detect plant pathogens (Lindqvist *et al*., 1998; Aegerter *et al*., 2002; Kernaghan *et al*., 2008; Sundelin *et al*., 2009). Amplification of target nucleic acid sequences is performed using selected primers (McCartney *et al*., 2003). The ribosomal RNA (rRNA) gene region is the main nucleic acid

sequence used for identification of plant pathogenic fungi and oomycetes (Robideau *et al*., 2011). The ribosomal subunits in the rRNA region are encoded as repeat units in tandem arrays in the chromosome. Each repeat unit contains a transcribed region and a non-transcribed spacer region. The transcribed region involves three genes: the large subunit gene (28S), the small subunit gene (18S) and the 5.8S gene, all of which are highly conserved (Figure 1.8) and separated by internal transcribed spacer (ITS) regions (McCartney *et al*., 2003). The length and sequence of ITS regions can vary between species due to the rapidly evolving nature of these regions (White *et al*., 1990 cited in Ristaino *et al*., 1998). Lee and Taylor (1992), in a study using a *"Phytophthora palmivora* complex" showed that there is more ITS sequence variation at the inter-species level, with low intra-species variation. Therefore, the ITS region has become the most widely sequenced DNA region for identification of fungi/oomycetes to the species level. Furthermore, the ITS sequences of oomycetes are dissimilar to those of true fungi (Ingram and Robertson, 1999; Aegerter *et al*., 2002) and have been used for the detection of *P. sparsa* DNA (Lindqvist *et al*., 1998; Aegerter *et al*., 2002; Sundelin *et al*., 2009).

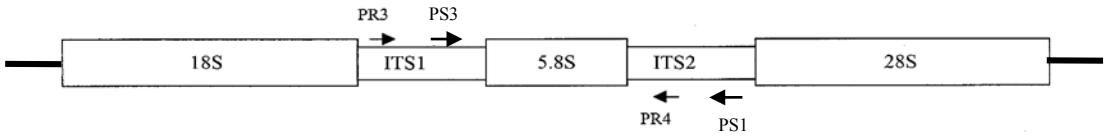

Figure 1.8 Schematic representation of the rRNA gene region of fungi/oomycetes. Open boxes represent the ribosomal genes. Arrows representing the position of *Peronospora sparsa* specific primer pairs, PR3, PR4 (Lindqvist *et al*., 1998) and PS3, PS1 (Aegerter *et al*., 2002) in the ITS regions are also indicated.

To achieve amplification of target DNA from a pathogen, suitable primers need to be designed. When designing the primers, the conserved regions should be targeted in order to detect the presence of similar genes among species, genera, families or kingdoms. Genes in the rRNA gene cluster (18S, 5.8S and 28S gene regions) are highly conserved and universal primers designed for these regions can amplify DNA from any fungus/oomycete (Fox, 1993). The design of species-specific PCR primers enables amplification of the target genes only from particular species.

1.8.2 Development of species specific PCR for *P. sparsa*

Lindqvist *et al*. (1998) reported that ITS1 and ITS2 sequences were highly conserved in *P. sparsa* isolates from arctic bramble and designed two primers, PR3 and PR4 (Figure 1.8) which amplified a 560 bp product only from *P. sparsa* and *P. sparsa*-infected arctic bramble. The detection sensitivity was 0.25 pg of *P. sparsa* DNA. Similarly, Aegeter *et al*. (2002) used two primers (PS1 and PS3) designed to amplify the ITS region of *P. sparsa* and used these to detect *P. sparsa* in rose tissue. This PCR could detect 2.0 pg *P. sparsa* DNA. A nested PCR method

was developed by Dodd *et al*. (2007) using two primer pairs, ITS4, ITS5 and PR3, PR4, in a nested PCR approach. The sensitivity of this method was not stated, although it was mentioned that this nested PCR method had significantly increased sensitivity compared with the previous standard PCR method. Nested PCR generally improves sensitivity of detection relative to standard PCR, especially when there is a low amount of target DNA (Gupta *et al*., 2013).

The published PCR methods (Lindqvist *et al*., 1998; Williamson *et al*., 1998; Aegerter *et al*., 2002) for detection of *P. sparsa* infection of *Rubus* and *Rosa* species are qualitative rather than quantitative. A quantitative PCR (qPCR)/real time PCR (RT-PCR) method was developed by Hukkanen *et al.* (2006) to enable the quantification of *P. sparsa* infection in plant tissue and could detect as little as 37 fg of *P. sparsa* spore DNA. Primers were designed to amplify a 94 bp and 140 bp fragment for *P. sparsa* (targeting the ITS1 region) and infected host plants (targeting the 5.8S region), respectively.

1.9 Control of downy mildew

Proper management practices for control of downy mildew disease in boysenberry can reduce the impact of the disease. Pathogen-free stocks should be selected, especially for planting into areas where *Rubus* species have not been grown previously (Tate, 1981). As wild *Rubus* species, including blackberry and native *Rubus* spp., were found by Hall and Shaw (1987) to be susceptible to *P. sparsa*, it is recommended that these alternative hosts are eliminated from around boysenberry crops. Further, the removal of growing dibs (rooted ends of primocanes, the means of natural propagation), root suckers and early primocanes can reduce incidence of the disease. Immediately after harvest, old floricanes (second year fruit bearing cane) should be destroyed to reduce the carryover of inoculum to the next season. Weed control is also an important practice as this reduces humidity and retention of wetness on the foliage during rainfall, factors which favour development of the pathogen (Tate, 1981; Walter *et al*., 2004). In addition, selection of disease resistant cultivars can also be recommended. In North America and Europe rose breeding research programs are generally focussed on disease resistance rather than new cultivars, mainly due to consumer demand for disease resistant roses, high costs of controlling the disease, and to decrease the frequency of agrichemical use that can be detrimental to the environment and human health (Debener and Byrne, 2014). However, there is no evidence of resistance in boysenberry/youngberry cultivars and whether some are less susceptible than others is not known.

Chemical desuckering using desiccating sprays of dinoseb applied up to late flowering is important in controlling dryberry disease. Primary spore sources are destroyed by this cultural practice and it opens up the base of each plant, desiccating tender growth and increasing aeration while reducing free moisture and humidity. In order to reduce spore sources further, periodic application of systemic fungicides between desuckering sprays is recommended (Tate, 1981). Application of agrichemicals plays a major role in eliminating *P. sparsa* primary

inoculum sources to prevent the infection of leaves, flowers and berries. Agrichemicals based on chlorothalonil, copper oxychloride, mancozeb and metalaxyl-M are the only fungicides registered for control of *P. sparsa* in boysenberries in New Zealand (Novachem Agrichemical Manual, 2015). However, of the phosphorous acid agrichemicals only Foscheck has been recommended as a liquid fertiliser for berryfruit (Novachem Agrichemical Manual, 2015). Phosphorous acid (an aqueous solution of mono- and di-potassium phosphite), an agrichemical currently being used has been shown to be an effective fungicide against dryberry in boysenberries (Walter *et al*., 2004). Although the acceptable rates of copper for organically grown crops is 3 kg of copper/ha (Bio Gro standard), effective control of the disease was not achieved with this rate (Richards, 2002). In addition, Tate and Van Der Mespel (1983) showed that copper oxychloride is phytotoxic to boysenberry.

Walter *et al*. (2004) evaluated the effectiveness of different agrichemical treatments for downy mildew control in boysenberry in the 2000/01 and 2001/02 growing seasons in New Zealand. They found that two applications of metalaxyl-M, three applications of phosphorous acid or three applications of azoxystrobin plus a single application of dichlofluanid significantly reduced fruit losses due to downy mildew at low disease pressure. However, only the phosphorous acid treatment (three applications) gave acceptable downy mildew control (less than 20% fruit loss) at high disease pressure.

O'Neill *et al*. (2002) reported that a mixture of cymoxanil + mancozeb + oxadixyl and fluazinam provided good control of *P. sparsa* on both rose and blackberry. Further, chlorothalonil and metalaxyl mixed with either thiram or mancozeb were also successful at controlling downy mildew on blackberry (O'Neill *et al*., 2002). In California, pre-planting treatment with the systemic fungicides metalaxyl or mefenoxam was highly effective at reducing *P. sparsa* infection of rose when outbreaks occurred at bud-break (Aegerter, 2001). In Mexico, seven fungicide programs that tested mefenoxam, potassium phosphite, azoxystrobin, captan, mancozeb, copper sulfate, *Bacillus subtilis*, and a sanitising agent indicated that only programs which began with potassium phosphite (3-4 applications) on a 10-14 day schedule after bud break significantly reduced dryberry on fruits compared to the untreated control. This was not affected by any fungicide (either biological or chemical) that was applied later in the season (Rebollar-Alviter *et al*., 2012). However, more recently 3-4 applications of potassium phosphite (10-14 day schedule) followed by azoxystrobin applications close to harvest has been reported to effectively control the disease on blackberry in Mexico (A. Reboller, pers. comm., 2014). In addition, one or two applications of mefenoxam as a drench (after bud break) followed by potassium phosphite, azoxystrobin and pyraclostrobin close to harvest also provided effective disease control on blackberry (Monika Walter, pers. comm., 2014). Systemic fungicides metalaxyl or mefenoxam applied as pre-planting dips at rates of 100-10,000 mg a.i./litre were shown to effectively control downy mildew disease in rose in California (Aegerter *et al*., 2002). Under high disease pressure, fosetyl-Al and a combination of metalaxyl and mancozeb were

effective in controlling *P. sparsa* in rose rootstock cuttings (Aegerter, 2001). Since protectant fungicides such as mancozeb are subject to weathering, their effects against the pathogen are lost once the fungus has penetrated the host (rose) tissue. This problem is further exacerbated by the short generation time of *P. sparsa* and rapid disease development during warm and wet weather (Aegerter, 2001). Hukkanen *et al*. (2008) reported that euparenM and bion (syn. Actigard) gave the best control of *P. sparsa* in artic brambles, whereas aliette, phosfik, and phostrol gave moderate protection against the disease. Bion contains the active ingredient benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester, an activator of the plant defences found to induce accumulation of phenolics. Unlike other agrichemicals which only provide protection against the pathogen, bion is reported to increase the resistance of plants to *P. sparsa* infection through the induction of plant defense mechanisms (Hukkanen *et al*., 2008).

According to the Fungicide Resistance Action Committee (2005), *Peronsopora* species are categorized as being at 'medium' risk of developing resistance to fungicides. Therefore, rotation of fungicides is recommended to minimise the risk of resistance development in *P. sparsa* isolates. It was recommended that fungicides in Group 4 (mefenoxam/metalaxyl-M and oxadixyl) be used in combination with other fungicides as they are at particularly high risk for developing resistance when used as foliar sprays (Plantwise, CABI, 2011).

1.10 Aim and Objectives

The aim of this research was to study the biology of *P. sparsa* and the drivers of disease epidemics in boysenberry, specifically to improve understanding of the disease cycle and the favourable environmental conditions that trigger pathogen infection. As the pathogen cannot be cultured on artificial culture media, the research initially focussed on development of a method for production of spores. Further, since disease-free plant material was not available for experiments, methods for producing clean propagation material were investigated. Finally, this research aimed to identify chemical products able to reduce spore infection and systemic disease progression. To achieve these aims, five objectives were developed as outlined below.

Objective 1: To determine the factors affecting sporulation and infection of *Peronospora sparsa* on detached tissues as well as glasshouse- and commercially-grown field plants.

Objective 2: To improve understanding of the source and progression of *Peronospora sparsa* infection in systemically infected plants using Polymerase Chain Reaction (PCR).

Objective 3: To identify the environmental factors that enhances expression and development of *Peronospora sparsa*-initiated dryberry disease and boysenberry flower and berry infections.

Objective 4: To investigate methods for producing *Peronospora sparsa* free propagation material and the use of chemical products to limit spore infection and systemic disease in boysenberries.

Objective 5: To develop an improved molecular detection system for *Peronospora sparsa*.

CHAPTER 2

Factors affecting sporulation and infection

2.1 INTRODUCTION

The environmental factors that favour *P. sparsa* disease development in boysenberry have not been well studied. Ellis *et al*. (1991) reported that, in New Zealand, downy mildew disease of raspberry, blackberry and hybrids including boysenberry was favoured by warm (18-22°C) and wet weather in mid to late spring and autumn. In rose leaves, the optimal temperatures for infection and colonisation by *P. sparsa* was reported to be 15 to 20°C and 20 to 25°C, respectively, with infection requiring only 2 h of leaf wetness (Aegerter *et al*., 2003). In a study by Breese *et al*. (1994) using *P. rubi* (syn. *P. sparsa*) isolates from *Rubus* and *Rosa* hosts from both the UK and New Zealand, spores were observed to germinate at temperatures of 2°C to 24°C, with the optimum varying between isolates but generally being between 6 and 18°C. No germination was observed for any isolates at temperatures above 26°C. Further, disease incidence on leaf discs of tummelberry (blackberry × red raspberry) inoculated with *P. rubi* was highest at 15°C, with infection occurring over a range from 2 to 28°C. *Peronospora sparsa* sporulation on *Rubus* plants has been reported to be inhibited at temperatures above16-18ºC (Shaw, 1982), Similarly, Breese *et al*. (1994) reported greater sporulation by the pathogen at 10ºC than at 20ºC on tummelberry leaf discs. However, there is little information on more specific temperature and relative humidity requirements for *P. sparsa* spore (sporangiospore) germination, infection, sporulation and colonisation on boysenberry, and no information on optimum spore inoculum concentration for infection. There is also limited information about the relative susceptibility of different boysenberry cultivars although hybrid cultivars of blackberry × red raspberry were also reported to be more susceptible than blackberry and raspberry cultivars (Breese *et al*., 1994).

To undertake experiments that investigate the effect of environmental factors on sporulation and infection, reliable methods for the production of inocula must first be developed. As an obligate biotroph, *P. sparsa* is difficult to maintain in the laboratory as it cannot be cultured on artificial media. To provide inoculum for experiments requires maintenance of the pathogen on plants, which is both time and space consuming and is difficult to manage during plant dormancy over winter periods. A number of different techniques needed to be investigated to resolve this. A long term storage method could provide spore inoculum for experiments. Breese *et al.* (1994) reported that *P. sparsa* isolates stored as freshly sporulating blackberry, tummelberry or rose leaf discs or leaflets in sealed plastic boxes at -70ºC remained viable for up to 4 months, but longer storage was not reported. In addition, they also reported that *P. sparsa* viability was retained when sporulating leaf discs were stored in liquid nitrogen. However, no information was provided on spore viability after different storage periods. For the related pathogen, *P.*

viciae, spores on sporulating pea leaves stored dry at -80°C retained viability for at least 1 year (Gill and Davidson, 2005). Glycerol has also been widely used as a cryopreservative protectant for different microorganisms, including oomycetes (Hubálek, 2003). However, there is limited information on effective methods which retain viability and infectivity of *P. sparsa* spores during long-term storage.

To enable effective control strategies to be developed, information is required regarding the timing of spore release and the environmental conditions conducive for spore production and infection. However, most of the reported studies focussed on the environmental factors needed to cause infection in controlled *in vitro* conditions or in the field, and none have been used to develop an *in vitro* spore production method. The overall aim of this chapter was to determine the factors affecting the germination, infection, colonisation, and sporulation of *P. sparsa.* These factors included (1) the effects of temperature and relative humidity on germination, infection, colonisation and sporulation of *P. sparsa* (2) the effects of spore number and leaf age on *P. sparsa* infection and (3) the susceptibility of different boysenberry cultivars on infection and sporulation.

2.2 MATERIALS AND METHODS

2.2.1 Inoculum potential in the field

2.2.1.1 Timing of spore release in the field

Spore traps were set up in three boysenberry gardens in the wider Nelson area (Appendix A.1), with two grower properties in Motueka (Lower Moutere and Upper Moutere) and one grower property near Richmond. The spore traps were set up from October to December in 2010 and September to December in 2011. The cultivars were Mapua in Lower Moutere and Richmond, and Tasman in Upper Moutere. The trapping slides were placed in boysenberry rows which were left unsprayed, with the five slides (2010) or four slides (2011) per property being replaced with new slides each week. In 2011, the slides were placed in the Lower Moutere grower property in row 163/164, bay numbers 3, 5, 7 and 9 (of the 11 total bays in the row), in the Upper Moutere grower property in row 1/2, bay numbers 3, 5, 7 and 9 (of the total 9 bays in the row), and in the Richmond grower property in row 2/3, bay numbers 2, 4, 6 and 8 (of the total 9 bays in the row). The same rows were used in 2010 except for the Richmond grower property where row 122/123 was used, because the rows used in 2010 had been sprayed with phosphorus acid. Each bay had three to five boysenberry plants depending on the property.

The clean glass microscope slides used for trapping the sporangiospores (called ‘spores’ in this thesis) had been coated with Vaseline® dissolved in hexane (Vaseline®: hexane 1:7 W/V) on one side and allowed to dry in a fume hood. The slides were placed horizontally in the field by clipping the slides onto strings using small bull-dog clips (Figure 2.1).

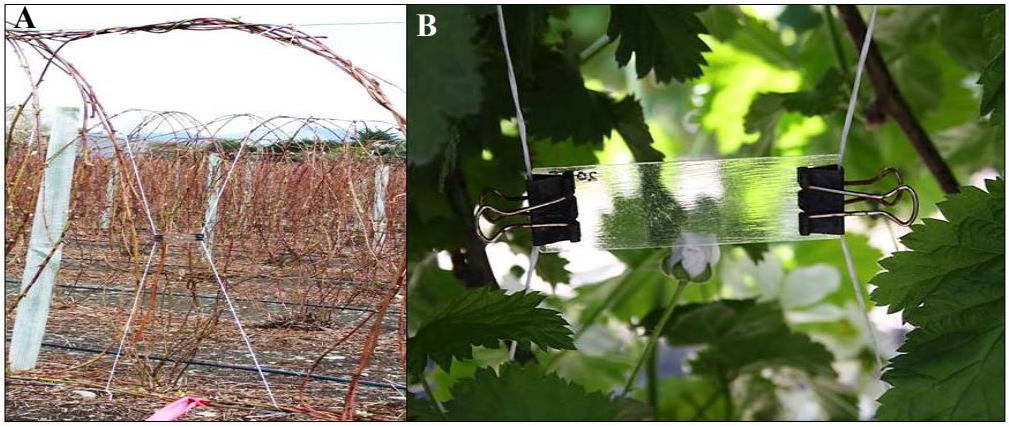

Figure 2.1 Vaseline®-coated spore trap slides placed in the boysenberry canopy of field plants for trapping spores of *Peronospora sparsa* in 2011, shown in A) Crop canopy in September, and B) the crop canopy in November.

After collection, the slides were brought back to the laboratory at Lincoln University for assessment. Each slide was divided into 12 horizontal transects, which was the number of fields of views at x 10 magnification across the slide. Two of these transects, selected using a random number table, were examined using a bright field microscope, with all of the spores characteristic of *P. sparsa* counted. These counts were used to determine the average number of spores per slide. For the 2011 assessment, confirmation of presence of *P. sparsa* spores on the slides at selected times was carried out using species-specific PCR as described in Chapter 3.

In an attempt to determine environmental factors which promoted spore production/release weather data was obtained from the NIWA (National Institute of Water and Atmospheric research, New Zealand, http://cliflo.niwa.co.nz/pls/niwp/wgenf.genform1_proc) weather stations, using data from the two closest weather stations that provided all the four weather parameters i.e. wind speed, rainfall, RH and temperature. The Riwaka Electronic Weather Station (Ews) was the closest weather station for both grower properties in Motueka and, was approximately 13.8 km from Lower Moutere grower property and 25.5 km from Upper Moutere grower property. The Nelson Automatic Weather station (Aws) was approximately 14.8 km from the Richmond grower property.

Comparisons between the spore counts obtained in 2010 and 2011 for the same grower properties were made with respect to the sporulation potential across both years and with respect to weather conditions. In addition, the relationship with dryberry production in 2011 was also assessed.

The spore counts were $\log_{10}(x+1)$ transformed prior to analysis using the General Linear Model (GLM) in Minitab Ver.16. The grower property was considered as the random factor. When factors were significant, means were compared between treatments using Tukey's honest significant difference (HSD) at $P \leq 0.05$. Only means which met this standard were referred to as being 'significantly' different.

2.2.1.2 Disease assessment under field conditions

The incidence of dryberries in the row (untreated) where the spore trap slides were placed was determined for all three grower properties in late December 2011. The number of dryberries per bay was assessed from all plants and used to calculate the number of dryberries per plant. This was conducted in all four bays where the spore trapping slides were located at each grower property. There were three to five plants per bay in the Lower Moutere grower property whereas there were five and four plants, respectively, in the Upper Moutere grower and Richmond grower properties. In 2011, assessments were made on the 23 and 29 December.

2.2.1.3 Potential sources of inoculum under field conditions

In the third week of November, 2011, leaf symptoms were assessed in the row where the spore traps were placed for each of the three grower properties. Five plants taken at random in each row were observed for leaf lesions on both the upper and lower leaf surfaces. Fifteen symptomatic leaves were collected from both the Lower Moutere (cv. Mapua) and the Upper Moutere (cv. Tasman) properties. Floricane (15), flower (10) and berry (24) (unripe green) samples from symptomatic plants were also collected from the Lower Moutere grower property. Since no symptoms were observed on the boysenberry plant material at the Richmond grower property, primocane (15), flower (10) and berry (24) (unripe green) samples were collected from asymptomatic plants. The samples were transported to Lincoln University using cold insulated boxes and incubated at 100% RH at 15 and 20ºC for 14 days to induce sporulation. The plant material was assessed under a stereo microscope and incidence of sporulation was recorded. This provided information on the potential sources of spore inoculum.

2.2.2 Sporulation potential of naturally infected boysenberry plant tissues at different temperatures and relative humidities

The boysenberry ("*Rubu*s mapua" - *Rubus ursinus* x *idaeus)* plantlets (150) propagated by tissue-culture, which were obtained from a commercial nursery in New Zealand in 2011, were found to be systemically infected with *Peronospora sparsa* since characteristic angular lesions along the veins of leaves were observed on all plants. These systemically infected plants were used to provide infected plant material to determine the effect of temperature and relative humidity on spore production. The plantlets were potted up into 12-14 month potting mix (Appendix A.2) in 2.5 L clean plastic pots, with half placed in the greenhouse and the remainder in the shadehouse.

2.2.2.1 Sporulation potential of leaves

Experiment 1- sporulation potential on symptomatic leaves

To determine the best environmental conditions for spore production, leaves showing characteristic *P. sparsa* lesions (Figure 2.2) were harvested from systemically infected boysenberry plants (cv. Mapua at a nursery field site prior to planting in the field) from the Lower Moutere property (17 November, 2011) and from plants in the shadehouse at Lincoln University (early December, 2011).

Figure 2.2 Leaves showing characteristic *Peronospora sparsa* leaf lesions harvested from systemically infected boysenberry plants (cv. Mapua) at a nursery field site prior to planting in the field) in Lower Moutere.

New fully expanded leaves and mature leaves from the bottoms of the plants were harvested. The leaves were incubated under a combination of different temperatures (10, 15 or 20ºC) and relative humidities (RH) (80, 90-95 or 100%) and assessed for spore production. Both whole symptomatic leaves and 12 mm diameter leaf discs cut with a 12 mm cork borer from lesion areas were used. These were placed on the lids of sterile Petri dishes, placed on racks in plastic containers (32 cm x 26 cm x 8 cm) containing different salt solutions, used to produce different relative humidities. Ammonium sulfate ($(NH_4)_2SO_4$; 333.33 g/L 300 mL) salt solution was used to produce 80% RH, potassium nitrate (KNO_3; 551.67 g/L, 300 mL) salt solution was used to produce 90-95% RH (Dhingra and Sinclair, 1985) and H_2O (300 mL) was used to produce 100% RH. The salt solutions were saturated and with excess salt added to ensure the solutions remained saturated. The relative humidity in each chamber was allowed to equilibrate for one week prior to setting up the experiment for shadehouse leaves whereas due to time constraints no equilibration time was allowed for field collected samples. There were six replicate leaves per treatment, and 15 leaf discs for field material and six for shadehouse material, per treatment. The leaves were placed abaxial side facing upwards, one leaf per Petri dish lid. Similarly the leaf discs were placed abaxial side facing upwards, with five discs on each of three Petri dish lids for field material, and one disc on each of six Petri dish lids for shadehouse material (for each treatment). Therefore each container had six leaves and fifteen (field samples) or six (shadehouse samples) leaf discs. The Petri dish lids containing leaves or leaf discs were

arranged at random on the racks of each plastic container. There was one replicate container for each RH and temperature. The containers were arranged at random in the three incubators set at 10, 15 and 20ºC. The effect of temperature and RH on lesion expansion and sporulation was assessed. Lesion expansion was assessed by measuring lesion diameters initially and after 14 days using digital callipers (Mitutoyo Absolute Digimatic, Mitutoyo Corporation, Kawasaki, Japan). However, since the lesions were largely irregular; the maximum diameter/length of each lesion was measured.

Sporulation was determined by observing the leaves and leaf discs under a stereo microscope after 14 days. Spore numbers were then enumerated using 12 mm diameter leaf discs cut from the full lesion areas which developed on each leaf. These were washed in 5 mL of sterile water, centrifuged at 4696 x *g* for 10 min, and the pellet re-suspended in 0.5 mL sterile water. The numbers of spores in the suspension were counted using a haemocytometer. The numbers of spores per mm^2 lesion area was calculated for each of the 12 mm discs cut from the lesion. The same method was used to assess spore production on the leaf disc lesions. The efficiency of the washing procedure was assessed for the shadehouse samples by counting the number of spores which remained on the leaf discs after washing using the stereo microscope.

Experiment 2- sporulation potential on asymptomatic leaves

To determine the sporulation potential of asymptomatic young leaves a similar experiment was conducted using asymptomatic leaves detached from the primocanes of the systemically infected boysenberry plants (cv. Mapua) obtained from the Richmond grower property on the same day (17 November, 2011). Six replicate leaves (three leaves for each of two sizes, 4 cm long at the mid rib and 6 cm long at the mid rib) were placed separately in Petri dishes lids either with the abaxial side or the adaxial side of the leaf facing upwards and arranged at random on the racks of plastic containers, and incubated in 100% RH at 15ºC.

2.2.2.2 Sporulation potential of canes

Naturally infected symptomatic boysenberry cane (floricane) samples were collected from the Lower Moutere grower property and asymptomatic primocane samples were collected from the Richmond grower property on 17 November, 2011. The symptomatic floricane samples were cut aseptically into 10 cm segments. The bottom 2 cm of each cane segment was immersed into 10 mL sterile water in a sterile Universal bottle (28 mL). Each Universal bottle was sealed using Parafilm® (Bemis Inc., Oshkosh). The cane segments were then incubated in a humidity chamber consisting of a polystyrene tray within a new plastic bag. To provide 100% relative humidity, moistened paper towels were placed within the humidity chamber. The cane segments were incubated at 15ºC and 20ºC with six replicates per treatment. After 3, 6, 9 and 12 days, the cane segments were observed under a stereo microscope for sporulation. When profuse sporulation was observed, the length of the cane segment showing sporulation was measured

using a digital calliper. This was repeated for the asymptomatic primocane samples to determine the potential for sporulation.

In addition to determining the sporulation potential along the cane length one long symptomatic floricane (160 cm long) was collected in the field (Lower Moutere) and cut into three sections of approximately 60 cm (top), 45 cm (middle) and 55 cm (base) for ease of transportation to Lincoln University.

The sections were labelled to distinguish the different sections, and to identify the base and top. Each section was reduced to a 44 cm section by removing 11 cm from the upper part of the bottom cane, 1 cm from the lower part of the middle cane, and 16 cm from the lower part of the top cane. They were labelled as base, middle and top. Each section was cut into four 11 cm segments, which each had a 1 cm angle cut at the base to differentiate the base from the top. Just prior to placing the bottom section of each segment in Universal bottles containing water the 1 cm slope portions were removed so as to retain only 10 cm. The opening of each Universal bottle was sealed using Parafilm® and the Universal bottles containing the canes incubated at 15°C in 100% RH. The sporulation potential along the length of the cane was determined as previously described.

2.2.2.3 Sporulation potential of fruit

In order to assess the sporulation potential of fruit, 12 unripe berries for each of two different bunch positions, which represented different maturity stages (king berry and non-king berry) were collected from the organic site (where no fungicides had been applied) in the Richmond grower property on 17 November, 2011. To reduce *Botrytis* growth, berries were surface sterilised with 0.25% sodium hypochlorite solution for 30 s followed by a 5 min sterile water wash. The berries were bisected lengthwise, and the halves were each placed separately cut surface down in a small (6 cm diameter) Petri dish lid, and placed in 100% RH chambers and incubated at 15 or 20ºC. Six replicates were set up per treatment.

After 3, 6, 9, 12 and 14 days incubation the berries were observed for spore production under a stereo microscope. When profuse sporulation was observed on the majority of berries from at least one treatment, the presence/absence of sporulation was recorded on all berries and treatments.

To study the effect of surface sterilisation on sporulation potential of fruit, 24 berries at a similar maturity level being unripe, fully expanded (but not coloured) were collected from another organic property (Lower Moutere grower property) on the same day. Half of the berries were surface sterilised with 0.25% sodium hypochlorite solution for 30 s followed by a 5 min sterile water wash, whilst the other half were left not sterilised. The berries were cut in half and incubated at either 15 or 20ºC in a 100% RH chamber and assessed for sporulation as previously described. Six replicates were set up for each treatment. The experiment was repeated with ripe

berries collected from the same two grower properties and another in Upper Moutere in December 2011. The ten replicate berries per grower property were not surface sterilised and were incubated at 15°C and 100% RH and assessed for sporulation as previously described.

2.2.3 *In vitro* production of asexual spore inoculum

2.2.3.1 Surface sterilisation methods for non-symptomatic plants

To test the most effective methods for reducing microbial contamination on leaves a range of surface sterilisation methods were tested using non-symptomatic systemically infected leaves from shadehouse/greenhouse plants. Four treatments were assessed, including 10% bleach (for 30 s), 70% ethanol (for 2 s) both being followed by sterile distilled water for 5 min, a wash only treatment with sterile distilled water (twice for 5 min each time) and no washing (control). Six replicates were used for each treatment. The treated leaves were placed in sterile Petri dishes for 20 min in a laminar flow hood to dry. Once the leaves had dried, each side of each leaf (upper and lower) was pressed onto potato dextrose agar (PDA; Difco^{TM}, New Jersey, USA), one PDA dish for each side of the leaf. The PDA dishes were sealed with cling film and incubated at 20°C, with a 12h/12h light/dark regime for one week in a randomised complete block design in the incubator. The numbers of bacterial and fungal colonies per leaf on the PDA plates were counted. The experiment was repeated using nutrient agar (NA; Oxoid Ltd, Basingstoke, Hampshire, England) in addition to PDA as the recovery of bacteria was very low in the first experiment. Analysis of variance (ANOVA) was used to analyse data in GenStat Ver.16. When factors were significant, means were compared between treatments using Fishers least significant difference (LSD) at $P \leq 0.05$. Only means which met this standard were referred to as being 'significantly' different.

2.2.3.2 Development of a method for producing spore inoculum on pathogen free leaves

To provide spore inoculum of specific *P. sparsa* isolates for subsequent experiments, a method needed to be developed for producing inoculum on pathogen free leaves. Young healthy leaves with no disease lesions were collected from boysenberry plants (cv. Mapua) growing in the greenhouse. The leaves were surface sterilised using the treatments described in Section 2.2.3.1 and placed onto 1.5% water agar (w/v) (Appendix A.3.3) with the abaxial surface upwards. The abaxial surface of each leaf was inoculated with three drops (20 µL each) of freshly prepared spore suspension, prepared by shaking sporulating leaf discs (12 mm diameter) cut from naturally infected leaves from plants (cv. Mapua) grown in the Lincoln University shadehouse, in sterile water. The spore concentration was adjusted to $1x10^5$ spores/mL based on haemocytometer counts and used within 30 min of preparation. One drop was placed close to the midrib whereas the other two drops were placed closer to the veins on either sides of the midrib (Figure 2.3). To retain high RH, the Petri dishes were sealed with cling film, which had

been shown in a preliminary test to be a suitable method for sealing the Petri dishes, and could be done without disrupting the spore drops placed on the leaves.

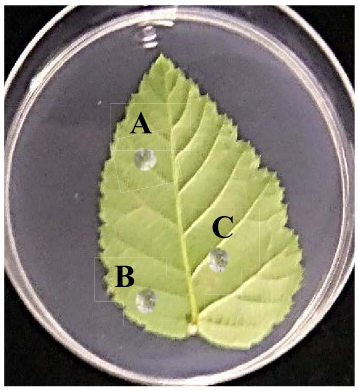

Figure 2.3 Surface sterilised boysenberry leaf placed on 1.5% water agar. The abaxial side was inoculated close to the (A) midrib and (B, C) closer to the veins on either sides of the midrib, each with 20 µL freshly prepared *Peronospora sparsa* spore suspension.

Six replicates per sterilisation treatment were set up and these were arranged in a randomised block design, with three trays, each tray containing two replicates of each treatment. These were then incubated at 20ºC at 12h/12h day/night condition for 24 h. On the second day the trays containing the Petri dishes were placed inside three clean plastic bags which were folded loosely under each tray, and placed in the same position in the incubator for further incubation. The leaves were incubated for 1 month, and observed weekly for development of lesions.

The time to first appearance of lesions and the size of lesions for the different treatments and with respect to the location inoculated on the leaf were assessed. The lesions were measured using a digital calliper as previously described. The best method was used to produce spores for subsequent experiments.

2.2.3.3 Optimum conditions for spore germination

A fresh spore suspension was prepared by shaking sporulating leaf discs (12 mm diameter) cut from naturally infected leaves from plants (cv. Mapua) grown in the Lincoln University shadehouse in sterile water. The spore concentration was adjusted to $1x10^4$ spores/mL based on haemocytometer counts and used within 30 min after preparation.

Experiment 1: *The effect of different substrates on spore germination*

Leaf discs, microscope slides and 1.5% water agar (WA) were inoculated with a 20 µL drop of a 1×10^4 /mL spore suspension to determine their effect on spore germination. For leaf discs, each leaf disc (12 mm) was surface sterilised using a sterile water wash (found to be the best in Section 2.2.3.1) and the abaxial (lower) side was inoculated with a 20 µL drop (approximately 200 spores) of the spore suspension and incubated on 1.5% WA to provide high humidity. For microscope slides, each slide was surface sterilised with 70% ethanol, which was allowed to dry in a laminar flow cabinet, inoculated with two 20 µL drops of the spore suspension and the

slides were placed onto 1.5% WA to provide high humidity. For the 1.5% WA substrate, six 20 µL drops of spore suspension were placed on a 1.5% WA plate with the location of the inoculations marked on the lid of the Petri dish. All treatments were incubated at either 10, 15 or 20°C in the dark for 24 and 48 h. Germination of the spores on the leaf discs was assessed using a sticky cellotape strip to remove the spores from the surface with the strip then being mounted in a lacto-glycerol cotton blue (LGCB) (Appendix A.3.4) drop placed on a microscopic slide. For the microscope slides and 1.5% WA plates, a drop of LGCB was placed on the spore droplets on the slide or 1.5% WA and covered with a coverslip. The total number of spores and the number of germinated spores were counted under x10 magnification using a bright field microscope. A spore was assessed as having germinated when the length of the germ tube was at least half the diameter of the spore. Six replicates per treatment (18 treatments) were used. Results were analysed using GLM in Minitab Ver.16. When factors were significant, means were compared between treatments using Tukey's HSD at $P \leq 0.05$. Only means which met this standard were referred to as being 'significantly' different.

Experiment 2: *Verification of spore germination counts using different methods*

The experiment was repeated to determine whether the enumeration method affected the spore germination counts. In this experiment, leaf discs only were used, inoculated as described in Experiment 1 and incubated at 10, 15 and 20°C in the dark. The total spores numbers and the numbers germinated were counted after 24 h incubation using one of three methods: (1) a sticky cellotape strip was used to remove the spores from the leaf discs and mounted in a LGCB drop on a microscope slide and assessed as described in Experiment 1. (2) The region of the leaf inoculated with the spore suspension was flooded with a LGCB drop. Spore germination was assessed using a stereomicroscope. (3) The leaf discs were cleared in a 1:1 solution of glacial acetic acid and 95% ethanol (Obanor, 2006) for 24 h, stained with LGCB and spore germination enumerated at x40 magnification using a bright field microscope. Six replicate leaf discs were used for each treatment. ANOVA was used to analyse data using GenStat Ver.16. When factors were significant, means were compared between treatments using Fishers least significant differences (LSDs) at $P \leq 0.05$. Only means which met this standard were referred to as being 'significantly' different.

Experiment 3: *Verification of the cellotape strip method for assessing spore germination*

An experiment was conducted to assess whether the germination counts were affected by the numbers of germinated and non-germinated spores adhering to the cellotape strip. Ten leaf discs were inoculated with 20 µL of freshly prepared *P. sparsa* spore suspension (200 spores each) using the method described in Experiment 1 and incubated at 20°C in the dark. The total number of spores and the number germinated were counted after 24 h incubation using sticky cellotape strips, followed by clearing of each leaf disc separately in 10 mL of 1:1 solution of glacial acetic acid and 95% ethanol for 24 h (Obanor, 2006). After 24 h in the solution they

were removed and stained with LGCB and total number of spores and the number of germinated spores was enumerated at x10 magnification using a bright field microscope.

2.2.4 Optimum conditions for *P. sparsa* infection

2.2.4.1 Effect of temperature and infected material (leaves/leaf discs) on *P. sparsa* infection, sporulation and lesion size

Leaves and leaf discs (12 mm) with no obvious symptoms were sourced from boysenberry plants from the shadehouse at Lincoln University. These were then washed with sterile water and inoculated with a 20 µL drop of spore suspension ($1.5x10^4$ spores/mL) prepared in sterile water and used within 2 h of being produced.

The leaves/leaf discs were incubated at one of three temperatures (10, 15 and 20°C) and 12h/12h light/dark on 1.5% WA. After 14 days, the number of leaves/leaf discs which produced symptomatic lesions and with sporulation (incidence) was assessed. Lesion diameter was measured using a digital calliper. Spore concentration was determined as described in Section 2.2.2.1 Experiment 1. The pellets were each re-suspended in 0.5 mL sterile water and spores counted using a haemocytometer. Sporulation score was assessed using a sporulation scale (0=no spores, 1=1-5 spores, 2=6-12 spores, 3=13-50 spores, 4=51-100 spores, 5=101-1000 spores and 6= more than 1000 spores), with the six leaves and eighteen leaf discs per treatment.

Statistical analysis

Data which included lesion size and numbers of spores per lesion were analysed by GLM in Minitab Ver.16. When factors were statistically significant, means were compared between treatments using Tukey's HSD at $P \leq 0.05$. Only means which met this standard were referred to as being 'significantly' different. The scale data were analysed by the non-parametric Kruskal-Wallis test in the Minitab Ver.16. When overall significance occurred, pairwise comparisons were conducted using a Mann-Whitney U test with a Bonferroni adjustment to correct the significance level. With respect to the lesions and sporulation incidence, the leaves/leaf discs in the population which formed a sequence of Bernoulli trials were statistically analysed by generalized linear modelling procedure in GenStat Ver.16. A Bernoulli distribution defined as a binary variable was assumed with logit link function to test for the main effects and interactions. Lesion development data was analysed using accumulated analysis of deviance to provide parameter estimates of significance for sporulation incidence for leaf types and incubation temperatures with the means presented with the 95% confidence intervals.

2.2.4.2 Effect of spores concentration on infection

Fresh suspensions of five different spore concentrations (10 fold dilutions from 10^1 to 10^5 spores/ mL) were prepared in sterile water and used within 3 h of being produced. Leaf discs (12 mm diameter) washed with sterile water (Section 2.2.3.1) were placed onto five 1.5% WA

plates. Each 1.5% WA plate contained two replicate leaf discs and each was inoculated with a 20 µL drop of one of the spore concentrations. There were also five replicate WA plates with two discs each that were inoculated with sterile water as a control treatment. The number of spores per 20 µL droplet equated to 0.2, 2, 20, 200 and 2000 for the 10^1, 10^2, 10^3 10^4 and 10^5 spores/mL concentrations, respectively. Ten replicate leaf discs were arranged at random for each spore concentration and control as two leaf discs from each of the designated spore concentration in each replicate 1.5% WA plate. The leaf discs were incubated in the optimum conditions determined in Section 2.2.4.1 (15°C, 12h/12h light/dark). After nine and twelve days incubation, disease incidence was determined as the number of leaf discs with visible symptoms whereas sporulation score was determined using the sporulation scale described in Section 2.2.4.1. The lesion incidence, sporulation incidence and sporulation scale data were statistically analysed as described in Section 2.2.4.1.

2.2.4.3 Effect of leaf maturity on susceptibility to *P. sparsa* infection

Mature (6 cm in length along the mid rib) and young (4 cm in length along the mid rib) asymptomatic leaves were detached from both shadehouse and greenhouse (cv. Mapua) plants (Lincoln University, New Zealand), washed with sterile water and leaf discs (12 mm) cut from them. Eighteen leaf discs were set up for each treatment and arranged in a randomised design. Each of the 18 leaf discs were placed on separate 1.5% WA plates and inoculated with 20 µL of freshly prepared spore suspension (1.8 x 10^4 spores/mL) of *P. sparsa* and incubated at the optimum temperature (15°C; 12h/12h light/dark) determined in Section 2.2.4.1 for 14 days until symptoms developed and sporulation was observed. For both leaf ages, controls consisting of 18 leaf discs inoculated with sterile water were also set up. After 10 and 14 days incubation, disease incidence (presence/absence) and sporulation score using the sporulation scale was assessed as described in Section 2.2.4.1. The lesion incidence, sporulation incidence and sporulation scale data were statistically analysed as described in Section 2.2.4.1.

2.2.4.4 Susceptibility of different *Rubus* spp. cultivars to *P. sparsa* infection

Leaves of four different *Rubus* spp. which comprised a youngberry, a blackberry (Karaka Black) and two boysenberry cultivars (Tasman and Mapua), were collected from plants growing at the Riwaka Plant and Food Research station site, stored at 4°C in the fridge in new plastic bags for two days and held in a chilly bin overnight while being brought down to Lincoln University. The leaves were washed with sterile water and 12 mm diameter leaf discs cut from each cultivar, 12 leaf discs per cultivar. The leaf discs were arranged eight per 1.5% WA plate with two leaf discs per cultivar/species in each Petri plate. Each disc was then inoculated with a 20 µL drop of a freshly prepared spore suspension (1.5x10^4 /mL) and incubated under optimum conditions for infection (15°C and 12h/12h light/dark; Section 2.2.4.1). Three separate 1.5% WA plates each with two leaf discs per cultivar inoculated with 20 µL drops of sterile water was

used as the control treatment. Disease incidence, lesion size and spore production were assessed after 14 days as previously described (Section 2.2.4.1).

The lesion incidence and sporulation incidence data were statistically analysed as described in Section 2.2.4.1. Lesion size and numbers of spores per lesion were analysed using one way ANOVA separately using GenStat Ver.16. When factors were significant, means were compared between treatments using Fisher's protected LSD at $P \leq 0.05$. Only means which met this standard were referred to as being 'significantly' different.

The experiment was repeated at the Riwaka research station using leaf discs cut from freshly detached leaves of the same cultivars as well as the cultivar Navaho to determine if transportation had affected susceptibility.

2.2.5 Production of oospore inoculum and its infection capability *in vitro*

In the first experiment, twelve leaf discs were washed in sterile water, inoculated with the optimum numbers of asexual spores of *P. sparsa* (200/20 µL) and incubated on 1.5% WA in Petri dishes (six per plate) at the optimum temperature (15ºC) found for infection. After 14 days inoculation to allow asexual sporulation they were further incubated for another 2 weeks to assess the production of sexual spores (oospores) on the artificially inoculated leaf discs. Leaf discs were then mounted on clean glass slides and observed under a bright field microscope for presence of oospores.

In the second experiment, leaf discs treated as above were used to prepare a suspension of oospores (200 spores/20 µL), which were harvested as described for asexual spores (Section 2.2.2.1 Experiment 1). To test their infection capability, the oospores were inoculated onto 30 washed leaf discs (abaxial side) (200 spores/20 µL), which were incubated on 1.5%WA (six per plate) at 15ºC 12h/12h light/dark conditions.

2.2.6 Evaluation of methods for long term storage of inoculum

Six storage methods were assessed: (1) the leaf discs cut from sporulating areas of the leaf stored dry or (2) in 20% glycerol, and (3) asexual spores were suspended in 20% glycerol. These three types of preparations were stored in vials at either -20°C or -80°C. After 1, 2, 4 or 6 months storage, vials containing the spore preparations were removed from storage and the spore germination and capability to infect leaf discs were evaluated as described in Sections 2.2.4.4 (Appendix A.13). Long term storage of the sexual spores (oospores) was not studied as the oospores were not able to infect the leaf discs according to the results of the previous experiment (Section 2.3.5).

2.2.7 Effect of optimum incubation conditions on lesion expansion of systemic and spore initiated lesions

Young leaves with naturally occurring systemic and spore initiated lesions (Figure 2.4) were detached from plants growing in the Lincoln University shadehouse and from these 12 mm diameter leaf discs were cut to include the lesion.

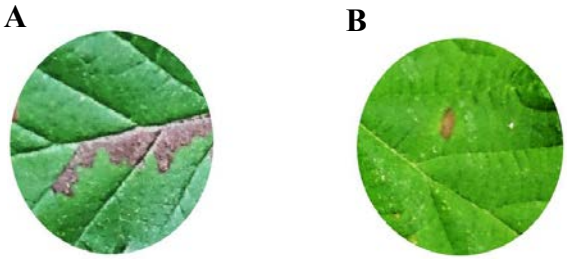

Figure 2.4 Boysenberry leaf discs (12 mm diameter) cut from leaves with (A) systemic lesions and (B) spore initiated lesions.

The leaf discs were then placed on 1.5% WA plates and incubated at the optimum conditions determined in Section 2.2.4.1 (15ºC 12h/12h light/dark) for 14 days. Eighteen leaf discs per lesion type were used, three per WA plate. The lesion sizes were measured using a digital calliper, initially and after 3, 5, 7, 10 and 14 days. As the lesions were irregular in shape (Figure 2.5), the maximum length and maximum width of each lesion was measured to estimate the maximum lesion area by multiplying both measures. Results were analysed by ANOVA repeated measures using GenStat Ver.16.

Figure 2.5 Measurement of the *P. sparsa* lesion area on the boysenberry leaves due to *P. sparsa* infection. x and y arrows indicate the maximum length and the width of the lesion area, respectively. The lesion area calculated is within this square area.

2.3 RESULTS

2.3.1 Inoculum potential in the field

2.3.1.1 Timing of spore release in the field

Spores characteristic of *P. sparsa* were observed trapped on the Vaseline® coated slides placed in the boysenberry rows in the three growers properties in both the 2010 and 2011 seasons (Figure 2.6).

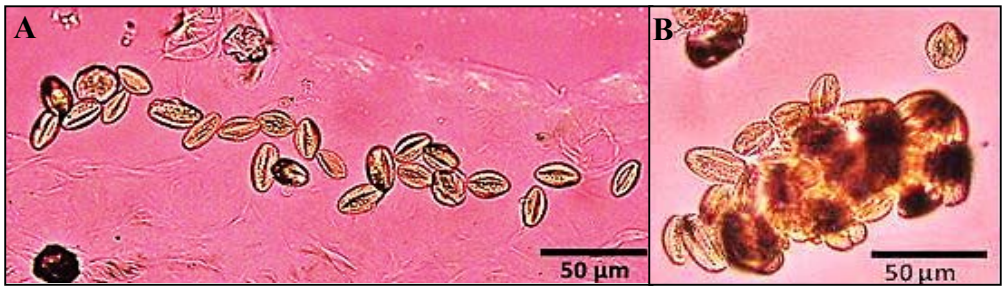

Figure 2.6 (A) Spores characteristic of *Peronospora sparsa* trapped on a Vaseline® coated slide placed in the canopy of boysenberry plants within a planting row in a grower property, and (B) example of spores found in clusters which caused potential errors in the spore counts.

In 2010, there was a significant effect of time (date) (P<0.0001) on the mean $\log_{10}$ spore counts, which was mainly associated with significantly higher mean $\log_{10}$ spore counts for the two assessments in mid-November (5-12 and 12-19 November) compared with the 9-15, 15-23 and 23-29 of October and 25-2 and 2-9 in December (Table 2.1). However, the mean $\log_{10}$ spore counts in the 29-5 and 19-25 of November were not significantly different from other assessment times except for the 9-16 December. There was no significant (P=0.111) effect of the grower property on the overall numbers of spores trapped. There was a significant (P<0.0001) interaction between assessment time and grower property, which was associated with differences in date when the large numbers of spores were trapped in the three properties. During 29 October to 5 of November, significantly more spores were trapped on the Richmond property (mean of 3.2 $\log_{10}$ spores) compared to the other two properties, whereas in the 5-12 November significantly higher counts were from both Richmond (mean of 3.7 $\log_{10}$ spores) and Lower Moutere (mean of 3.0 $\log_{10}$ spores) compared with the Upper Moutere (mean of 0.7 $\log_{10}$ spores). On 12-19 of November there was no significant difference in the number of spores trapped at the three grower properties.

In 2011, there was a significant effect of time (P=0.001) on the mean $\log_{10}$ spore counts with significantly more spores on 5-12 of November compared with all other assessment times, and on 24 October to 8 November, on 8-16 of October and 12-19 of November compared with all other assessment times apart from 29 October to 5 November and 19-25 November (Table 2.2). There was also a significant effect of the grower property (P<0.0001) on the mean $\log_{10}$ spore counts, with significantly higher mean $\log_{10}$ spore counts in the Upper Moutere and Lower Moutere grower properties than from the Richmond property. The interaction between

assessment time and grower property was also significant (P<0.0001). During 8-16 October, significantly more spores were trapped in the Upper Moutere property (mean of 1.8 $\log_{10}$ spores) compared with at the Richmond property (mean of 0.5 $\log_{10}$ spores), with numbers from the Lower Moutere property (mean of 0.7 $\log_{10}$ spores) not being significantly different from either. For the 29 October to 5 of November, significantly more spores were trapped at the Richmond property (mean of 1.4 $\log_{10}$ spores) compared with the Upper Moutere property (mean of 0.2 $\log_{10}$ spores), with again the Lower Moutere property (mean of 0.9 $\log_{10}$ spores) not being significant from either. The spore numbers trapped at the three properties did not differ significantly from each other for any other time. Full statistical analysis is presented in Appendix A.4.1 and A.4.2.

Table 2.1 The numbers of spores ($\log_{10}$) trapped on the Vaseline® coated glass slides per week over the trapping period October-December in 2010 in three boysenberry growers' properties in the Nelson region.

Assessment time	Spore counts ($\log_{10}$)			
(Date)	Lower Moutere	Upper Moutere	Richmond	Date effect
5-9 Oct	2.0 abcdefg[1]	2.6 abcde	1.3 cdefg	2.0 BC[1]
9-15 Oct	1.1 defg	1.6 cdefg	0.3 fg	1.0 D
15-23 Oct	0.6 fg	2.2 abcdef	0.9 efg	1.2 CD
23-29 Oct	1.2 defg	0.4 fg	2.2 abcdef	1.3 CD
29-5 Nov	1.1 defg	0.3 fg	3.2 abc	1.6 BCD
5-12 Nov	3.0 abcd	0.7 efg	3.7 ab	2.5 AB
12-19 Nov	3.1 abcd	3.8 a	3.0 abcd	3.3 A
19-25 Nov	0.8 efg	1.8 bcdefg	1.8 abcdefg	1.4 CD
25-2 Dec	0.3 fg	2.2 abcdef	0.7 efg	1.1 CDE
2-9 Dec	0.8 efg	0.9 efg	0.8 efg	0.8 DE
9-16 Dec	0.3 fg	0.3 fg	0.0 g	0.2 E
Grower Property effect	1.3	1.5	1.6	

[1]Values within the rows and columns followed by the same letter are not significantly different according to Tukey's test (P= 0.05). The main effect of the assessment date (A-E) and interaction between the assessment date and grower property (a-g) was significant (P <0.0001). The main effect of grower property was not significant (P=0.111).

Table 2.2 The number of spores (log_{10}) trapped on the Vaseline® coated glass slides per week over the trapping period September-December in 2011 in three boysenberry growers' properties in the Nelson Region.

Assessment time (Date)	Spore counts (log_{10})			
	Lower Moutere	Upper Moutere	Richmond	Date effect
9-15 Sep	0.2 h[1]	0.0 h	0.0 h	0.1 E[1]
15-24 Sep	0.3 fgh	0.0 h	0.0 h	0.1 DE
24-8 Oct	1.5 abcde	1.5 abcdef	0.4 efgh	1.1 B
8-16 Oct	0.7 bcdefgh	1.8 ab	0.5 efgh	1.0 B
16-22 Oct	0.0 h	0.6 bcdefgh	0.3 h	0.4 CDE
22-29 Oct	0.3 gh	0.0 h	0.0 h	0.1 DE
29-5 Nov	0.9 bcdefgh	0.2 h	1.4 abcdefg	0.8 BC
5-12 Nov	1.7 abc	2.1 a	1.6 abcd	1.8 A
12-19 Nov	1.4 abcdefg	1.5 abcdef	0.7 bcdefgh	1.2 B
19-25 Nov	0.5 cdefgh	1.1 abcdefgh	0.3 gh	0.6 BCD
25-2 Dec	0.5 efgh	0.5 defgh	0.0 h	0.3 CDE
2-10 Dec	0.0 h	0.0 h	0.0 h	0.0 E
10-17 Dec	0.0 h	0.0 h	0.0 h	0.0 E
Grower property effect	0.6 C[1]	0.7 C	0.4 D	

[1]Values followed by the same letter are not significantly different according to Tukey's HSD at 95% confidence level. The main effect of the assessment date (A-E) (*P*<0.0001); grower property (C and D) (*P*=0.001) and interaction (a-h) (*P*<0.0001) between the assessment date were significant (*P* <0.0001).

The total amount of rain recorded weekly in 2011 from both the Nelson Automatic Weather Station (close to the Richmond property) and the Riwaka Electronic Weather Station (close to the Upper and Lower Moutere properties) was greater than that recorded in 2010 (Figure 2.7A and 2.8A). The total weekly rain pattern from September to December in 2010 was similar for both weather stations. A similar amount of rain was recorded for the two weather stations in 2011, apart from the 2-10 of December when approximately 300 mm, was recorded from the Nelson weather station compared with only 100 mm from the Riwaka weather station.

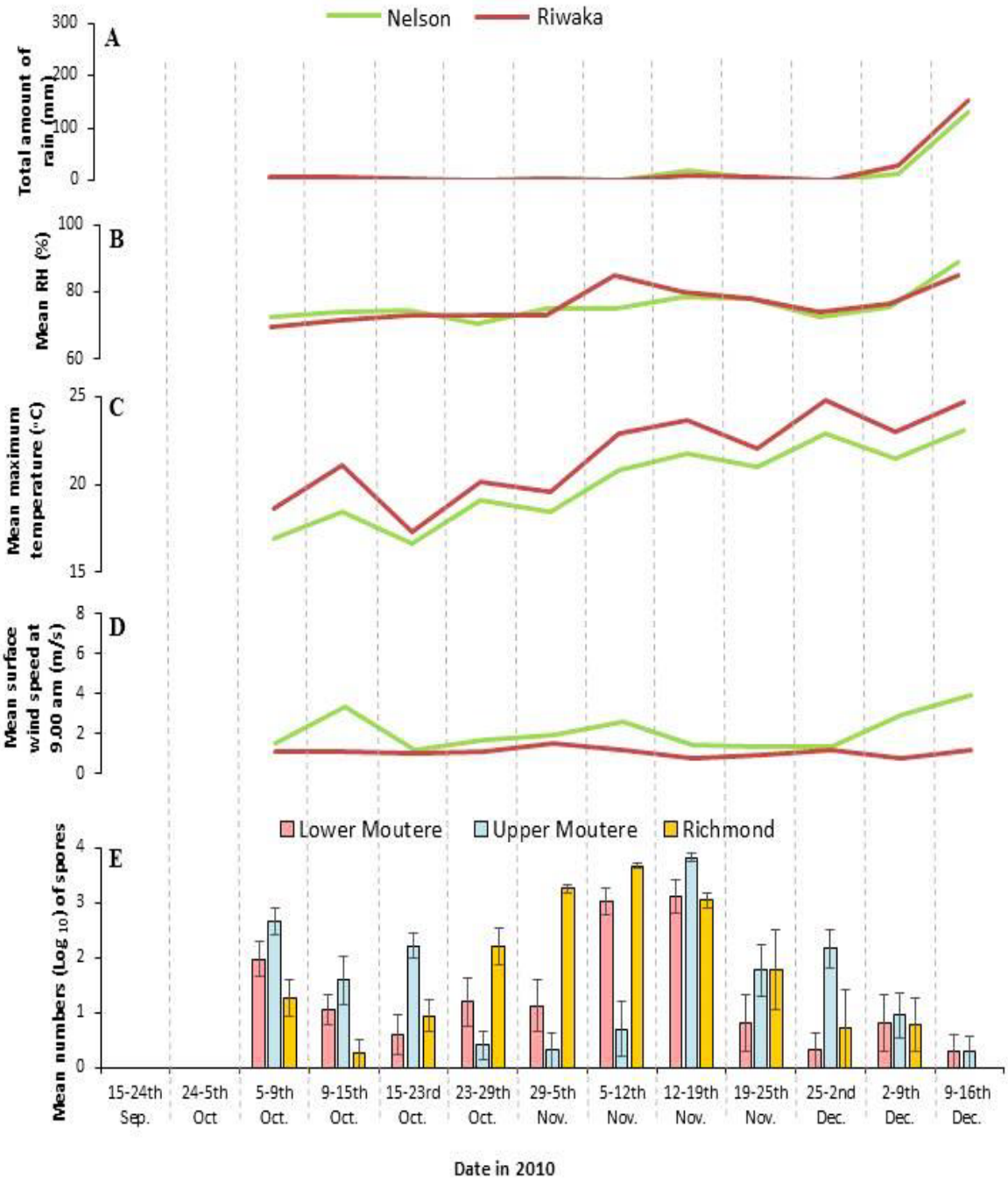

Figure 2.7 (A) Total amount of rain (mm), (B) mean RH (%), (C) mean maximum temperature (ºC) and (D) mean surface wind speed (m/s) at 9.00 am, recorded during the weekly spore trapping periods and (E) mean number (log_{10}) of spores characteristic of *Peronosparsa sparsa* trapped on Vaseline® coated glass slide placed in three boysenberry grower properties, Lower Moutere and Upper Moutere and Richmond, at weekly intervals between October and December in 2010. Mean of four glass slides per property per week, with error bars representing the standard errors of weekly mean (SEM) spore numbers. Weather data collected from the Nelson and Riwaka weather stations representing the weather conditions in the Richmond grower property and the two Motueka grower properties (Lower and Upper Moutere), respectively.

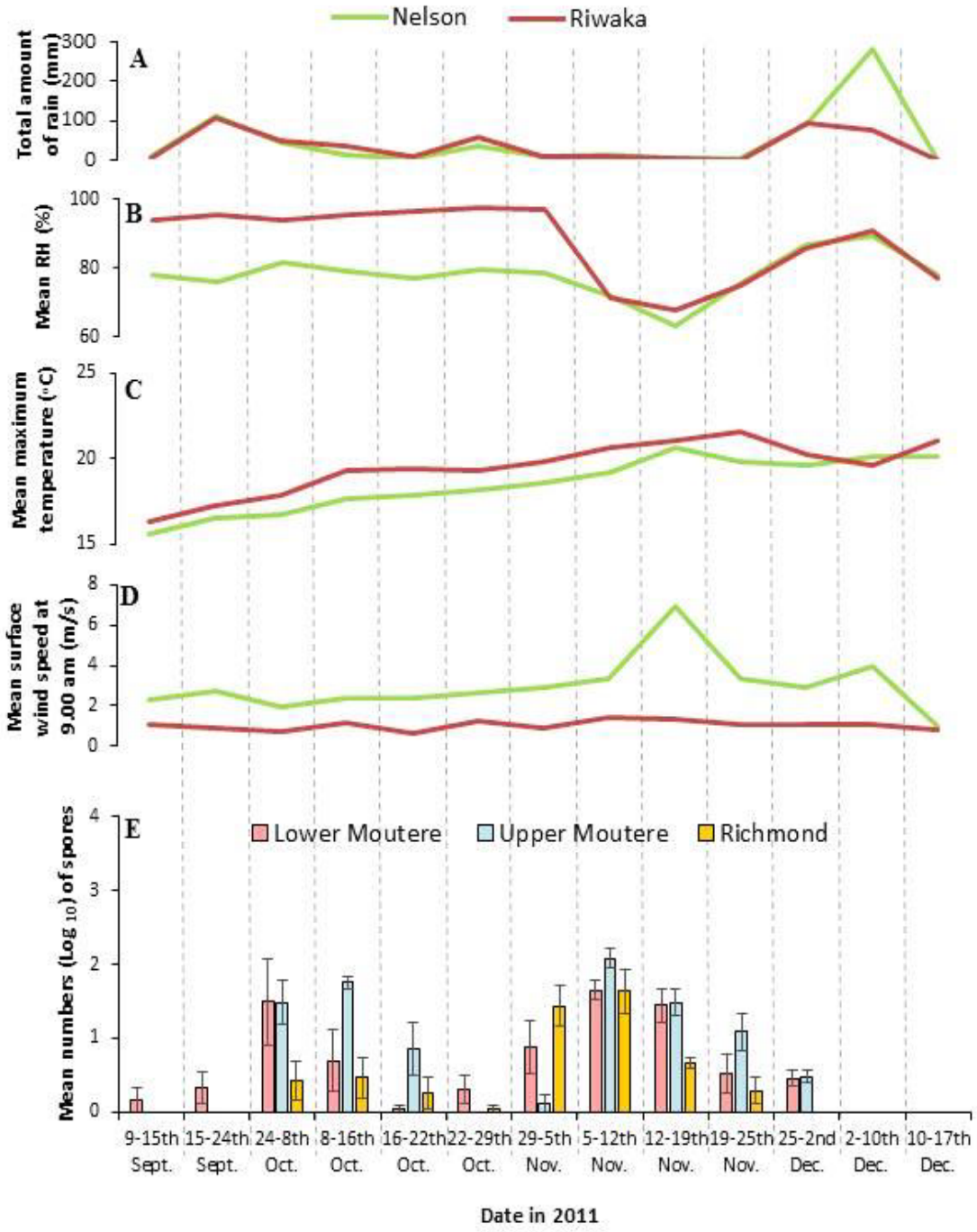

Figure 2.8 (A) Total amount of rain (mm), (B) mean RH (%), (C) mean maximum temperature (ºC) and (D) mean surface wind speed (m/s) at 9.00 am, recorded during the weekly spore trapping periods and (E) mean number (log_{10}) of spores characteristic of *Peronospara sparsa* trapped on Vaseline® coated glass slides placed in three boysenberry grower properties, Lower Moutere and Upper Moutere and Richmond, at weekly intervals between September and December in 2011. Mean of four glass slides per property per week, with error bars representing the standard errors of weekly mean (SEM) spore numbers. Weather data collected from the Nelson and Riwaka weather stations representing the weather conditions in the Richmond grower property and the two Motueka grower properties (Lower and Upper Moutere), respectively.

According to the climate data obtained from the NIWA weather station the frequency of days with rain in September 2010 was 55% which was greater than the rain threshold (r; 38.7%) determined by Kim *et al*. (2014). It was 26 and 23% in October and 23 and 13% in November in Nelson and Riwaka weather stations, respectively (Figure 2.9). In contrast, in 2011 the frequency of days with rain in September recorded at both weather stations was always below the rain threshold value (27 and 37% Nelson and Riwaka weather stations, respectively) although the frequency of days with rain for the next consecutive months reached or exceeded this value.

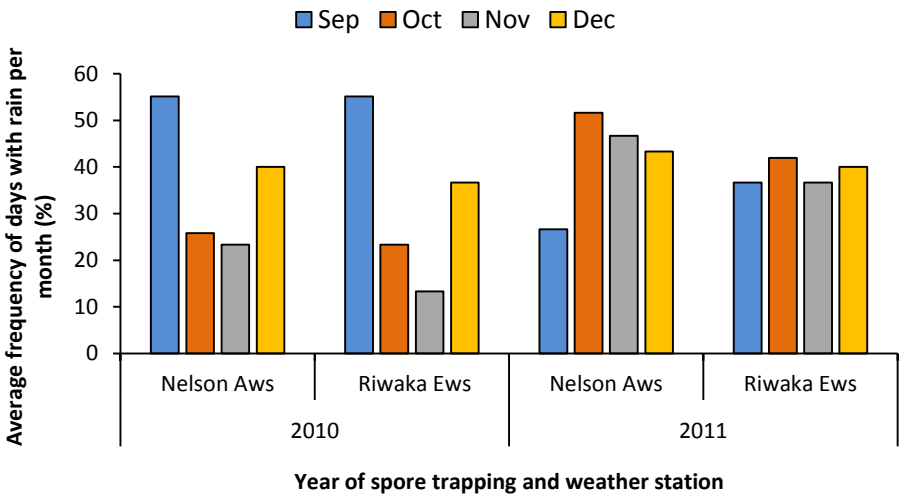

Figure 2.9 The average frequency (%) of days per month (Sept-Oct) with rain in 2010 and 2011 during the spore trapping experiment at the three grower properties based on the data from the Nelson and Riwaka weather stations.

The mean weekly % RH recorded in 2011 at the Riwaka weather station was higher than that recorded in 2010 for the first part of the assessment period (September to 29-5 of November) and slightly higher for the Nelson weather station during this period. In 2011 the RH was also greater than in 2010, for weather stations during the periods 25-2 and 2-10 of December, (Figure 2.7B and 2.8B). However the % RH for weeks 2-4 (5-12, 12-19 and 19-25) November and the 10-17 of December was higher in 2010 than in 2011 for both weather stations. In 2011, the % RH (95-70% and 85-65 for Riwaka and Nelson weather stations, respectively) fluctuated more broadly compared with 2010 when % RH varied over 70-85%, and 70-90% for Riwaka and Nelson weather stations, respectively. However, in 2011 whilst the RH recorded by the Riwaka station was always above 90% during September to October, the level recorded by the Nelson weather station was generally lower in both years. The decrease in RH recorded during the weeks 2-4 of November in 2011 at both weather stations corresponded with the greatest number of spores trapped. Further, the RH recorded at both weather stations in the 25-2 and 2-10 of December was above 85%, whilst in the previous year the RH was less than 80% during

the same period. The spore numbers trapped during the same period was higher in 2010 compared with 2011 (Figures 2.7E and 2.8E).

The mean weekly maximum temperature (Tmax.) recorded at both weather stations in 2010 fluctuated over 14-24ºC (Figure 2.7C). In 2011, the Tmax. recorded at both weather stations was between 16 and 21ºC with the pattern similar from both weather stations. In 2010 and 2011, Tmax fluctuated between 18 and 23ºC at both weather stations during November when the number of spores trapped from all three properties were highest. During December (early summer) in 2010 the Tmax recorded at both weather stations was always above 20ºC. Spores were trapped on all dates at all properties apart from the 9-16 in December at the Richmond property. In contrast, in 2011 the mean Tmax recorded at both weather stations during December was always near 21ºC, with spores trapped only on 25-2 of December in the Upper and Lower Moutere properties with none trapped at the Richmond property.

According to the climate data obtained from the NIWA weather station, the temperature threshold (t; 9.8 h) determined by Kim *et al*. (2014) as the average number of hours per day with temperature at 15-20ºC was only exceeded in Nelson in November (11.2 and 10.0) and December (13.1 and 14.7) in 2010 and 2011 respectively (Figure 2.10). In Riwaka the temperature threshold was only exceeded in December 2011 (11.2). The temperature threshold was not exceeded in any other month for either site.

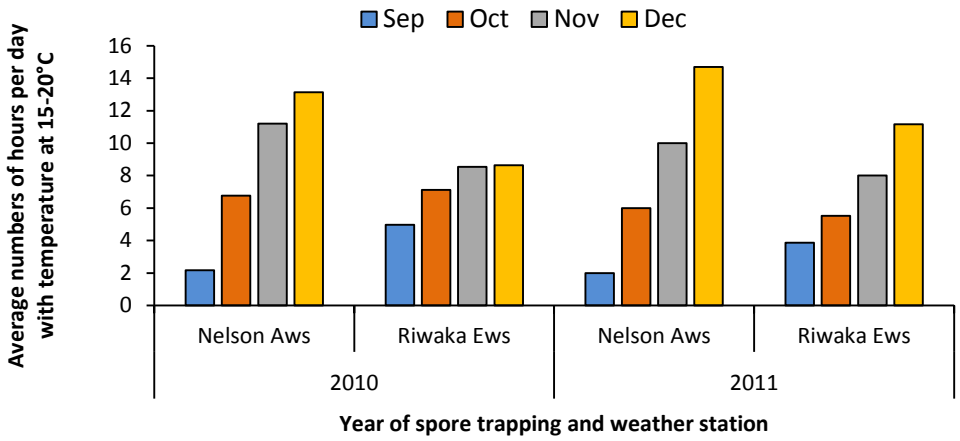

Figure 2.10 The number of hours per day with temperatures averaging 15-20ºC per month (Sept-Dec) in 2010 and 2011 during the spore trapping experiment at the three grower properties based on the data from the Nelson and Riwaka weather stations.

The mean surface wind speed recorded at 9:00 am at the Nelson weather station in 2011 (1-7 m/s) was greater than that recorded in 2010 (1-4 m/s) (Figure 2.7D and 2.8D). In 2010, the maximum mean wind speed of 4 m/s was recorded during the 9-16 in December corresponding to no spores being trapped at the Richmond site. Whereas, in 2011 the mean maximum wind

speed of 7 m/s was recorded during the 12-19 in November which corresponded with the third highest number of spores (0.7 $\log_{10}$ spores) trapped during the year at the Richmond site (Figures 2.8E). In contrast, the mean surface wind speed recorded at the Riwaka station was relatively constant (0-2 m/s) over the trapping period in both years.

2.3.1.2 Disease assessment under field conditions

There was a significant effect (P<0.001; Appendix A.4.3) of grower property on the number of dryberries per plant in 2011, with more per plant in the Upper Moutere property. Also the mean number of dryberries per plant assessed on the 29 Dec (P<0.001) was significantly higher than in the previous week (23 Dec) (Figure 2.11). The interaction effect of grower property and assessment time was highly significant (P<0.001) with the most dryberries from the Upper Moutere property at the second assessment time (29 Dec).

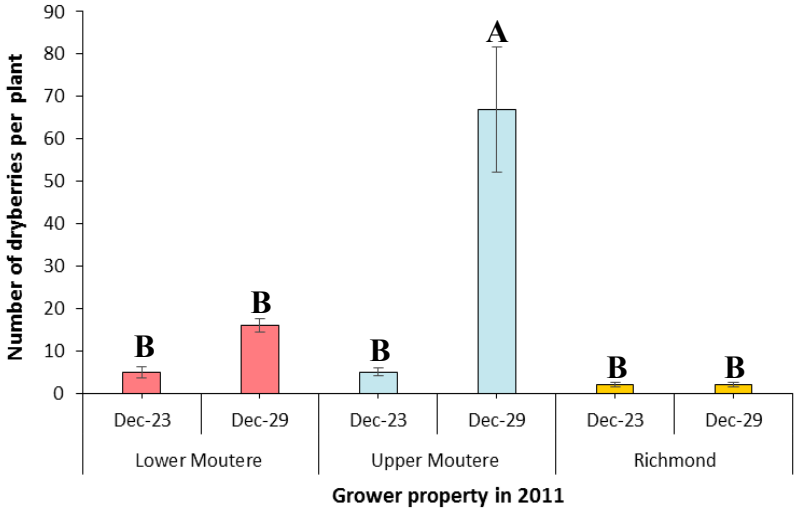

Figure 2.11 The mean numbers of dryberries per boysenberry plant in rows where the spore trapping slides were placed, assessed on the 23 and 29 December 2011, at three grower properties (Lower Moutere, Upper Moutere and Richmond). Bars with the same letters are not significantly different according to Tukey's HSD test (P<0.05). The main effect of grower property, assessment time and interactions are significantly different (P<0.001). Error bars represent the standard errors of mean (SEM) number of dryberries per plant.

2.3.1.3 Potential sources of inoculum under field condition

Symptomatic leaves were observed from the more shaded areas of the canopy, at both the Lower Moutere and the Upper Moutere properties. More symptomatic leaves were observed on boysenberry plants in the Lower Moutere property than the Upper Moutere property; however sporulation was not observed in the field. No symptomatic leaves were observed from the assessed rows at the Richmond property. Only leaves obtained from the Lower Moutere property showed sporulation on the symptomatic lesions after incubation under laboratory conditions, with sporulation observed on 100% of the leaves incubated at 15ºC and 83% at 20ºC (both at 100% RH). Leaves collected from the Upper Moutere grower property were contaminated with *Botrytis cinerea*.

Young floricane stems from the Lower Moutere property sporulated when incubated at 15ºC and 100% RH but not when incubated at 20ºC. No symptoms or sporulation was observed on asymptomatic primocanes or flowers.

The mature unripe fully expanded berries (not coloured) which were collected from the Lower Moutere property sporulated on their calices only when not surface sterilised with 10% bleach. Sporulation was observed on the calices of all berries incubated at 15ºC and 100% RH, whereas at 20ºC and 100% RH sporulation only occured on the calices of 67% of berries. No sporulation or symptoms were observed on drupelets. No sporulation was observed on any of the king berries or non-king berries (unripe) collected from the Richmond property and incubated at 15 or 20ºC at 100% RH after surface sterilisation with 10% bleach.

2.3.2 Sporulation potential of naturally infected boysenberry plant tissues at different temperatures and relative humidity

2.3.2.1 Sporulation potential of leaves

Experiment 1- sporulation potential on symptomatic leaves

All of the leaves and leaf discs incubated at 80% RH became dried at all three incubation temperatures and no lesion expansion or sporulation were observed (Appendix A.5), therefore, the data was not included in the results. Lesion expansion was measured only for leaves obtained from shadehouse plants. No statistical analysis was carried out due to the large number of zero values across all treatments, for both lesion expansion and sporulation. Mean lesion expansion for both mature and young leaves incubated appeared to be greater at 20°C and 100% RH than at in the other conditions (Figure 2.12).

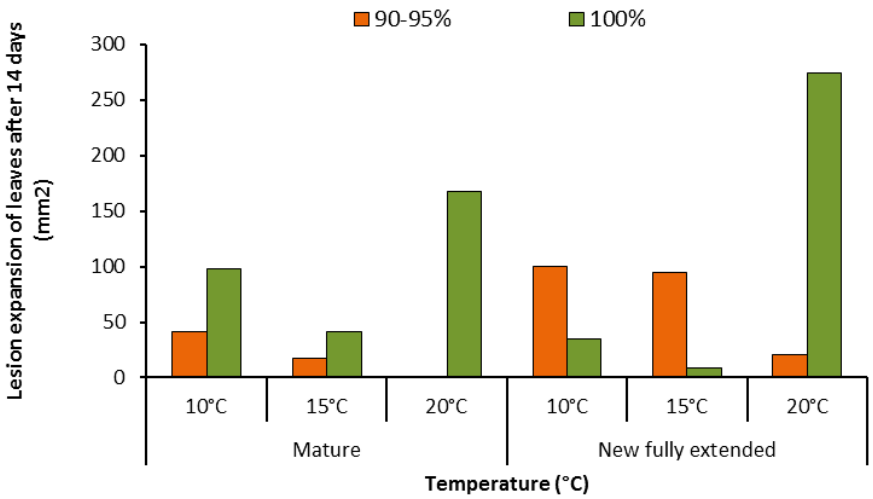

Figure 2.12 Lesion expansion (mm^2) on leaves obtained from *Peronospora sparsa* systemically infected boysenberry plants grown in the shadehouse (Lincoln University) at two maturity stages (mature or new, fully extended) assessed after 14 days incubation at three temperatures (10, 15, 20°C) and two relative humidities (90-95%, 100%).

In all cases, sporulation was observed only on the lower surfaces of the leaves/leaf discs. The maximum numbers of spores were observed on leaf discs cut from mature leaves obtained from the shadehouse plants and incubated at 15°C at 100% RH (mean of 762 spores/mm^2) followed by leaf discs cut from new but fully extended leaves also from shadehouse plants incubated at 15°C at 100% RH (mean of 567 spores/mm^2) (Table 2.3). At 100% RH spores were also produced on leaf discs cut from mature leaves incubated at 10°C and discs cut from new fully extended leaves incubated at 20°C. No spores were produced on any leaf discs incubated at 90-95% RH at any temperature, apart from discs cut from mature leaves incubated at 10°C.

For leaves, the maximum numbers of spores were produced on mature leaves incubated at 10°C at 100% RH (mean of 387 spores/mm^2), followed by new fully extended leaves incubated at 20°C and 10°C at 100% RH (means of 326 and 277 spores/mm^2, respectively) (Table 2.3). No spores were produced on mature leaves from shadehouse plants incubated at 20°C at either 90-95% or 100% RH or newly fully extended leaves incubated at 20°C at 90-95% RH.

Table 2.3 The mean numbers of spores/mm^2 lesion area produced on naturally infected boysenberry leaves or leaf discs of two ages (mature or new fully extended) from shadehouse (Lincoln University) plants after incubation at one of three temperatures (10, 15, 20°C) and two RHs (90-95, 100%) for 14 days.

		No. of spores per mm^2					
		Mature			New fully extended		
		10°C	15°C	20°C	10°C	15°C	20°C
Leaf discs	90-95%	63	0	0	0	0	0
	100%	166	762	0	0	567	92
Leaves	90-95%	72	39	0	148	50	0
	100%	387	153	0	277	94	326

*No statistical analysis was carried out due to the large number of 0 counts

For leaf discs cut from field collected leaves in the Lower Moutere property, maximum spore production (146 spores/ mm^2) was observed on discs from mature leaves incubated at 20°C at 90-95% RH followed by discs cut from mature leaves incubated at 10°C at 90-95% RH (102 spores/ mm^2) (Table 2.4). Spores were produced on all discs cut from field leaves apart from discs from mature leaves incubated at 15°C at 90-95% RH and from new fully extended leaves incubated at 10°C at 90-95% and 15°C at 100% RH. For field obtained leaves maximum spore production was observed on mature leaves incubated at 10°C at 100% RH (67 spores/mm^2) followed by mature leaves incubated at 10°C at 90-95% RH (30 spores/mm^2) (Table 2.4). No spores were observed on mature leaves incubated at 15°C at 90-95% RH or new, fully extended leaves incubated at 15°C at 100% RH).

Table 2.4 The number of spores/mm^2 lesion area produced on symptomatic naturally infected boysenberry leaves or leaf discs of two ages (mature or new fully extended) from field (Lower Moutere) plants after incubation at one of three temperatures (10, 15, 20°C) and two RHs (90-95, 100%) for 14 days.

		No. of spores per mm^2					
		Mature			New fully extended		
		10°C	15°C	20°C	10°C	15°C	20°C
Leaf discs	90-95%	102	0	146	0	7	1
	100%	46	43	7	1	0	6
Leaves	90-95%	30	0	15	7	11	4
	100%	67	2	17	10	0	8

*No statistical analysis was carried out due to the large number of 0 counts

Experiment 2- sporulation potential on asymptomatic leaves

No lesions developed on any of the asymptomatic leaves and no spores were produced after 14 days. Further incubation resulted in *Botrytis cinerea* infection and sporulation developing on the leaves.

2.3.2.2 Sporulation potential of canes

Sporulation occurred on symptomatic floricane segments incubated at 15°C, with sporulation observed on two of the six floricane segments after six days. At 20°C, no sporulation was observed on cane segments, even after 14 days incubation. On the sporulating floricanes, sporulation occurred on the calices of the flower buds or flowers, extending 2-3 cm down the soft (young) stems, including on the leaves (Figure 2.13A). The sporulated area eventually shrivelled. Microscopic preparations from the sporulating region on the canes confirmed the presence of characteristic *P. sparsa* spores and sporangiophores (Figure 2.13B and C). However, fungal structures characteristic of *B. cinerea* also grew on leaves and canes making assessment of *P. sparsa* sporulation difficult with longer incubation (Figure 2.13B).

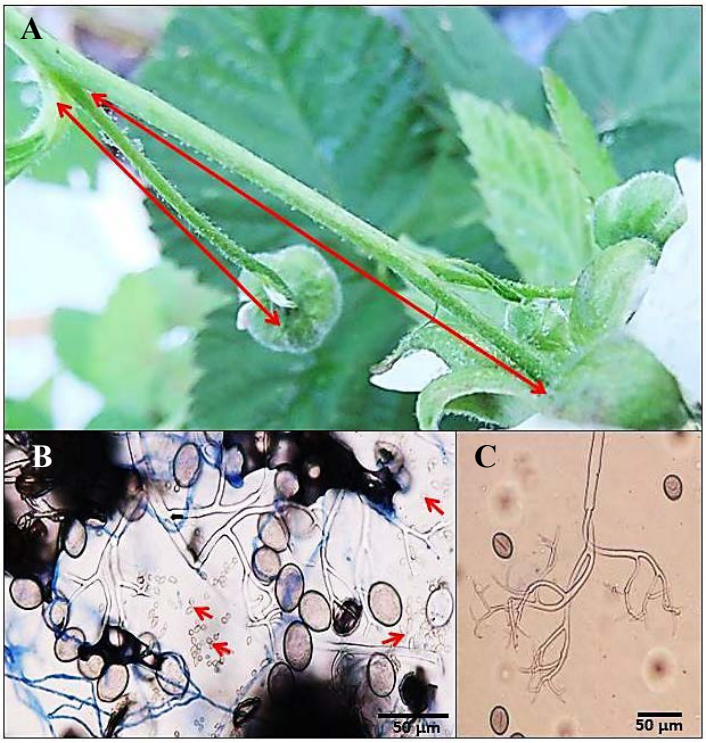

Figure 2.13 (A) Sporulation characteristic of *Peronospora sparsa* on the calyx and stem of the flower and flower bud of the systemically infected floricane incubated at 100% RH at 15°C [the red arrows indicate the length of sporulation of *P. sparsa* on the calyx of a flower (approx. 3 cm) and a bud (approx. 2 cm)], (B) spores and sporangiophores characteristic of *P. sparsa* on stem tissue with the contaminant *Botrytis cinerea* spores and mycelia indicated by red arrows, and (C) characteristic dichotomously branched sporangiophore of *P. sparsa* on calyx tissue.

For the symptomatic single floricane cut into sections and incubated at 15°C and 100% RH, no symptoms or sporulation developed on the cane, however, symptoms developed on the leaves. Symptoms occurred on some leaves from all the sections cut from the base, middle and top of the floricane. No symptoms or sporulation occurred on asymptomatic primocane sections incubated at either 15°C or 20°C.

2.3.2.3 Sporulation potential of fruit

No sporulation occurred on any of the surface sterilised or non-surface sterilised unripe berries collected from either of the two properties (Lower Moutere and Richmond). However, sporulation occurred on the calices of berries obtained from the Lower Moutere property that were not surface sterilised and incubated at either 15°C and 20°C. Sporulation was observed on the calices of all non-surface sterilised berries incubated at 15°C, and 67% of berries incubated at 20°C. Ripe berries collected in December 2011 and incubated at 15°C were all contaminated with *B. cinerea* which sporulated profusely, and no *P. sparsa* sporulation was observed.

2.3.3 *In vitro* production of asexual spore inoculum

2.3.3.1 Surface sterilisation methods for non-symptomatic plants

In Experiment 1, when the leaves were surface sterilised and pressed onto PDA to determine the efficacy of the different sterilisation methods, the number of fungal colonies from the upper leaf surfaces was significantly (P=0.002; Appendix A.6.1) higher than from the lower leaf surfaces (Table 2.5). There was a significant effect (P=0.013) of sterilisation method on the number of fungal colonies, with all treatments reducing the numbers of fungal colonies relative to the control. Treatment with 0.25% hypochlorite significantly (P<0.05; Table 2.5) reduced the number of fungal colonies compared with sterile water and the untreated control, but the effect of this treatment did not differ from the ethanol treatment. Low numbers of bacterial colonies were recorded from all treatments and, therefore, data for bacterial colonies were not statistically analysed. In Experiment 2, when the leaf surfaces were pressed onto both PDA and NA, fungal colony numbers were again significantly higher (P<0.001; Appendix A.6.2) on the upper than the lower leaf surfaces (Table 2.6). The number of fungal colonies on both culture media was significantly affected (P<0.001 on PDA and P=0.001 on NA) by sterilisation treatment, being lower for ethanol and hypochlorite than for sterile water and the untreated control (Table 2.6). Only a few bacteria developed on PDA and NA plates and no analysis was carried out due to the large number of zero counts (Appendix A.6.3; Table 2.6).

Table 2.5 The effects of three leaf sterilisation methods on the numbers of fungal and bacterial colonies growing on PDA after pressing the upper and lower surfaces of treated leaves onto the surface of the agar. Mean of six replicate leaves per treatment.

	No. of Fungal colonies[1]			No. of Bacterial colonies[2]	
Sterilisation treatment	Upper	Lower	Sterilisation treatment effect	Upper	Lower
Control	84.75 C	45.25 B	65.00 b	0.67	0.25
Sterile water	24.25 AB	8.00 A	16.12 a	0.08	0.25
70% Ethanol	7.42 A	3.75 A	5.58 a	0.92	0.67
10% Bleach	2.33 A	2.42 A	2.38 a	0.08	0.00
LSD 18.20			LSD 9.10		
Leaf surface effect LSD 12.87	29.7 c	14.9d			

[1]Values followed by the same letter are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. The main effect of sterilisation treatment (fungi) (a-b) was highly significant (P<0.001; LSD=9.10); and leaf surface (c-d) was significant (P=0.002; LSD=12.87); and interaction between them (A-C) was also significant (P=0.013; LSD=18.20).

[2]The main effect of sterilisation treatment (bacteria) or interactions were not statistically analysed due to low number of bacterial colonies and large number of zero counts.

Table 2.6 The effects of three leaf sterilisation methods on the numbers of fungal and bacterial colonies growing on PDA and NA after pressing the upper and lower surfaces of treated leaves onto the surface of the agar. Mean of six replicate leaves per treatment.

Culture medium	Sterilisation treatment	No. of Fungi colonies[1]			No. of Bacterial colonies[2]	
		Upper	Lower	Sterilisation treatment effect	Upper	Lower
PDA	Control	225.33 A	100.67 B	163.00 c	3.83	1.17
	Sterile water	93.83 BC	47.00 CD	70.42 b	0.17	0.17
	70% Ethanol	8.50 D	5.67 D	7.08 a	3.00	1.33
	10% Bleach	6.33 D	7.67 D	7.00 a	0.00	0.00
	LSD 43.50					
LSDs 21.75		83.5 d	40.2 e	LSD 30.76		
NA	Control	252.00 A	135.50 B	193.75 c	37.83	13.83
	Sterile water	163.00 B	60.50 C	111.75 b	5.83	5.17
	70% Ethanol	4.33 C	3.67 C	4.00 a	6.67	4.00
	10% Bleach	3.33 C	4.83 C	4.08 a	0.00	0.00
	LSD 50.23					
LSDs 25.11		105.7 d	51.1 e	LSD 35.52		

[1]Values followed by the same letter are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. The main effect of sterilisation treatment (fungi) on PDA (a-c) was highly significant ($P<0.001$; LSD=30.76); and leaf surface (d-e) was significant ($P<0.001$; LSD=21.75); and interaction of them (A-D) was also significant ($P<0.001$; LSD=43.50). Also the main effect of sterilisation treatment (fungi) on NA (a-c) was highly significant ($P<0.001$; LSD=35.52); and leaf surface (d-e) was significant ($P<0.001$; LSD=25.11); and interaction of them (A-C) was also significant ($P=0.001$; LSD=50.23)

[2]The main effect of sterilisation treatment (bacteria) or interactions were not statistically analysed due to the low number of bacterial colonies and large number of zero counts.

2.3.3.2 Development of a method for producing spore inoculum on pathogen free leaves

Results were not statistically analysed as no infection was observed in the majority of replicates for each treatment. Infection incidence (3 out of the 6 replicates) was slightly higher in the leaves surface sterilised with 70% ethanol than in the leaves from other methods (Figure 2.14). With 70% ethanol, lesions were first observed 9 days post-inoculation (dpi), whereas for all other treatments the first sign of symptoms was at 14 dpi. The remaining trends were for lowest incidence of infection on leaves treated with 0.25% hypochlorite, and the sterile water treatment was similar to the untreated control.

Symptoms appeared first as faded purplish angular shaped lesions, and after a further one to two days sporulation was observed. When the lesion matured they were a deep purplish colour, and finally ended up as a brown area surrounded by a yellow margin (Figure 2.15).

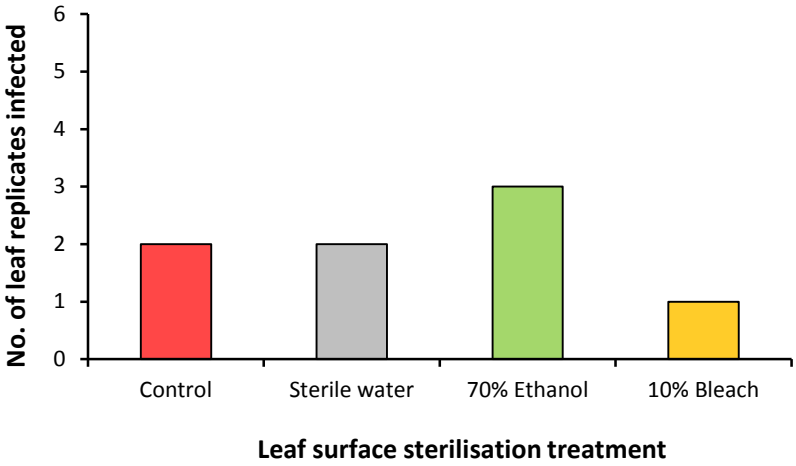

Figure 2.14 The number of leaves (out of 6) which developed lesions when inoculated with *Peronospora sparsa* after different surface sterilisation treatments.

The mean lesion size on the treated leaves (180, 66, 45 mm^2 in 70% ethanol, 10% bleach and sterile water treatments, respectively) were larger than those produced on the untreated control (21 mm^2). Spore production was not assessed since increased incubation to allow for more lesions to develop resulted in the lesions growing into each other and becoming contaminated by *B. cinerea*. However, in general spore production appeared to be greatest on leaves surface sterilised with 70% ethanol. Therefore, 70% ethanol was used to sterilise leaves prior to use for subsequent spore production, whereas, sterile water was used to wash leaves prior to use in infection studies as this had reduced numbers of fungal contaminates and was unlikely to affect the susceptibility of the leaves to infection. There was no difference between leaf regions with regards to symptom development or spore production. However, once the lesion had established, lesion expansion and spore production occurred along the veins of the leaves.

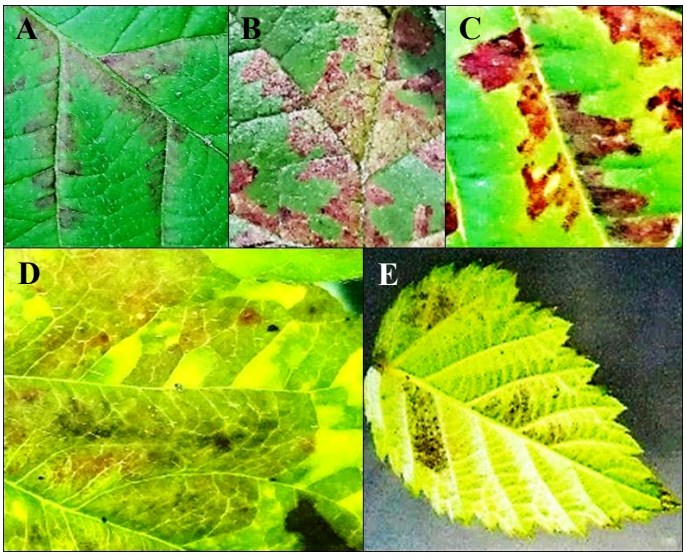

Figure 2.15 Stages of maturity of lesions caused by *Peronospora sparsa* infection on boysenberry leaves (A-D) young purplish angular lesion developing into a brownish lesion area surrounded by a yellow margin, and (E) sporulation on abaxial side of the leaf after 14 days.

2.3.3.3 Optimum conditions for spore germination

Experiment 1: *The effect of different substrates on spore germination*

There was a significant effect ($P<0.001$; Appendix A.7.1) of temperature on spore germination, with spore germination significantly higher at 20°C compared with 15°C and 10°C (Table 2.7). There was also a significant effect ($P<0.001$) of substrate on spore germination with more spore germination on leaf discs compared with microscopic slides and 1.5% WA, with no significant difference between the latter two substrates. The interaction between temperature and substrate was also significant ($P<0.05$) with spore germination significantly higher at 20°C on leaf discs compared with 15 and 10°C, with no significant difference between the latter two temperatures. In contrast, incubation temperature had no effect on spore germination on either glass slides or 1.5% WA. There was no effect ($P=0.897$) of incubation time on spore germination (Table 2.7). Therefore, spore germination was assessed on water washed leaf discs incubated at 20°C for 24 h in the dark in the remaining experiments.

Table 2.7 Mean percentage germination of *Peronospora sparsa* spores on leaf discs, microscopic slides or 1.5% water agar and incubated at three temperatures (10, 15 and 20°C) after 24 and 48 h incubation in the dark. Means of six replicates for each treatment are presented.

Substrate	% Germination[1]						
	10°C		15°C		20°C		Substrate effect
	24 h	48 h	24 h	48 h	24 h	48 h	
Leaf disc	45.5	47.1	60.9	49.9	68.9	71.5	57.3 A
Means	(46.3 b)		(55.4 b)		(70.2 a)		
Microscopic slide	13.0	17.7	15.9	18.9	29.7	23.5	19.8 B
Means	(15.4 c)		(17.4 c)		(26.6 c)		
1.5% WA	13.0	14.9	13.8	17.8	16.2	17.7	15.6 B
Means	(14.0 c)		(15.8 c)		(16.9 c)		
Temperature effect	25.2 D		29.5 D		37.9 C		

[1]Values followed by the same letter are not significantly different according to Tukey's HSD at $P<0.05$. The main effect of the substrate (A-B) and temperature (C-D) were highly significant ($P=0.000$); and interaction between them (a-c) also significant ($P=0.01$). The main effect of incubation time or interactions of incubation time and temperature or substrate was not significant ($P=0.897$ and $P=0.613$, 0.652, 0.327, respectively).

Experiment 2: *Verification of spore germination counts using different methods*

The effect of the method used to enumerate spore germination was highly significant ($P<0.001$). The percentage germination obtained using the cellotape strip method was significantly ($P\leq0.05$) higher compared with the other two counting methods (Table 2.8). There was a significant effect ($P=0.018$; Appendix A.7.2) of temperature on percentage germination, with germination being significantly higher at 20°C than at 10°C, but not compared with 15°C. Additionally, the percentage germination obtained after 24 h incubation at 20°C and in the dark, in this experiment, was similar to that recorded for the leaf discs in Experiment 1.

Table 2.8 Effect of three enumeration methods (light microscopy of sticky cellotape strip, observation by stereomicroscopy with intact leaf discs, and after clearing them) on the percentage germination of spores incubated on leaf discs at three temperatures (10, 15 and 20ºC) after 24 h. Means of six 20 µL spore droplets per replicate for each treatment are presented.

Method	% Germination[1]			
	10°C	15°C	20°C	Method effect
Cellotape strip	46.7	60.8	68.5	58.6 A
Stereomicroscopy	27.1	28.7	29.1	28.3 B
Leaf clearing	32.3	36.0	43.4	37.2 B
LSD (Interaction)13.65				LSD 7.88
Temperature effect	35.4 C	41.8 CD	47.0 D	
LSD 7.88				

[1]Values followed by the same letter are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. The main effect of the method (A-B) was highly significant ($P<0.001$; LSD=7.88) and temperature (C-D) was also significant ($P=0.018$; LSD=7.88); interaction between them was not significant ($P=0.333$; LSD=13.65).

Experiment 3: *Verification of the cellotape strip method for assessing spore germination*

The mean percentage spore germination after 24 h incubation at 20ºC on the leaf discs assessed using the cellotape method was 60.6% (Appendix A.7.3). These leaf discs were cleared and the germination of the spores remaining on the leaf discs determined as being 10.0%. However, of the calculated 200 spores applied per inoculum drop, only 71.5% were recovered either by the cellotape method or observed after clearing the leaf discs.

2.3.4 Optimum conditions for *P. sparsa* infection

2.3.4.1 Effect of temperature and infected material (leaves/leaf discs) on *P. sparsa* infection, sporulation and lesion size

The lesion size, number of spores per lesion and sporulation score were significantly affected ($P=0.009$, $P=0.006$ and $P=0.049$, respectively) by tissue type (leaves and leaf discs), with all being greater on leaf discs compared with leaves (Table 2.9). Disease incidence was not significantly affected ($P=0.111$; Appendix A.8.1) by tissue type. Disease incidence was significantly affected ($P=0.03$) by temperature (Table 2.10). The incidence of infection at 15°C was greater than at 10 and 20°C. There was also a significant effect of temperature on lesion size, number of spores per lesion and sporulation score ($P=0.009$, $P=0.006$ and $P=0.003$, respectively) which were all higher at either 15°C or 20°C than at 10°C. There were no significant interactions between tissue type (leaves and leaf discs) and temperature on lesion size ($P=0.069$) or number of spores per lesion ($P=0.362$). There was a significant interaction ($P=0.004$) between temperature and tissue type on sporulation, with sporulation being significantly higher on leaf discs at 15°C than at 10°C, but not with any other treatment. Full statistical analysis is presented in Appendix A.8. To provide for spore production for the remainder of the experiments, inoculated leaf discs were incubated at 15°C in 100% RH.

Table 2.9 The effects and interaction effects of temperature (10, 15 and 20°C) and boysenberry leaf tissue type (leaves or leaf discs) on the incidence (proportion of six leaves or 18 discs) and lesion size (mm^2), number of spores produced per lesion or sporulation score (1-6 scale) of *Peronospora sparsa* infection of boysenberry leaf tissues 14 days after inoculation.

Leaf tissue type	Temp. (°C)	Incidence	Lesion size (mm^2)	No. of Spores/ lesion	Sporulation Score[2,3]	Effect of tissue type[1];		
						lesion size	spores per lesion	sporulation score[2,3]
Leaves	10	0.33	3.5	150.0	0.00 GH	8.4 A	552.8 C	0.00 E
	15	0.50	10.5	791.7	2.50 GH			
	20	0.50	11.0	716.7	2.50 GH			
Leaf discs	10	0.39	7.7	522.2	0.00 H	24.0 B	1342.4 D	5.00 F
	15	0.89	45.3	2116.7	6.00 IG			
	20	0.78	19.0	1388.3	5.50 GH			

[1]Mean values followed by the same letter are not significantly different according to Tukey's HSD at $P \leq 0.05$. The main effect of the tissue type on lesion size (A-B) and number of spores produced per lesion (C-D) were significant (P=0.009 and P=0.006, respectively). No significant interaction between tissue type and temperature on lesion size and number of spores per lesion (P=0.069 and P=0.362, respectively). Significant interaction between tissue type and temperature on sporulation score (P=0.004).
[2]Median values for the sporulation scores were obtained from the Kruskal-Wallis non parametric test
[3]There was a significant effect of tissue type (E-F; P=0.049) and a significant interaction (G-I; P=0.004) between tissue type and temperature on sporulation score.

Table 2.10 The effect of the temperature (10, 15 and 20°C) on the incidence (proportion of six leaves or 18 discs) and lesion size (mm^2), number of spores produced per lesion or sporulation score (1-6 scale) of *Peronospora sparsa* infection of boysenberry leaves or leaf disc tissue 14 days after inoculation.

Temp. (°C)	Incidence[4]	Lesion size (mm^2) [1]	No. of Spores/ lesion[1]	Sporulation Score[123]
10	0.38 (0.19-0.57)	5.6 a	336.1 c	0.00 e
15	0.79 (0.63-0.95)	27.9 b	1454.2 d	5.00 f
20	0.71 (0.53-0.89)	15.0 ab	1052.5 cd	5.00 f

[1]Mean values followed by the same letter are not significantly different according to Tukey's HSD at $P \leq 0.05$.
The main effect of the temperature on lesion size (a and b) and number of spores produced per lesion (c and d) were significant (P=0.009 and P=0.006, respectively).
[2]Median values for the sporulation scores were obtained from the Kruskal-Wallis non parametric test.
[3]There was a significant effect of temperature (e-f; P=0.003) on sporulation score.
[4]There was a significant effect of temperature (P=0.003) on incidence of *P. sparsa* infection and the confidence intervals are provided in parentheses.

2.3.4.2 Effect of spores concentration on infection

Spore concentration, assessment time (dpi) and the interaction between concentration and time significantly affected (P <0.001, P<0.001, P=0.026, respectively) disease incidence (Table 2.11; Appendix A.9.1). At 9 dpi, lesions were only observed on leaf discs inoculated with 200 and 2000 spores /20 µL droplet. At 12 dpi, lesions were produced on 20% of leaf discs inoculated with 20 spores compared with 100% and 80% infection of leaf discs inoculated with 200 and 2000 spores, respectively. In addition there was a significant effect of spore concentration (P<0.001; Appendix A.9.2) and a significant interaction between spore concentration and assessment time (P<0.001; Appendix A.9.2) sporulation score, with the highest sporulation score for leaf discs inoculated with 200 spores at 12 dpi or 2000 spores at 9 or 12 dpi.

Table 2.11 Incidence (proportion of ten leaf discs) and sporulation score of *Peronospora sparsa* infection on leaf discs of *Mapua* boyensenberry plants grown in the Lincoln University shadehouse and inoculated with six different spore concentrations incubated at 15ºC for 9 and 12 days assessment periods.

	Incidence[2]			Sporulation score [1]		
Spore conc. (spores/20 μL)	9 dpi	12 dpi	Spore conc. effect on incidence[2]	9 dpi	12 dpi	Spore conc. effect on sporulation score
0.2	0.00 (0.000-0.308)	0.00 (0.000-0.308)	0.00 (0.000-0.168)	0.0 a	0.0 a	0.0 A
2	0.00 (0.000-0.308)	0.00 (0.000-0.308)	0.00 (0.000-0.168)	0.0 a	0.0 a	0.0 A
20	0.00 (0.000-0.308)	0.20 (0.025-0.556)	0.10 (0.012-0.317)	0.0 a	0.0 ay	0.0 A
200	0.50 (0.187-0.813)	1.00 (0.692-1.000)	0.75 (0.509-0.913)	2.5 ac	5.5 bc	5.0 B
2000	0.80 (0.444-0.975)	0.80 (0.444-0.975)	0.80 (0.563-0.942)	5.0 byc	5.0 bc	5.0 B
Assessment time effect on incidence	0.26 (0.146-0.403)	0.40 (0.264-0.548)				

[1]Median values for the sporulation scores were obtained from Kruskal-Wallis non parametric test . There was a highly significant effect of spore concentration (A-B; *P*=0.000) and interactions between spore concentration and assessment time (a-b; *P*=0.000) on sporulation score (0-6 scale).
[2]Confidence intervals at 95% significant level are indicated in parentheses for incidence data.

2.3.4.3 Effect of leaf maturity on susceptibility to *P. sparsa* infection

Disease incidence was significantly affected by source of leaves (shadehouse or glasshouse) (*P*<0.001), leaf age (*P*<0.001) and assessment time (*P*=0.006) (Table 2.12; Appendix A.10.1). Disease incidence was higher on leaves sourced from the shadehouse compared with those from the glasshouse, and on young leaves compared with mature leaves, and at14 dpi compared with 10 dpi. However, there was no significant interactions between source of leaves, leaf age or assessment time (*P*=0.986). Sporulation score was significantly affected by source of leaves (*P*<0.001), leaf age (*P*<0.001) and interaction (*P*<0.001) of leaves, leaf age and assessment time (Appendix A.10.2), with sporulation only observed on young leaf discs detached from shadehouse plants at 14 dpi (Table 2.12). For subsequent spore production discs obtained from young leaves collected from boysenberry plants grown in the shadehouse plants were used as a source of leaves.

Table 2.12 Incidence (proportion of leaf discs developing lesions) and sporulation score (0-6 scale) of *Peronospora sparsa* infection on discs obtained from leaves at two maturity stages (mature and young) from boysenberry plants grown in the greenhouse and shadehouse assessed 10 and 14 days post inoculation (dpi) after incubation at 15°C. Means are for 18 leaf discs per treatment.

Source	Age	Incidence			Sporulation score[1]		
		10 dpi	14 dpi	Source effect on incidence[2]	10 dpi	14 dpi	Source effect on sporulation score[1]
Greenhouse	Mature	0.00	0.06	0.06 (0.015-0.136)	0.00 a	0.00 a	0.00 A
	Young	0.00	0.17		0.00 a	0.00 a	
Shadehouse	Mature	0.06	0.11	0.32 (0.214-0.440)	0.00 a	0.00 a	0.00 B
	Young	0.44	0.67		0.00 a	4.50 b	
Age effect	Mature	0.06 (0.015-0.136)			0.00 C		
	Young	0.32 (0.214-0.440)			0.00 D		
Assessment time effect[2]	10dpi	0.13 (0.059-0.224)					
	14dpi	0.25 (0.155-0.366)					

[1] Median values for the sporulation scores were obtained from Kruskal-Wallis non parametric test (Appendix A.10.2a).[2] The confidence intervals for incidence of infection are provided in parentheses.

2.3.4.4 Susceptibility of different *Rubus* spp. cultivars to *P. sparsa* infection

No lesions were observed on any of the control leaf discs inoculated with sterile water; thus they were not included in the analysis. Cultivar had a significant effect on disease incidence (P<0.001; Appendix A.11.1); the maximum incidence of 0.92 was seen with youngberry (Table 2.13) with zero incidence on Karaka Black. Lesion size was significantly affected (P<0.001; Appendix A.11.2a) by cultivar, with lesions on youngberry significantly larger compared with the other cultivars. The effect of cultivar on the number of spores produced per lesion was also significant (P<0.001; Appendix A.11.2b), with more produced on youngberry compared with the other cultivars. None of the leaf discs inoculated at Riwaka Plant and Food laboratories produced symptoms or sporulation. Most leaves were contaminated with *Epicoccum* spp. which grew from the leaf discs onto the WA medium.

Table 2.13 Disease incidence (proportion of leaf discs developing lesions) and lesion size (mm^2) and number of spores per lesion on four different *Rubus* cultivars after inoculation with *Peronospora sparsa* spores and incubation at 15ºC for 14 days.

Rubus cultivar	Incidence[2]	Lesion size (mm^2)	No. of spores per lesion
Karaka Black	0.00 (0.000-0.265)	0.00 A	0 C
Tasman	0.83 (0.516-0.979)	26.16 A	6375 D
Mapua	0.67 (0.349-0.901)	30.82 A	4375 CD
Youngberry	0.92 (0.615-0.998)	67.42 B	14125 E
P value (Cultivar) $_{incidence}$	<0.001		
LSD		24.71	4925.2

[1]Values followed by the same letter are not significantly different according to Tukey's HSD at $P \leq 0.05$. The main effect of the cultivar (A-B) on lesion size was significant t (P<0.001; LSD=24.71) and no. of spores produced per lesion was also significant (P<0.001; LSD=4925.2).
[2] The confidence intervals for incidence of infection are provided in parentheses.

2.3.5 Production of oospore inoculum and its infection capability *in vitro*

In the first experiment, oospores were observed on all leaf discs incubated at 15ºC after 30 days, (2 weeks after asexual sporulation was observed) (Figure 2.16). However, inoculation of the leaf discs with the oospore suspension prepared from the first experiment did produce lesions and no sporangiospores was observed even after 28 days incubation.

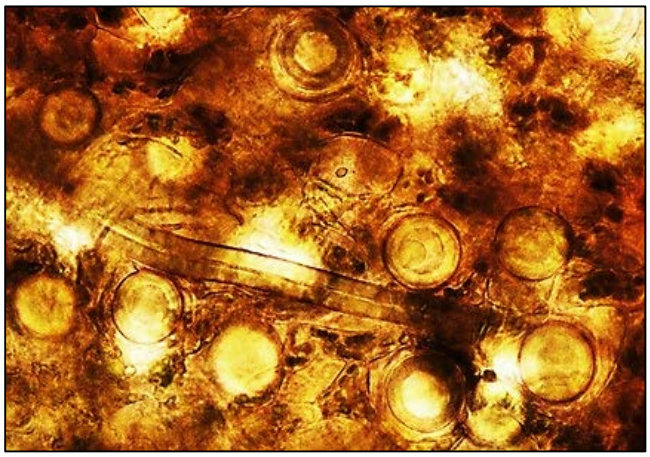

Figure 2.16 Oospores of *Peronospora sparsa* produced on leaf discs inoculated with *P. sparsa* sporangiospores after incubation at 15ºC on 1.5%WA for 30 days at 12h/12h light/dark condition.

2.3.6 Evaluation of methods for long term storage of inoculum

Storage methods had no significant effects on spore germination or infection. Storage time and temperature significantly (both $P<0.001$) affected spore viability and infection, being greatest after 1 month and at -80°C. Germination decreased from over 40-60% after 1 month of storage to 0.4-2.6% after 4 months (Table 2.14). Infection incidence decreased from over 80% after 1 month of storage to less than 5% after 4 months (Figure 2.17). The full data is presented in the published paper in Appendix A.12.

Table 2.14 Mean percent germination of *Peronospora sparsa* spores after different storage periods at -80 or -20°C for different storage methods: spores were suspended (SS) in 20% glycerol, leaf discs cut from sporulating regions were stored either dry (LD) or in 20% glycerol.

	-80ºC			-20ºC			Temperature × time[1]	
Months	SS in 20% glycerol	LD	LD in 20% glycerol	SS in 20% glycerol	LD	LD in 20% glycerol	-80ºC	-20ºC
1	61.5	47.6	50.8	44.3	40.8	39.6	53.3 a	41.6 b
2	22.0	13.2	13.3	4.3	12.6	14.6	16.2 c	10.5 c
4	5.0	1.2	0.7	0.9	1.1	0.7	2.3 d	0.9 d
6	2.6	1.2	1.2	0.4	0.5	1.5	1.7 d	0.8 d
Temperature × method								
	22.8 A	16.5 B	15.8 B	12.5 B	13.7 B	14.1 B		

[1]Values followed by the same letter are not significantly different. For temperature × method (A-B) and temperature × time (a-d), differences between means were highly significant ($P=0.006$, $P=0.004$, respectively). Means within a column followed by the same letters are not significantly different according to Tukey's HSD at $P<0.05$

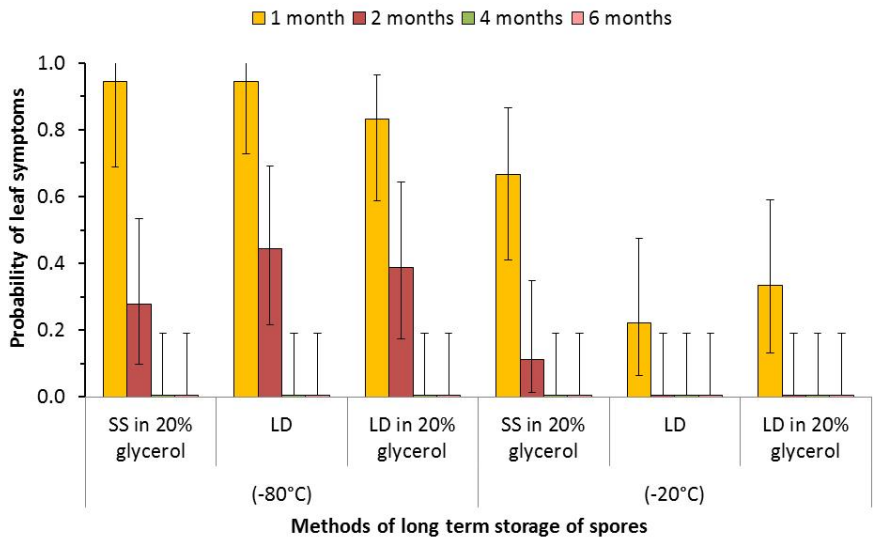

Figure 2.17 Probability of lesions (infection) on leaf discs (LD), resulting from *Peronospora sparsa* spores stored for different periods at either -80 or -20°C, using three different storage methods: leaf discs cut from sporulating regions stored dry (LD), leaf discs in 20% glycerol or spores suspended (SS) in 20% glycerol. Error bars represent the 95% confidence intervals of the probability of lesions (infection). Refer to Appendix A.12 for details of the statistical analysis.

2.3.7 Effect of optimum incubation conditions on lesion expansion of systemic and spore initiated lesions

There was no significant effect (P=0.277) of time on lesion expansion rate for either systemic or spore initiated lesions. However, lesion expansion was significantly greater (P = 0.002) for systemic lesions compared with spore initiated lesions (Table 2.15; Appendix A.13).

Table 2.15 Expansion rate (mm^2/day) of *Peronospora sparsa* systemic and spore initiated lesions which developed on leaf discs obtained from shadehouse plants incubated at 15°C on 1.5% WA after different incubation times

Incubation time (days after)	Lesion expansion rate (mm^2/day)		
	Systemic lesion	Spore initiated lesion	Incubation time effect
3	0.999	0.171	0.585
5	0.817	0.187	0.502
7	0.833	0.188	0.510
10	1.030	0.221	0.626
14	1.383	0.212	0.797
Lesion type effect[1]	1.012 A	0.196 B	
LSD 0.503			

[1]Values followed by the same letter are not significantly different at $P \leq 0.05$. The main effect of the lesion type (A-B) was significant (P=0.002; LSD=0.503) and there was no significant effect of incubation time (P=0.277) or interaction between lesion type and incubation time (P=0.346).

2.4 DISCUSSION

Aerial spores were trapped in boysenberry gardens from early spring (September) to early summer (December), but there were differences in spore numbers over time. The weather data indicated a relationship between rainfall pattern, humidity and temperature on the dispersal of spores in the field. The release of *P. sparsa* spores seemed to be related not to the amount of rain, but rather to the frequency (%) of rainy days in early spring (September). This agreed with the findings of Kim *et al*. (2014), who used temperature and rainfall data, along with disease incidence recorded over 11 years, to develop the 'Fuzzy Peronospora Sparsa' model to predict high disease risk periods. They identified the high disease risk seasons as those in which the number of days with rainfall was >38.7% in any month. In the current study, although the frequency of days with rain in September 2010 was greater than 38.7%, it was less than 26% for the next two months. However, it was during this dry period after September that most spores were trapped. This suggested that the high frequency of rainy days promoted spore production and subsequently spore release was less reliant on rain events. Similar results were reported by Walter *et al.* (2012b); the peak in the number of spores trapped on paraffin coated slides in November 2002 and 2007 coincided with >36% of days with rain in September, which resulted in dryberry incidences of over 10%. In 2011 data from the current study showed that the frequency of days with rain in September recorded at the Nelson weather station was less than 38.7% (26.7%), whereas at the Riwaka station the frequency was 36.7%. Thus, the difference in rainfall days in September is likely to be the reason for the difference in the disease level between Moutere (high level in Dec 29) and Richmond. However, since Lower Moutere had an intermediate disease level there may also be local differences, or a combined rain/temperature effects which affect spore release.

Temperature is also reported to influence *P. sparsa* disease incidence (Shaw, 1982). The temperature range for the 2010 and 2011 seasons were similar and, therefore, the lower number of spores trapped in 2011 indicate that spore release is influenced by a combination of environmental parameters rather than temperature alone. Kim *et al*. (2014) reported that risk of high incidence of this disease was associated with a combination of temperature and the number of rainfall days. If the mean number of hours per day of 15-20ºC (t) was ≥9.8 h in a month and ≥38.7% of the days had some rainfall (r) then the disease risk would be high. In the current study, in 2011 both conditions were satisfied in November (t=10 and r=47%) and December (t=15 and r=43%) according to the Nelson weather station, and in December the conditions were only satisfied at the Riwaka weather station (t=11 and r=40%). However, the greatest disease incidence was from the Upper Moutere property which was associated with the Riwaka weather station and the Richmond (Nelson weather station) grower had the lowest disease incidence. This suggested that in addition to the combination of t and r, disease incidence may be related to timing of the conducive conditions and to local differences in climatic factors such as diurnal variation. They may also reflect differences in grower management practices, such as

cane training, and presence of shelter belts. Differences in the environmental conditions recorded at the weather stations and that actually experienced at the grower property might also have resulted in errors with interpreting the effect of environmental factors on spore counts. Although all three grower properties are located in the Nelson region and as such are not expected to vary dramatically in environmental conditions, local variations between grower properties, and also within properties, are likely to influence not only spore production and release but also spore infection and hence disease incidence.

Relative humidity (RH) during early spring also seemed to affect spore release. In 2010, the RH during October and November fluctuated between 70 and 85% with rainfall relatively low during this period, but following a wetter September (National Institute of Water and Atmospheric research, New Zealand). In contrast, there was greater frequency of rainfall during October to December in 2011, and the RH fluctuated over a wider range (60 to 95%). However, the means were calculated for the period of time (4-5 days) that the slides were in the field, with the environmental conditions likely to fluctuate not only within that period but also within a day. Therefore, a dry period (1 h) within this period is likely to facilitate spore dispersal from sporangiophores. This is in agreement with the known biology of spore release for oomycetes. The hygroscopic twisting of *Phytophthora infestans* and *Peronospora tabacina* sporangiophores in changing RH has been reported to enhance spore dispersal through the dry, active mechanisms of spore dispersal common in Mastigomycotina (Lacey, 1996; McCartney and West, 2007). In addition a study conducted on *Pseudoperonospora cubensis,* the causative agent of the downy mildew in cucurbits, reported that sporangiophores twist as they desiccated, resulting in the sporangia detaching so they are easily dispersed by the air currents (Erhardt, 2009). The greater number of spores trapped in November 2010 than in November 2011 may have been associated with the wetter September in 2010 (Appendix A.4.4.1). Erhardt (2009) reported that the mechanism by which sporangia are released from sporangiophores is facilitated by an early wet period where high moisture levels promote spore production with a subsequent dry period facilitating spore dispersal.

Wind speed did not appear to affect *P. sparsa* spore release. It was greater in 2011 than 2010 in Nelson however there were more spores trapped in 2010. Wind speed fluctuated in both years in Nelson whereas it was generally consistent in Riwaka. Wind speed was not considered by Kim *et al*. (2014) to be an environmental variable which influenced disease incidence. In contrast, wind speed was reported to affect spore release from the potato canopy for *Phytophthora infestans* spores, with higher wind speed correlating to more spores for the same moisture conditions (Harrison and Lowe, 1989 cited in Erhardt, 2009). Even moderate winds were effective at dispersing spores of *Peronosporaceae* members (excluding *P. cubensis*) upwards from the bottom of the canopy (Aylor, 1990 cited in Erhardt, 2009). In the current study, only the surface wind speed was considered, with wind direction not determined. The direction of the wind at the study sites may have varied during the assessment period and may have reduced

spores impacting on the Vaseline® coated slides in some months, resulting in inconsistent data. Further work is required to determine the effect of wind speed and direction on spore release and, therefore, disease incidence in the field.

For the Upper Moutere property, the high number of spores trapped during 2011 corresponded to higher dryberry incidence in December. In contrast, low dryberry disease at the Richmond site corresponded with low spore numbers at this site. Similarly, more leaf lesions were observed in the boysenberry gardens at the Upper and Lower Moutere properties than the Richmond property in November, 2011. However, much lower dryberry incidence was recorded at the Lower Moutere property although the prediction based on the weather data and the spore numbers recorded was similar to those for Upper Moutere. In addition to the environmental conditions, differences in the susceptibility of the boysenberry cultivars grown at the different properties may have also influenced disease incidence. The cultivar grown at the Lower Moutere and the Richmond properties was 'Mapua', whilst 'Tasman' was grown at the Upper Moutere property which was more susceptible to *P. sparsa* infection than Mapua in the *in vitro* study.

The Vaseline coated slides used to trap spores were a relatively cheap and easy method to provide information on spore release and therefore disease risk. However, as identified their accuracy may have been affected by wind speed and direction. Another limitation of the Vaseline coated slides is likely to be associated with counting errors. The spore counts were made only along two horizontal transverse sections (total 12 fields of view) in each glass slide and because spores were not evenly scattered across each glass slide, often being clustered in some areas (Appendix A.1b), this may have led to counting errors. There are other methods available to traps spore. Solar-powered spore traps with two vertical spinning rods that can sample 62 litres of air/minute have been used to trap *P. cubensis* spores in New York (Tancos *et al*., 2012). However, comparisons cannot be made because that study did not count spores but rather extracted DNA for PCR assessment. Another study using rotorod spore traps to collect spores of *P. cubensis* in cucurbits was also for DNA extraction (Smart *et al*., 2013). Future work could include comparison of different spore trapping methods such as using Burkard, Rotorod and Hirst spore traps.

In vitro spore production was optimal on leaf discs cut from mature or new fully extended leaves when compared to whole leaves detached from shadehouse plants or those grown in the field. This indicated that leaf discs are more susceptible for infection/sporulation of *P. sparsa* than detached whole leaves, which may be due to the physiological effects of wounding the plant tissue (Macnicol, 1976). In addition, changes in the expression of defence related genes occurs in leaf discs when compared to the intact leaves (Ramiro *et al.*, 2007). Ramiro *et al*. (2007) reported that expression of transcription factors for WRKY, a family of genes activated by many biotic and abiotic stresses, was suppressed in leaf discs challenged by *Hemileia*

vastatrix the coffee rust fungus when compared to whole leaves. Whether results on detached plant material are representative of spore infection of attached plant tissue warrants investigation and will be studied in Chapter 4.

Temperature and moisture (RH) affected sporulation by *P. sparsa* on lesions on young or mature detached leaves/ leaf discs. The optimum conditions varied slightly depending on the source of the leaf material. The optimum incubation conditions for material detached from shadehouse was 100% RH at 15°C compared with 90-95% RH at 20°C for field sourced material. This may be due to time constraints in setting up the experiment. The RH in the boxes for the field leaf samples was not given time to equilibrate to the specified RH prior to incubation of the leaf tissue. In contrast, for the shadehouse leaves, the RH boxes containing the different saturated salt solutions were set up one week prior to incubating the leaves allowing the RH to equilibrate, and as such provide a more reliable result of the effect of RH on sporulation.

The optimum temperature of the temperatures tested for sporulation of *P. sparsa* on boysenberry leaf discs was 15ºC. Sporulation was also observed at 10 and 20ºC across 90-100% RH. Breese *et al*. (1994) also reported maximum sporulation by *P. rubi* (syn. *P. sparsa*) at 14.7ºC on tummelberry leaf discs. They also showed that greater sporulation occurred at 10ºC than 20ºC, which was not observed consistently in the current study. Similarly, Shaw (1982) showed that temperatures above 16-18ºC reduced sporulation on *Rubus* plants. This was also observed for *P. tabacina* on tobacco leaves (Cohen, 1976 cited in Rotem *et al*., 1978). These authors hypothesised an interaction of light and temperature. When temperatures are ≥15ºC light activates the accumulation of compounds in the irradiated tissues which inhibit sporulation and this was demonstrated on detached leaves. However, they did not define examples of the compounds. In the current study, constant temperature and humidity were maintained and dry dark proceeding cycles were not tested. However, *P. sparsa* tissue colonisation, as indicated by lesion expansion, was favoured by higher temperatures (20ºC) than the temperature optimum for infection and sporulation. This agreed with the findings of Aegeter *et al*. (2003) who reported greatest lesion expansion on rose at temperatures of 20 to 25ºC.

The *in vitro* study demonstrated that sporulation was enhanced by high humidity, with most spores being produced at 90-95% or 100% RH, whereas, no sporulation was observed at 80% RH. It was not clear whether sporulation by *P. sparsa* under humid conditions is enhanced by free leaf moisture or a RH close to saturation. Maximum sporulation of a related downy mildew pathogen, *P. trifoliorum* on alfalfa was at RH below 100% (Fried *et al.*, 1977 cited in Rotem *et al*., 1978). Further, free leaf moisture has been shown to inhibit sporulation of *P. tabacina* on tobacco and *Pseudoperonospora cubensis* on cucumber (Fried *et al.*, 1977 cited in Rotem *et al*., 1978). In the present study data loggers were not used to precisely measure the RH in the

humidity chambers and differences between the RH of the air and at the leaf surface, as well as presence of condensation, may have directly affected sporulation.

Results also indicated that moisture (RH) and temperature both affect sporulation on naturally infected leaves. Sporulation of *P. sparsa* under high relative humidity was observed at all temperatures (10, 15 and 20ºC) used in the study. The selection of the temperatures used in the study was based on the study conducted by Breese *et al*. (1994) who showed that sporulation occurred on *P. sparsa* inoculated tummelberry (blackberry and red raspberry hybrid) leaf discs incubated at 2-28ºC, with maximum numbers of leaf discs that produced spores being at 14.7ºC. Unlike the current study, the number of spores produced was not assessed in the study of Breese *et al.* (1994) only the number of discs showing sporulation. Further, the effect of RH was not investigated by these authors. This is the first reported study examining the combined effect humidity and temperature on *P. sparsa* sporulation. However, in the field a constant temperature and RH would not occur and what effects temperatures beyond the range studied here, and their interaction with RH, would have on sporulation is unclear.

There seemed to be more sporulation from lesions on mature leaves than from lesions on new fully extended young leaves for both shadehouse and field samples. However, the frequent presence of 'zero' data meant that statistical analysis was not possible. When young and mature leaves were inoculated and observed for lesion development and sporulation, there was more infection on young than mature leaves but no effect of leaf age on sporulation; however field observations showed sporulation on leaves within or under the canopy, which were always the older leaves. The classification of leaf age was relatively broad in the current study with the age of plant from which the leaves were removed not taken into account. Future work should explore the effect of tissue and plant age on sporulation. A PCR method to determine the presence of the pathogen in different tissue was developed in Chapter 3 and will facilitate such studies by confirming whether the plant tissue is infected.

Germination increased with increasing temperature to 20ºC. MacLean and Baker (1951) cited in Francis (1981) reported that the optimum temperature for *P. sparsa* spore germination was at 18ºC (65ºF), whereas they did not germinate at 4ºC (40°F) or at 26ºC (80°F) and over. These authors also reported that the spores remained viable if held at 4ºC for 24 h, but not if held at 26ºC. Breese *et al.* (1994) assessed UK isolates (from *Rubus* and *Rosa* spp.) for germination between 2 to 20ºC and reported similar germination at 15ºC and 20ºC, with maximum germination of the New Zealand isolate (PrNZ) at 10 to 15ºC. Thus, it is likely that the effect of temperature on germination of different isolates varies and it would have been interesting to investigate whether there is a correlation between the mean climate from which isolates are sourced (Canterbury, Nelson and Whakatane) and germination at a particular temperature. The isolate used in the current study was from the infected tissue culture plants grown in the Lincoln University, Canterbury.

In the current study, different media were evaluated for germination of spores and this has not previously been reported. The results indicated that germination was greater on leaf discs than on glass slides and agar. However, assessment of spore germination on leaf discs by the cellotape method may have been biased because the cellotape seemed to pick up more germinated spores than non-germinated spores. Leaf clearing probably gave the best assessment of germination, but was a more laborious method. Therefore, germination on leaf discs with assessment using the cellotape method was used for germination assessments throughout the study to maintain uniformity.

In vitro experiments were used to assess the effect of temperature on infection by *P. sparsa*. The optimum temperature for infection of leaves by *P. sparsa,* as indicated by subsequent spore production and lesion size, was 15 to 20ºC under high humidity. Similar results were reported by other researchers, with optimum infection of detached rose leaves by *P. sparsa* occurring between 15 and 20ºC (Aegerter *et al.*, 2003), and at 15ºC on Tummelberry (Breese *et al.*, 1994), although these researchers reported infection to occur over a wider temperature range (2-28ºC) than investigated here. This is the first study to investigate the number of spores required to initiate infection by *P. sparsa.* The optimum number of spores of those tested was 200, for infection and subsequent sporulation from the lesions produced under optimum incubation conditions. Inoculation with a lower concentration of 20 spores produced lesions on 20% of the leaf discs but they did not sporulate. Whether longer incubation of these lesions would have resulted in subsequent sporulation was not determined. The relatively high spore concentration required to infect these tissues may indicate that some spores were not able to germinate as was reported by Gomez and Filgueira-Duarte (2012) for *P. sparsa* on rose. They reported that spores which were either small or non-turgid did not germinate. In the present study small spores were observed in the inoculum suspension (approximately 10%) and it is likely that these were not fully mature and unable to germinate. Also some spores were non-turgid (approximately 8%), possibly because they were old spores which were nonviable. According to the *in vitro* germination studies present in this chapter 6-14 of the 20 spores would be likely to germinate.

Maturity of the detached boysenberry leaves affected susceptibility to *P. sparsa* with incidence and subsequent spore production higher on young leaf discs than mature ones. Younger leaves are likely to have more functional stomata than older leaves according to the study conducted on Cucurbitaceae leaves (Adebooye *et al*., 2012) and geraniums (Schletz, 2008). The role of leaf stomata, which occur mostly on the abaxial side of the leaf (Appendix A.14.2) was shown in the current study; inoculation of the adaxial side of a leaf disc with *P. sparsa* spores did not result in any infection or sporulation compared with 100% infection and subsequent sporulation when inoculation of the abaxial side was carried out (Appendix A.14.1). In contrast, *P. sparsa* was reported to infect and sporulate on both sides of rose leaves (Caro, 2014). However, direct penetration between the epidermal cell walls with appressorium of *P. sparsa* on rose was reported (Castillo *et al*., 2010), which may explain the differences between these hosts. In the

current study, the pathogen may preferentially infect via the stomata, and this may partly explain why infection and sporulation were higher on the young leaf discs and with abaxial inoculations.

In the current study, leaves were inoculated with spore suspension droplets and subsequent observations showed reduced contact of the droplet with the cuticle due to the leaf hairs. A study on the influence of leaf hairs during the interaction of *Plasmopara viticola* on leaves of four *Vitis* species reported that hairs (trichomes and bristles) on the adaxial leaf surface acted to repel water from the leaf surface preventing successful penetration of the host by the germ tubes in more tolerant species (Kortekamp and Zyprian, 1999). However, leaf hairs may also increase humidity close to the leaf surface which may enhance *P. sparsa* sporulation. The actual mechanisms for the difference in susceptibility warrants further investigation to identify traits that could be selected for in boysenberry breeding programmes to decrease infection.

The susceptibility to *P. sparsa* of detached leaves from different *Rubus* cultivars varied in the current study. Youngberry was the most susceptible *Rubus* cultivar compared with Mapua, Tasman and the blackberry cultivar, Karaka Black, which showed no infection of the leaves. Youngberry was also reported by Hall and Shaw (1987) to be extremely susceptible to downy mildew with regards to both foliage and fruit infections under New Zealand field conditions. Breese *et al*. (1994) also reported that hybrid berries including a boysenberry were more susceptible than the blackberry or raspberry cultivars tested on both detached leaves and on plants grown in plastic tunnels. However, they did not state which cultivar of boysenberry they used, or whether this was a true boysenberry or a youngberry cultivar. The different susceptibility of cultivars and species needs to be tested further under field conditions in New Zealand before any recommendations can be made to growers.

The current study demonstrated that lesion expansion due to *P. sparsa* systemic infection was greater than for spore initiated lesions. The hypersensitivity reaction is known to be active against biotrophic pathogens, such as mildews, in external (spore) infection (Michelmore *et al*., 1988 cited in Bahcevandziev *et al*., 2015). Callose-like deposits or papillae containing hydroxyproline-rich glycoproteins, phenolics and silica generate antimicrobial conditions at sites of penetration by Peronosporales (Baka, 2008). Thus, expression of host immune mechanisms against spore infection might have reduced colonisation of *P. sparsa* into neighbouring tissues, thereby retarding the expansion rate of the spore initiated lesions. However, both types of infection caused expanding lesions and sporulation, so disease management practices are needed to control both systemic and spore infection in the field (Chapter 5).

To improve the uniformity of inoculum, surface sterilisation methods for leaves were assessed. The results showed that ethanol slightly increased the number of leaves which became infected possibly because it reduced the efficacy of the waxy cuticle on the leaf surface, thereby

increasing the ability of *P. sparsa* to infect the tissue. Surface sterilisation with sodium hypochlorite slightly decreased infection. In subsequent experiment it was decided to rinse leaves using a sterile water wash to avoid any inhibitory or stimulatory effect from the sterilant used.

Assessment of the surface sterilants by the leaf-press method showed that the number of contaminant fungal colonies isolated on agar from the adaxial side of the boysenberry leaves was always greater than from the abaxial (lower surface) side. This was probably related to there being a greater chance for airborne fungal spores to settle on the upper surface unlike the lower where the leaf hairs also acted as a barrier to aerial contaminants (Kortekamp and Zyprian, 1999). The number of bacterial contaminants was very low for both leaf surfaces, which indicated that boysenberry leaves are not favourable to epiphytic bacterial colonisation. This was surprising as bacteria are known to be the most numerous colonists of leaves generally, in numbers averaging 10^6–10^7 cells·cm^{-2} (up to 10^8 cells·g^{-1}) of leaf (Andrews and Harris 2000, Hirano and Upper 2000, and Lindow and Brandl 2003 cited in Penuelas *et al*., 2012). The exact reason for apparent conflict with the current study is unknown but may indicate biochemical inhibition of bacterial growth or that boysenberry leaf extracts are not conducive to bacterial growth.

Viability of *P. sparsa* spores during storage decreased with increasing storage time over 1 month, with none of the storage methods tested resulting in maintenance of spore viability and ability of the spores to infect boysenberry leaves beyond 2 months storage. In contrast, Breese *et al.* (1994) reported that *P. sparsa*, both from *Rubus* (blackberry and tummelberry) and *Rosa* species, retained viability for 1-4 months when stored as sporulating leaf discs or leaflets in a sealed plastic box at -70ºC. However, no information regarding the relative infectivity of the spores after different storage periods was provided. Long term storage of *P. sparsa* spores suspended in dimethylsulphoxide by cryopreservation using liquid nitrogen was also reported by these authors, but again no information on the infectivity after different storage periods was provided. *Peronospora viciae* spores stored dry as sporulating infected pea leaves at -80°C retained viability for at least 1 year (Gill and Davidson, 2005), infecting approximately 62% of pea seedlings compared with 93% seedling infection when fresh spores were used. These authors suggested that sufficient spores should be stored to compensate for the decrease in infectivity. They also reported that spores suspended in dimethylsulphoxide, glycerol, glycerol and skim milk or in sterilised water and stored at -80°C did not remain viable. From these studies, it appears that different methods may be effective for different species of downy mildew pathogens.

The relationship between spore viability and ability of the spores to infect and cause leaf lesions was expected. According to the *in vitro* study, at least 20 spores of *P. sparsa* were required to initiate infection and produce a lesion. Therefore, the numbers of lesions that developed during

the assessment period were probably proportional to the numbers of viable spores applied to the leaf discs. However, although they remained viable, some storage methods reduced capacity for infection. Spores stored as sporulating leaf discs, both dry and in glycerol at -20°C, retained reasonable viability (approx. 40%) after 1 month storage. However, spores with reduced viability (4.3-13%), such as those stored as leaf discs at -80°C after 2 months, also resulted in reduced infection. Likewise, although viability of spores stored for 2 months on leaf discs at -20°C was similar to those stored at -80°C, resulting infection was much less from the spores stored at -20°C. It has been suggested that the spores of Peronosporales are relatively susceptible to damage and, therefore, lose viability due to their relatively thin and fragile walls (Laviola *et al.*, 2006). Gulya *et al.* (1993) reported variable success of storage methods for different downy mildew species, with the spores of some species having extremely thin walls making them difficult to store. In this study, the shaking (mixing) and pipetting of the spores or osmotic shock due to the use of 20% glycerol (Laviola *et al*., 2006) may have contributed to the loss of viability due the physiological or mechanical stresses caused. However, the observation that the spores stored without the addition of glycerol were misshapen indicates that these spores were damaged during storage at low temperature. The addition of glycerol may also have protected the spores as it can penetrate cell walls and membranes as a cryoprotective additive even though the permeability is slow (Hubálek, 2003). In the present study, the leaf discs were not dried prior to storage, and this may have contributed to loss of viability, as Gill and Davidson (2005) recommended including filter paper to prevent condensation or ice formation, which could contribute to a reduction in spore viability. Since *P. sparsa* also produces thick-walled oospores, the use of oospores for long term storage may be effective and warrants testing.

Peronospora sparsa oospores did not cause infection of leaf discs at 15ºC under high humidity after 28 days. This was studied with the hope that they could be used for long term storage as none of the methods used with asexual spores resulted in long-term storage of *P. sparsa*. The potential reasons for lack of infection may be (i) the oospore inoculum lacked germinability (Van Der Gaag and Frinking, 1996), (ii) they needed longer periods of maturation to be capable of germination, (iii) they required 'triggers' to initiate germination (Jones, 1994) or (iv) conditions such as temperature, light, moisture level and time allowed for oospore production might have affected their germinability or (v) the oospore inoculum was not viable (Van der Gaag and Frinking, 1997). Van Der Gaag and Frinking (1996) reported that oospore age, conditions during formation and environmental conditions affected germinability. The oospores used for inoculation of leaf discs in this study were approximately two months old which may have been too old as was suggested by Van Der Gaag and Frinking (1996). The oospores they used successfully for germination studies were extracted from pea plants just 2-4 weeks after asexual spore inoculation. However, Jones (1994) reported that oospores might be either constitutively dormant, such that they need to mature before they are germinable, or

exogenously dormant, such that they require an external trigger such as cold period, dry/wet cycle, specific nutrient etc. to enable them to germinate. Their role in nature for a number of pathogens supports this conclusion. Thus, in this study it seems more likely that the oospores were not ready to germinate or had not been given the correct trigger to break the dormancy.

Conditions such as temperature, light, moisture level and time allowed for oospore production might also have affected the germinability of the oospores produced. In the current study the oospores were produced at 15ºC at 12 h/12 h light/dark conditions, whereas by Breese *et al*. (1994) used 18ºC with a 16 h photoperiod of photon flux density of *c*. 20 E $m^{-2}s^{-1}$ for *P. sparsa* in boysenberry. However, they did not test the viability or infective capabilities of the oospores. In contrast, Van Der Gaag and Frinking (1996) produced oospores by inoculating pea plants with asexual spores of *Peronospora viciae* f.sp. *pisi* and incubating them at 15ºC (100% RH) for 24 h followed by incubation further at 20ºC. Germination of these oospores of *P. viciae* f. sp. *pisi* was greatest at 5ºC and 10ºC compared with 15ºC and 20ºC in the dark (Van Der Gaag and Frinking, 1996) indicating reduced temperature and darkness might also favour oospore infection. Although it was reported that light conditions did not affect germination of oospores of *Peronosclerospora sorghi,* the light conditions during oospore production were not reported (Shetty and Safeeulla, 1980, cited in Van der Gaag and Frinking, 1997). The incubation conditions used in the current study may have affected the germination of the oospores and hence infection of the leaf discs. Loss of viability of the oospore inoculum at primary dormancy period after formation as was indicated by Van der Gaag and Frinking (1997). Further research is required to determine the optimum incubation conditions for oospores production and the factors which affect oospore germination and infection.

In conclusion, the spore trapping method allowed for assessment of spore release timing and hence the risk period of *P. sparsa* infection in boysenberry gardens under New Zealand conditions. The disease was initiated by rain in early spring which promoted sporulation with spore release and dispersal possibly being triggered by subsequent dryer periods occurring in mid-late spring and infection of the host tissue in late spring-early summer. This has significant implications for disease management decisions primarily with regards to the timing of fungicide applications in the field to protect boysenberry tissue from spore infection, which will be further investigated in Chapter 5. Sporulation from naturally infected leaves, stems/canes and calyxes under optimum conditions of 15ºC and high humidity were identified as providing for potential inoculum in the field. Sporulation however was not observed on flowers or berries. Berry infections resulting in dryberry disease results in major yield losses for the grower and whether this result from direct spore infection or systemic growth of the pathogen into the developing flower and/or berry tissue is not known and will be investigated further in Chapter 4. However, a method is required to establish the infection status of asymptomatic tissues, which could allow systemic infection and the factors which influence disease progression to be monitored.

Therefore the next chapter (Chapter 3) will aim to develop robust PCR methods which will allow detection of *P. sparsa* colonisation in asymptomatic tissue.

CHAPTER 3

Using species-specific PCR to identify the sources of infection

3.1 INTRODUCTION

Sporulation by *P. sparsa* on asymptomatic boysenberry tissues that had been detached from field plants and incubated under optimum conditions (Chapter 2) showed that the pathogen could exist as a latent infection within plants. This may also be true of tissue-cultured plant material. Aegerter *et al*. (2002) reported that there were no methods that growers could use to determine if non-symptomatic plants were infected. Thus, development of a robust and sensitive method for detection of latent infections by *P. sparsa* was required. DNA-based methods, such as the polymerase chain reaction (PCR) were considered by Aegerter *et al*. (2002) to be the most appropriate for the detection of obligate parasites such as downy mildews.

PCR based systems specific for the ITS sequences of the highly conserved ribosomal RNA (rRNA) gene region have been used for molecular detection of *P. sparsa* DNA. These have been applied in previous studies on rose in the United States (Aegerter *et al.*, 2002), arctic bramble (*Rubus arcticus*) in Finland (Lindqvist *et al.*, 1998) and blackberry in Denmark (Sundelin *et al.*, 2009). Lindqvist *et al*. (1998) developed two primers, PR3 and PR4 while Aegerter *et al*. (2002) used PS1 and PS3 primers, which amplified 560 and 660 bp products, respectively, of the rRNA gene from the ITS region of *P. sparsa*. The detection sensitivity for these assays was reported as 0.25 pg and 2.0 pg DNA, respectively. However, in New Zealand Dodd *et al.* (2007) were unable to replicate the detection sensitivity reported by Lindqvist *et al*. (1998), achieving a detection sensitivity of only 0.4 ng. Thus, a nested PCR method was developed by them. The nested protocol used two primer pairs, ITS4 and ITS5 (universal) and PR3 and PR4 (*P. sparsa* specific). A qPCR method with a detection sensitivity of 37 fg was also developed and used successfully in *P. sparsa* in arctic bramble, other *Rubus* spp. and roses (Hukkanen *et al*., 2006).

Currently, the PCR based tools can only identify the presence or quantity of *P. sparsa* DNA in plant tissue. They do not provide information on whether hyphal or spore structures are present in infected material, which is normally achieved by microscopy. However, microscopic examination of systemic pathogens has limitations, such as a lack of specificity and limitations to the amount of plant material analysed (Aegerter *et al*., 2002). Despite these limitations, epifluorescent microscopy can be used to examine plant-fungal interactions providing evidence of infection and colonisation of host tissue, fungal reproduction and presence of fungal inoculum. It has been successfully applied to *P. rubi* on tummelberry and other *Rubus* spp. (Williamson *et al*., 1995), and *P. tabacina* on *N. tabacum* (Hood and Shew, 1996). In contrast, microscopy has not been used to investigate the source of infection of *P. sparsa* in boysenberry

and this is important for understanding when and which plant tissues are most susceptible to infection. Improving understanding of the pathogen's disease cycle is crucial to improve the potential effectiveness of disease management strategies.

Thus, the objectives of this chapter were (i) to optimise and validate the molecular detection method developed by Dodd *et al.* (2007) of Plant and Food Research, (ii) to examine survival structures within boysenberry tissues by epifluorescent microscopy, and (iii) to apply the optimised molecular tools and epifluorescent microscopy to detect *P. sparsa* latent infection. As the goal of this chapter was detection of the presence of *P. sparsa,* rather than relative amounts of nuclear material, the nested PCR which had equivalent detection threshold to qPCR, was used as the molecular detection process.

3.2 MATERIALS AND METHODS

3.2.1 Optimising DNA extraction from systemically infected boysenberry plants

3.2.1.1 Plant Samples

A) DNA extraction buffer optimisation

Two 1 year old symptomatic boysenberry plants (cv. Mapua) that had been maintained in a shadehouse (Lincoln University) in 2.5 L pots containing 12-14 month potting mix (Appendix A.2) were selected. Six samples, each comprising a different plant tissue, were collected from each plant. The different tissues, collected in winter (July) 2012, were asymptomatic primocane tips, young canes/stems, old canes/stems, leaf buds, leaves, and symptomatic (but not sporulating) leaves. The tissues were stored in 20% glycerol as a cryoprotectant at -80ºC for two months prior to DNA extraction. These 12 samples were used to evaluate two buffers for DNA extraction from the diverse tissue types (Section 3.2.1.2).

B) Comparison with a commercial DNA extraction kit

Tissue samples were taken from four 2-3 year old symptomatic boysenberry plants (cv. Mapua) grown in a cage at the Riwaka Plant & Food Research Station. Five samples comprising one each of asymptomatic primocane tips, old canes/stems, roots, and symptomatic (but not sporulating) or sporulating leaves were taken from each of the four plants. The 20 tissue samples were collected in autumn (March), 2012. They were stored in 20% glycerol at -80°C for seven months prior to DNA extraction.

3.2.1.2 DNA extraction

A) Optimising DNA extraction buffer

Two buffers were evaluated for DNA extraction of Lincoln University shadehouse samples using the Plant & Food Research protocol (Dodd *et al*., 2007). The two buffers were (i) Plant &

Food Research CTAB (Cetyltrimethyl ammonium bromide/Hexadecyltrimethylammonium bromide) buffer (Dodd *et al.*, 2007), and (ii) Aegerter CTAB buffer (Aegerter *et al.*, 2002) (Appendix B.1).

Prior to extraction from each tissue type any *P. sparsa* spores adhering to the surfaces were removed by placing each of the tissues in 50 mL sterile water and agitating the sample vigorously by hand for 20 s, then discarding that solution and rinsing again with vigorous agitation in 50 mL sterile water containing a drop of detergent (Palmolive®), followed by a final wash with 50 mL sterile water.

The plant tissues (approximately 300 mg) were placed into 2.0 mL tubes containing 1 mL of either Plant & Food Research CTAB buffer or Aegerter CTAB buffer, both with 200 µL 5% sarcosyl (Appendix B.1), and 0.5 cm^3 stainless steel beads. They were disrupted using a Mini-BeadBeater cell disrupter (FRITSCH Pulverisette 23, John Morris Scientific Ltd, Idar-Oberstein, Germany) run for two 5 min pulses at 26 oscillation per second and cooled by placing the tubes on ice for 1 min. The homogenised samples were then incubated at 65°C for 1 h after which they were centrifuged at 11,844 *x g* for 5 min. Each resulting supernatant (700 µL) was transferred to a clean 2.0 mL tube. Each supernatant was purified by adding 800 µL chloroform:isoamyl alcohol (24:1), mixed by inversion 50 times, before centrifugation at 11,844 *x g* for 10 min. The aqueous layer (450 µL) from each tube was transferred to separate clean 2.0 mL tubes and an equal volume of isopropanol (99%) added to each tube to precipitate the DNA. The tubes were inverted 20 times and placed on ice for 10 min. The DNA was pelleted by centrifugation at 11,844 *x g* for 5 min. Each DNA pellet was washed with 200 µL of 70% ethanol followed by centrifugation at 11,844 *x g* for 1 min. Tubes were inverted onto a paper towel to dry and re-hydrated in 50 µL of 10 mM TE buffer (pH 8) (Appendix B.1). Prior to use in PCR, the quality (260:280 nm ratio) and concentration of DNA was measured using a NanoDrop Lite spectrophotometer (ThermoFisher Scientific) with three replicate readings for each sample and the average was recorded.

B) Comparison with a commercial DNA extraction kit

DNA extraction from boysenberry plant tissue samples from the Riwaka Station (Section 3.2.1.1B) was conducted using two different methods (i) Modified Plant & Food Research protocol (optimised in Section 3.2.1.2 A), and (ii) PowerPlant® DNA isolation kit (MO BIO Laboratories, Inc, Carlsbad, CA, USA).

(i) Modified Plant & Food Research protocol

The Plant & Food Research protocol (Dodd *et al.*, 2007) was used with the Aegerter CTAB buffer (Section 3.2.1.2 A) but without mercaptoethanol (Appendix B.1).

(ii) PowerPlant® DNA isolation kit

DNA was extracted from approximately 50 mg of plant tissue according to the manufacturer's instructions (Appendix B.2). The concentration and quality of the extracted DNA was determined by spectrophotometry using a NanoDrop Lite spectrophotometer (ThermoFisher Scientific) prior to use in PCR.

3.2.1.3 PCR amplification

A nested PCR method developed by Dodd *et al*. (2007; pers. comm. Dr Brenda Pottinger, 2011) was used. This method had been optimised for cycle number (Appendix B.3). In the primary PCR the ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (forward) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (reverse) universal primers (White *et al*., 1990) (Invitrogen Technologies, New Zealand) were used to amplify an approximately 820 bp fragment that encompassed the ITS1, 5.8S and ITS2 regions of fungal ribosomal RNA gene (Figure 3.1). Each PCR contained 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3 and 0.01% (w/v) gelatine), 1.5 mM $MgCl_2$, 200 µM of each dNTP and 0.2 µM of each primer, 1 U of FastStart Taq polymerase (Roche Molecular Biochemicals, Mannheim, Germany) and 1.0 µL of template DNA in a total volume of 25 µL. The non-template control contained sterile water instead of the template DNA. The amplification conditions were as follows: 3 min initial denaturing at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 50°C and 45 s at 72°C, and a final elongation period of 10 min at 72°C, using the Veriti 96-well Thermal cycler (AB Applied BioSystems).

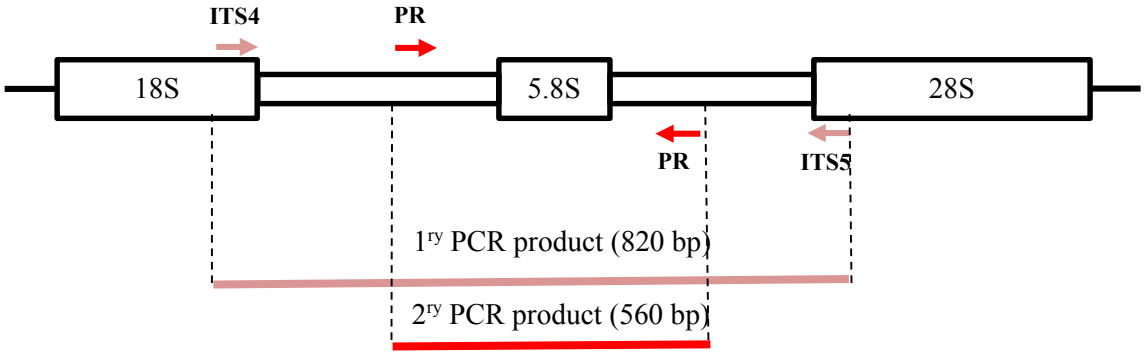

Figure 3.1 Schematic representation of the rRNA gene binding sites for primers used in the nested PCR. The open boxes represent the ribosomal genes. Arrows represent the positions and amplification directions from the universal primers ITS4/ITS5 and *Peronospora sparsa* specific primers PR3/PR4. The expected size of the PCR product is shown below.

The secondary PCR contained 1 µL of the primary PCR product as the template and the *P. sparsa* specific primers PR3 (5'-GGCTGGCTGCTACTGGGCA-3') (forward) and PR4 (5'-GCCGACTGGCCACGCGGA-3') (reverse) (Lindqvist *et al*., 1998), with the same amplification conditions. Ten µL of secondary reaction was separated by electrophoresis (10 V/cm, 45 min) in a 1.0 % agarose gel in 1 x Tris-acetate EDTA (TAE) buffer (40 mM Tris

acetate, 2 mM Na_2EDTA, pH 8.5). Each sample loaded into the gel was prepared by a 10 µL aliquot of secondary PCR product mixing with 2 µL of 6 x loading dye (0.025% bromophenol blue, 0.025% xylene cyanol FF, 40% (w/v) sucrose in water) to give a final volume of 12 µL. The molecular marker was prepared in a similar manner, except that the 10 µL PCR product was replaced by 10 µL of 1Kb + DNA Ladder™ (Invitrogen). The gel was stained in 0.5 µg/mL ethidium bromide (AmrescoR, OH, USA) for 10-15 min, destained with tap water for 5 min and visualised with UV light using a FireReader™ Gel Doc imaging system (UVITEC Cambridge, TLS Total Lab Systems Ltd). A 820 bp DNA band was expected for amplification of the conserved ITS region of oomycetes (*Peronospora* and *Phytophthora*) using primers ITS4 and ITS5, whereas, a 660 bp fragment for *P. sparsa* specific amplification using PR3 and PR4 specific primers was expected.

3.2.2 Sensitivity of the nested PCR method

Detection reliability in different plant tissues

Three symptomatic boysenberry plants were selected. The plants had been maintained in a greenhouse (Lincoln University) in 2.5 L pots, 12-14 month potting mix, and were nine months old. Primocane tip, leaf bud and leaf samples were collected from two canes of each plant in early winter (May). The 1.25 cm primocane tip segment was marked into three sections: (i) 0.25 cm from the base of the segment, (ii) 0.50 cm middle section and, (iii) 0.50 cm top-most section. The 0.25 cm base section from one cane sample and the 0.50 cm middle section from the other cane were used for DNA extraction from primocane tips. The top-most section was not used because a previous experiment (Appendix B.3), in which DNA extraction from primocane tips and amplification by PCR, showed that no *P. sparsa* was present. DNA was extracted from each leaf bud and leaf disc (12 mm diameter) separately. Tissue samples were detached the same day as DNA extraction, which was conducted according to Section 3.2.1.2.B(i). Nested PCR was conducted as described in Section 3.2.1.3 (Appendix B.3).

Establishing the detection sensitivity (limit of detection) of the nested PCR

DNA was extracted from *P. sparsa* spores using the method described in Section 3.2.1.2.B(i) except that the plant tissue was replaced with 200 µL of a *P. sparsa* spore suspension (1.5 x 10^6/mL). The extracted DNA was quantified by spectrophotometry. Nested PCR (Section 3.2.1) was conducted using 1 µL of template DNA, with stepwise 10 fold dilutions giving DNA concentrations ranging from 4 ng to 4 fg/ µL. Gel electrophoresis was conducted as described in Section 3.2.1. The PCR product (1 µL) was diluted with PCR water (6 µL) and loading dye (2 µL) prior to loading into the gel.

3.2.3 Comparison of two clearing methods for fluorescent staining technique to detect latent infection of *P. sparsa* in boysenberry tissues by fluorescence microscopy

Two different tissue clearing methods were tested for boysenberry tissues. The clearing methods were assessed for clarity of detection of *P. sparsa* in asymptomatic stem pieces (canes) and roots (both cross sections and longitudinal sections) obtained in August, 2012 from systemically infected asymptomatic 1 year old boysenberry plants (cv. Mapua) grown in the shadehouse (Lincoln University) in 2.5 L pots containing 12-14 month potting mix.

A) Fix specimen/clearing in NaOH

This method was based on the aniline blue fluorescence method developed by Williamson *et al*. (1995) for detection of *P. rubi* in *Rubus* spp. and some hybrids, including boysenberry. Thin (approximately 0.3 mm width) longitudinal sections and cross sections of cane or leaf material from the systemically infected asymptomatic boysenberry plants were cut using a scalpel. They were fixed in Carnoy solution (ethanol:chloroform:glacial acetic acid 6:3:1 v/v/v) for 30 min for soft tissues (leaves and stems), and overnight (approximately 12 h) for hard tissues (old stems). Specimens were placed in clean Universal bottles (28 mL) containing NaOH (1 N), and softened and cleared at 60ºC in for 1 h (approximately 15 min more for hard tissues). The incubation length was based on visual observations of plant tissues during clearing. The NaOH was discarded and the plant tissues were each rinsed in 15 mL sterile water which was poured gently along the internal wall of the Universals (softened tissues were fragile and were easily damaged) to remove residual NaOH. Then the specimens were transferred into a clean Petri dish lid, and a smooth paint brush used to pick up the individual tissue samples and transfer them onto a microscope slide for mounting. Excess water was removed by blotting the specimen gently with a clean Kimwipe™ tissue. Plant sections were stained with a drop of freshly prepared 0.1% aniline blue in 0.1 N K_2HPO_4 to act as a buffer. This replaced the K_3PO_4 described in the published protocol (Williamson *et al.,* 1995). The coverslip was placed on the top of the specimen and gently pressed to produce a squashed preparation for optimal resolution of the oomycete hyphae. The specimens were examined by fluorescence microscopy with an Olympus SZX12 microscope with a UV light source model U-LH100HG with a supplementary barrier filter (excitation wave length is 460-495 nm, emission is 510-550 nm). Image analysis was performed using the Cell/F (Olympus soft imaging solutions, Athens, Greece) image software.

B) Heat specimen/ clearing in KOH

This method was based on the KOH-aniline blue fluorescence method of Hood and Shew (1996) for detection of *Peronospora tabacina* on *N. tabacum* plants and some other true fungi on several other hosts. Thin (approximately 0.3 mm width) longitudinal sections and cross sections of cane or leaf material obtained from the same systemically infected asymptomatic boysenberry plants were autoclaved for 15 min at 121ºC (100 kPa) in 50 mL of 1 M KOH

followed by three rinses in 20 mL sterile water. The method for mounting, staining and observation of specimens was as described previously in Section 3.2.3A. The stain described by Hood and Shew (1996) was not used and was replaced with the 0.1% aniline blue dye described in Section 3.2.3A.

3.2.4 Application of the optimised nested PCR and/or fluorescence staining method(s)

3.2.4.1 To detect *P. sparsa* latent infection

Latent infection in asymptomatic boysenberry plant tissues (leaves, roots, stems, berries) was assessed using the optimised DNA extraction and nested PCR and/or fluorescence staining method (fluorescence microscopy).

A) Latent infection of *P. sparsa* in systemically infected tissues sampled over 12 months

Systemically infected one year old boysenberry plants (cv. Mapua) grown in the Lincoln University shadehouse were allowed to develop floricanes. Three replicate samples each of roots, shoot tips, stems/canes, leaves, leaf buds, flower buds, flowers and fruits/berries, as available, were collected at random in July (winter), September, October and November (spring), December (summer) and March (autumn) in 2012/2013 and stored in 20% glycerol at -80ºC for DNA extraction.

DNA was extracted as described in the Section 3.2.1.2.B(i) which was also used hereafter throughout the study followed by nested PCR as described in Section 3.2.1.3. Colonisation of plant tissue samples by *P. sparsa* was observed using fluorescence microscopy as described in Section 3.2.3.A.

B) Latent infection of *P. sparsa* in the berry/fruit

Asymptomatic berry samples at two maturity stages (five replicates each of green, hard, unripe and red, partially ripe) were collected from two year old systemically infected boysenberry (cv. Mapua) plants grown in the shadehouse (Lincoln University) in December, 2013. Samples were bisected into two portions horizontally, to divide the top section of each berry (contained drupelets and part of the torus) and bottom section (contained drupelets, the remaining torus, calyx and stem). Drupelets, torus, calyx and stem tissues were dissected from each portion using aseptic techniques to avoid contamination among different tissues of each berry (Figure 3.2). The samples were used fresh for DNA extraction [Section 3.2.1.2.B(i)] after washing, to remove any superficial *P. sparsa* spores, followed by the PCR (Section 3.2.1.3). Half of the calyx, 12 drupelets and 1 cm long stem were used for DNA extraction.

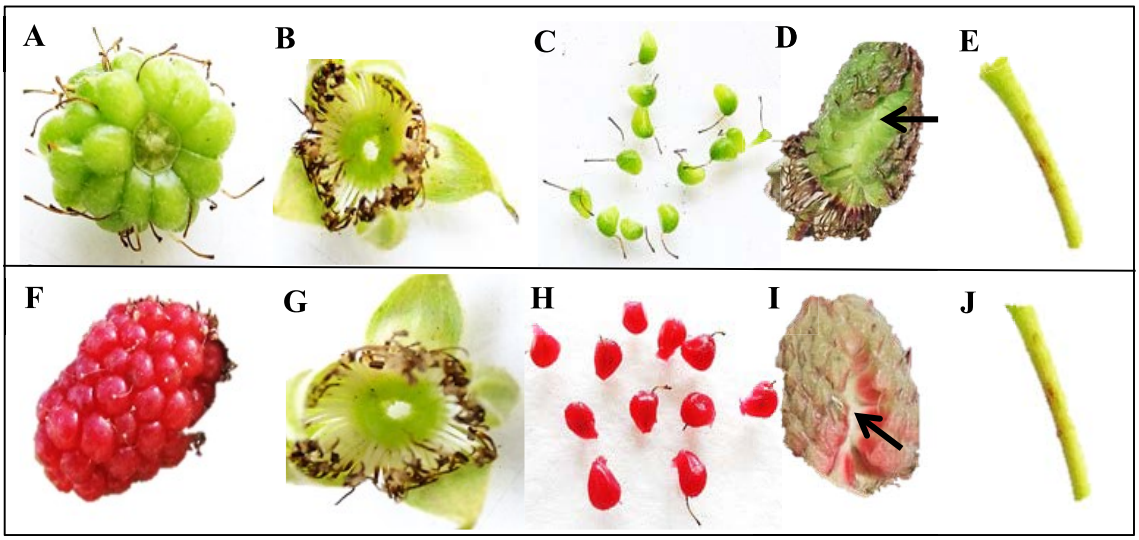

Figure 3.2 Green hard unripe berry tissues (A) full berry, (B) calyx, (C) drupelets, (D) torus (white middle portion indicated in arrow), (E) stem and (F-J) the same tissues of red partially ripe berry stage used for DNA extraction.

In order to evaluate whether all berry parts were infected by *P. sparsa* in symptomatic dryberries, two dryberries were detached at random from the same systemically infected boysenberry plants at the same maturity stage (red, partially ripe). They were dissected into stems, calyxes, torus and drupelets, DNA extracted [Section 3.2.1.2.B(i)] and PCR was conducted.

C) To determine the inhibitory effect of red berry tissue extract on PCR

DNA samples from two drupelets and three torus tissues from the red, partially ripe berries which gave negative PCR results in Section 3.2.4.1B were used to assess the limit of detection for *P. sparsa* by nested PCR in the red berry extract. These were chosen to determine whether they contained any inhibitory compounds that had been co-extracted and were able to inhibit the amplification of *P. sparsa* DNA. This was done by producing two templates for PCR, 1) combining 1 µL each of DNA extracts from the drupelet or torus tissue with 1 µL DNA extracted from spores and 2) DNA extracted from spores. The DNA from spores was added at different concentrations by serially diluting from 400 to 0.4 pg/ µL. Each template was amplified using the nested PCR (Section 3.2.1.3). The results of PCR in the presence and absence of berry extract at different *P. sparsa* concentrations were compared.

3.2.4.2 To assess detection sensitivity of the nested PCR for *P. sparsa* spores trapped on Petroleum jelly (Vaseline®) coated slides

To determine the detection sensitivity of the nested PCR method, representative slides (four slides per assessment time) were selected from the 2011 trapping season (Chapter 2). The set of four was chosen to includes slides which had zero, low (≤100), moderate (100-1000) and high (≥1000) spores per slide as determined by light microscopy (Section 2.2.1.1).

The slides were selected to be representative across the sampling period, but not for every week, and for each of the three grower properties. This resulted in the selection of 18 assessment times with four replicate slides each time, being six, eight and four different weekly traps from Lower Moutere, Upper Moutere and Richmond grower properties respectively. The slides were stored at 4ºC for approximately eight months until DNA extraction. The four slides from each sampling time/grower were washed together in a tube containing 10 mL sterile distilled water and held in a 60°C water bath for approximately 10 min. The resulting suspension was centrifuged at 4696 *x g* for 10 min. Samples of the supernatant were observed under a microscope at x 10 and x 40 magnification to ensure that no spores remained, such that the centrifugation had deposited all spores in the pellet. The supernatant was discarded and the pellet was mixed with 1.5 mL of 20% glycerol for storage at -80ºC prior to extraction of DNA for PCR. DNA was extracted from 350 µL of each re-suspended spore pellet sample (Section 3.2.1.2Bi). Nested PCR (Section 3.2.1.3) was used to detect the presence of *P. sparsa* DNA. Because the expected bands were faint or absent from the gel, a further electrophoresis was conducted with 10 representative samples of the DNA that was extracted from slides to verify presence/absence of DNA in the DNA extractions.

3.3 RESULTS

3.3.1 Optimising DNA extraction from systemically infected boysenberry plants

3.3.1.1 DNA extraction

A) Optimising DNA extraction buffer

DNA concentration, as determined by spectrophotometry, was higher when extracted using the Aegerter CTAB buffer (range 4.4-19.6 ng/µL) (Table 3.1) than with the Plant & Food Research CTAB buffer (range 1.6-4.6 ng/µL) for all asymptomatic tissues, including, primocane tips, young canes/stems, old canes/stems, leaf buds, leaves, and symptomatic (but not sporulating) leaves. Also the purity of the DNA extracted from the 12 samples using the Aegerter CTAB buffer was higher than with Plant & Food Research CTAB buffer, with an absorbance ratio (260:280 nm) closer to 1.8 (high purity DNA) and with the most pure DNA extracted from leaf buds (1.8) and cane (1.7 both old and young) samples.

Table 3.1 Evaluation of the Plant & Food Research CTAB buffer and Aegerter CTAB buffer for DNA extraction from systemically infected boysenberry plant tissues.

Boysenberry Tissue type	DNA concentration (ng/µL)		Absorbance ratio (260:280 nm)	
	Plant & Food Research CTAB buffer	Aegerter CTAB buffer	Plant & Food Research CTAB buffer	Aegerter CTAB buffer
Leaves (symptomatic)	2.3	4.4	1.3	1.5
Leaves (asymptomatic)	2.6	19.6	1.4	1.5
Primocane tips*	2.6	13.7	1.3	1.5
Young canes	1.7	10.9	1.4	1.7
Old canes	1.6	5.3	1.3	1.7
Leaf buds	4.6	11.3	1.4	1.8

*Except symptomatic leaves, other tissue types are asymptomatic.

B) Comparison with a commercial DNA extraction kit

Higher DNA quantity was extracted using the PowerPlant® DNA isolation kit (range 13.3-502.2 ng/µL) (Table 3.2) than with the modified Plant & Food Research CTAB method (range 6.2-270.4 ng/µL), which used the modified Aegerter CTAB buffer, for all 20 samples (four replicates each of sporulating leaves, symptomatic (but not sporulating) leaves, root shavings (asymptomatic), cane shavings (asymptomatic) and primocane tips (asymptomatic)). However, the purity of DNA was better using the modified Plant & Food Research CTAB method, in which the extraction buffer was a modified Aegerter CTAB buffer [Section 3.2.1.2.B(i)].

Table 3.2 Comparison of the Modified Plant & Food Research protocol and PowerPlant® DNA isolation kit for DNA extraction from systemically infected boysenberry plant tissues.

Boysenberry Tissue type	DNA concentration (ng/µL)		Absorbance ratio (260:280 nm)	
	Modified Plant & Food Research CTAB protocol	PowerPlant® DNA isolation kit	Modified Plant & Food Research CTAB protocol	PowerPlant® DNA isolation kit
Leaves (sporulating)	20.5	295.3	1.7	1.0
Leaves (symptomatic)	8.2	161.1	1.6	1.0
Primocane tips*	270.4	502.2	1.9	1.2
Canes	6.2	47.0	2.1	1.2
Roots	6.9	13.3	1.6	1.4

*Except leaves, other tissue types are asymptomatic.

3.3.1.2 PCR amplification

A) Optimising DNA extraction buffer

No visible bands were amplified by nested PCR using DNA extracted using the Plant & Food Research CTAB buffer as the template (Figure 3.3A). For DNA extracted using the Aegerter CTAB buffer, bands were observed for primocane tip (asymptomatic) and old cane (asymptomatic) samples (one of the two replicates each) and faint bands were observed for leaf (with symptoms but not sporulating) and asymptomatic young cane samples (Figure 3.3B).

B) Comparison with a commercial DNA extraction kit

For DNA extracted using the PowerPlant® DNA isolation kit, bands were only amplified from leaf samples (Figure 3.3C), but were not as bright as those amplified using DNA extracted with the modified Plant & Food Research protocol (Figure 3.3D). Bright bands were observed for all leaf samples (both sporulating and non sporulating) and 2-3 (of the four) replicates of root, cane and primocane tips with the modified Plant & Food Research protocol which used the modified Aegerter CTAB buffer (Figure 3.3D).

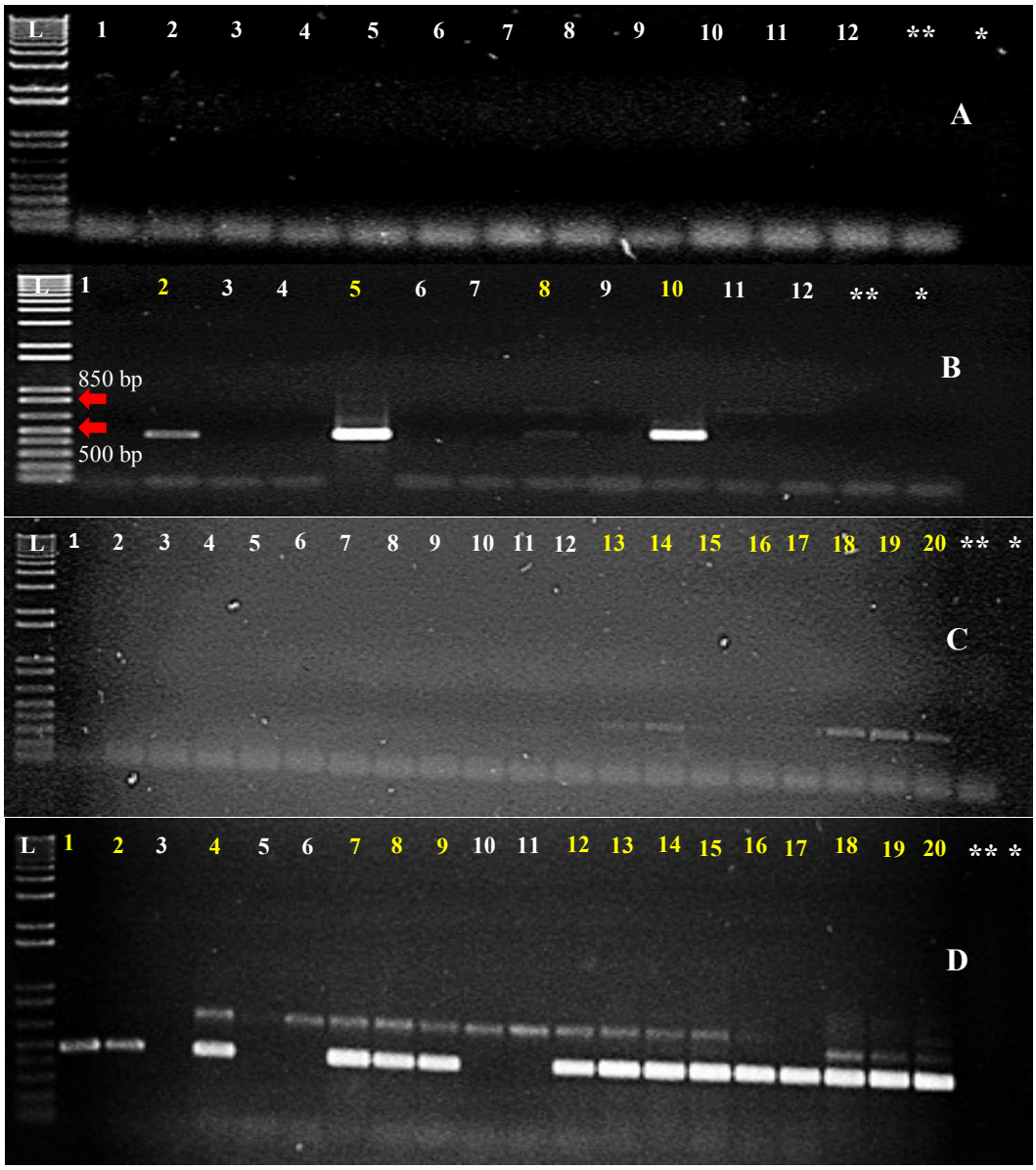

Figure 3.3 1% agarose gel of boysenberry plant tissue samples amplified using nested PCR from DNA extracted using (A) Plant & Food Research CTAB buffer, (B) Aegerter CTAB buffer (the Plant & Food Research protocol), (C) PowerPlant® DNA isolation kit and (D) modified Plant & Food Research protocol with modified Aegerter's CTAB buffer. Samples for (A and B) are symptomatic leaves (1, 2), asymptomatic leaves (3, 4), asymptomatic primocane tips (5, 6), asymptomatic young stem (7, 8), asymptomatic old stem (9, 10), asymptomatic leaf bud (11, 12). Samples for (C and D) are root samples (1-4), stem samples (5-8), asymptomatic primocane tips (9-12), symptomatic leaves (13-16), sporulating leaves (17-20). L=1+KB DNA size marker, control from primary PCR step of nested PCR (**), negative control (*). Samples which gave a positive PCR reaction for *Peronospora sparsa* are indicated in yellow.

3.3.2 Sensitivity of the nested PCR method

Establishing the detection sensitivity (limit of detection) of the nested PCR

The nested PCR method was sensitive enough to detect as little as 0.4 pg (400 fg) of *P. sparsa* genomic DNA that had been extracted from spores (Figure 3.4).

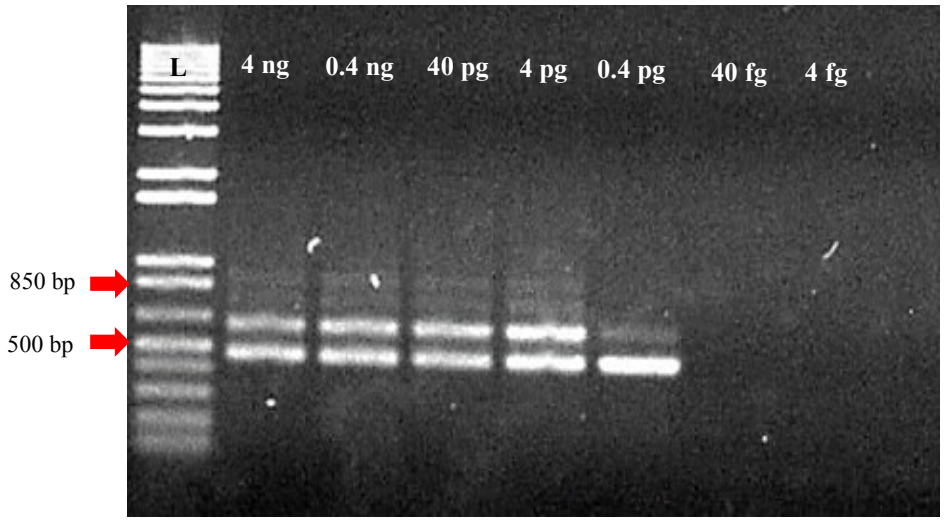

Figure 3.4 1% agarose gel of *Peronospora sparsa* spore genomic DNA (4 ng to 4 fg) amplified using nested PCR and DNA extracted using the modified Plant & Food Research protocol. DNA quantity is designated at the top of the gel. L=1+KB plus DNA ladder.

3.3.3 Comparison of two clearing methods for fluorescent staining technique to detect latent infection of *P. sparsa* in boysenberry tissues by fluorescence microscopy

The fixation and clearing of specimens with the NaOH method of Williamson *et al*. (1995) was more effective for observations of systemic hyphal growth than the method of heating in KOH of Hood and Shew (1996). The former method gave higher resolution of hyphae and excellent contrast to host plant tissue. It was also possible to visualise hyphae in longitudinal sections of root tissue samples under the general white light source in specimens prepared by this method (Figure 3.5F-H). Even though the plant tissues also fluoresced it did not interfere with the differentiation of the hyphae. Therefore, the fix specimen/clearing in NaOH method was used for fluorescence microscopy observations conducted in Chapter 4.

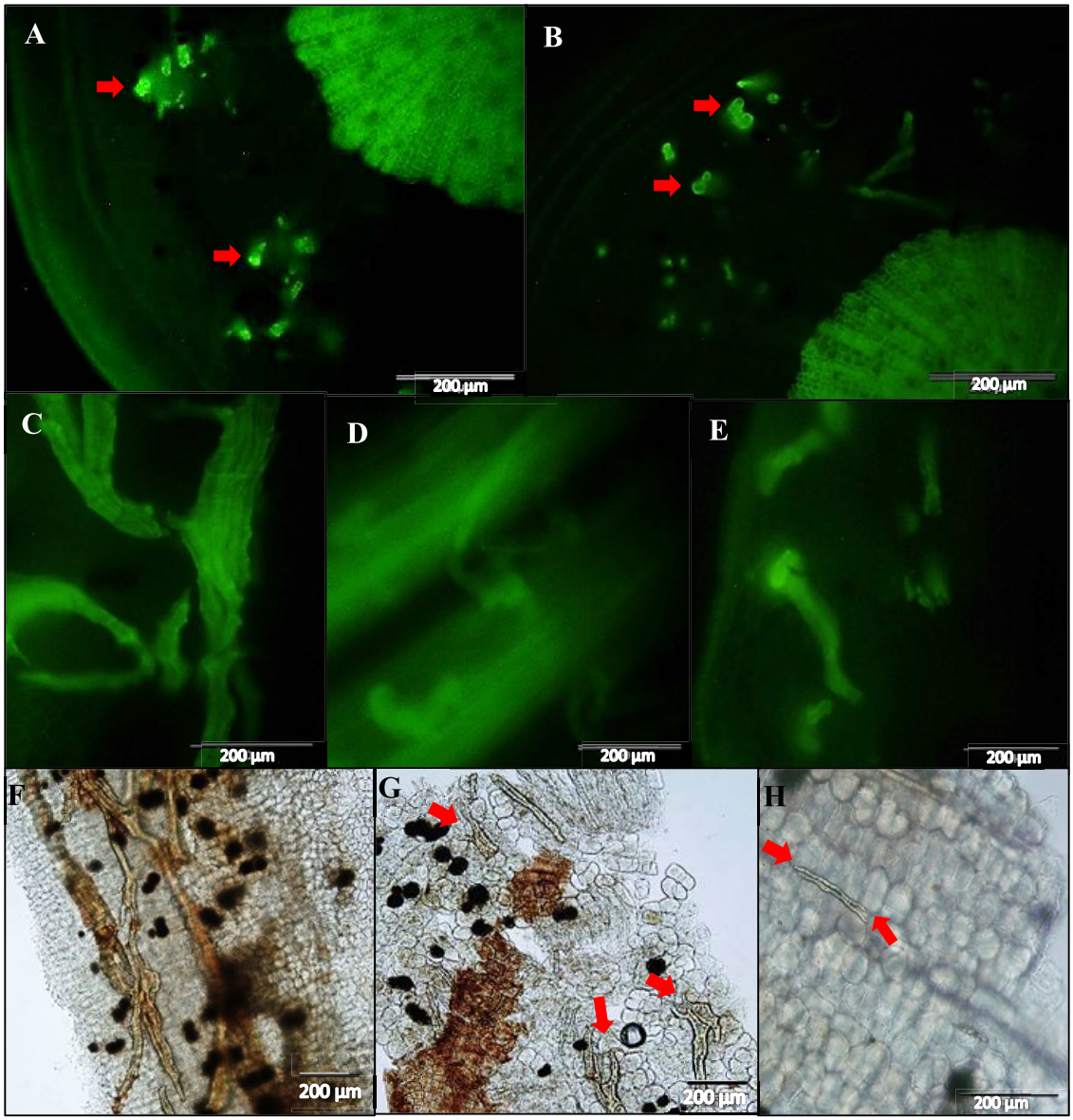

Figure 3.5 Evaluation of the clearing methods for fluorescence staining to detect oomycete structures in asymptomatic boysenberry tissues. Samples stained using the fix specimen/clearing in NaOH method in (A, B) cross-sections of root, with colonisation present in the cortex. Host cells penetrated by haustoria from the hyphal apex (indicated with red arrows), (C) hyphae like structures in cane cortex tissue (longitudinal section). Samples stained using the heat specimen/clearing in KOH method with hyphae in a (D) longitudinal section of cane and (E) cross section of root. Samples stained using the fix specimen/clearing in NaOH method under white light observation of longitudinal sections of root tissues with (F) heavy colonisation in the cortex and (G, H) with dichotomously branched haustoria (indicated with red arrows).

3.3.4 Application of the optimised nested PCR and/or fluorescence staining method(s)

3.3.4.1 To detect *P. sparsa* latent infection

A) Latent infection of *P. sparsa* in systemically infected tissues sampled over 12 months

From winter to autumn the pathogen was consistently detected in the cane (floricane) samples, being present in all three samples in winter and summer and two of the three samples in spring and autumn months (Table 3.3). The pathogen was not detected in asymptomatic leaves in the spring; however, two of the three leaves that were tested were positive for *P. sparsa* in winter,

summer and autumn months. The pathogen was detected in all three root samples in the summer, one in winter and autumn, and two of the three samples in spring. The pathogen was detected in one of the three leaf buds, one or two flower buds and two flowers of each triplicate in the spring samples. The pathogen was detected in only one of the three berries at the green unripe hard stage and all three of the red partially ripe berries tested (Table 3.3).

Although the pathogen was detected consistently in the cane tissue samples throughout the spring months, with one of the canes positive in mid spring (October) and two of the three canes in early (September) and late (November) spring, *P. sparsa* was only detected in tip samples in October (2 out of 3) (Table 3.3). No infection of the leaves was detected in early and mid-spring months with a low detection frequency (only 1 out of 3) present in November. In early spring the pathogen was detected in two of the three leaf buds sampled, and in mid and late spring in one out of the three leaf buds sampled. *Peronospora sparsa* was detected in one and two of the three flower buds sampled in mid spring (October) and late spring (November), respectively.

Table 3.3 Evaluation of the latent infection of *Peronospora sparsa* in a range of systemically infected boysenberry plant tissues over four seasons. Numbers of plant tissue samples (n=3) that were positive for *P. sparsa* DNA using the nested PCR.

Boysenberry plant tissue type (asymptomatic)	Number of samples testing for *P. sparsa* DNA positive					
	Winter (Jul.)	Spring*			Summer (Dec.)	Autumn (Mar.)
		Sept. (early)	Oct. (mid)	Nov. (late)		
Tips	2	0	2	0	1	1
Canes	3	2	1	2	3	2
Leaf buds	-	2	1	1	-	-
Leaves	2	0	0	1	2	2
Flower buds	-	NT	1	2	-	-
Flowers	-	-	NT	2	NT	-
Green berries	-	-	-	-	1	-
Red berries	-	-	-	-	3	-
Roots	1	2	2	2	3	1

* Samples were collected in all three months during the spring. NT not tested, - tissue type not present

B) Latent infection of *P. sparsa* in the berry/fruit

All tissues from the five dissected berries detached at the green unripe hard stage were positive for *P. sparsa* using the nested PCR. Only 27% (n=30) of the tissues from the red partially ripe berries produced a positive PCR result, but all five berries had at least one tissue sample positive using PCR. Calyx/sepals of four berries (of the five) and drupelets of three berries (of the five) were infected, of which two drupelet samples were from the top half of the berry and the other two from the bottom half, close to the stem of the berry. None of the torus or stem samples were *P. sparsa* positive.

All samples from the symptomatic dryberries, except one torus sample, gave a positive PCR result (Figure 3.6). Of the symptomatic dryberries, all the four tissue types in one berry were positive for *P. sparsa* and all tissues except the torus sample in the other.

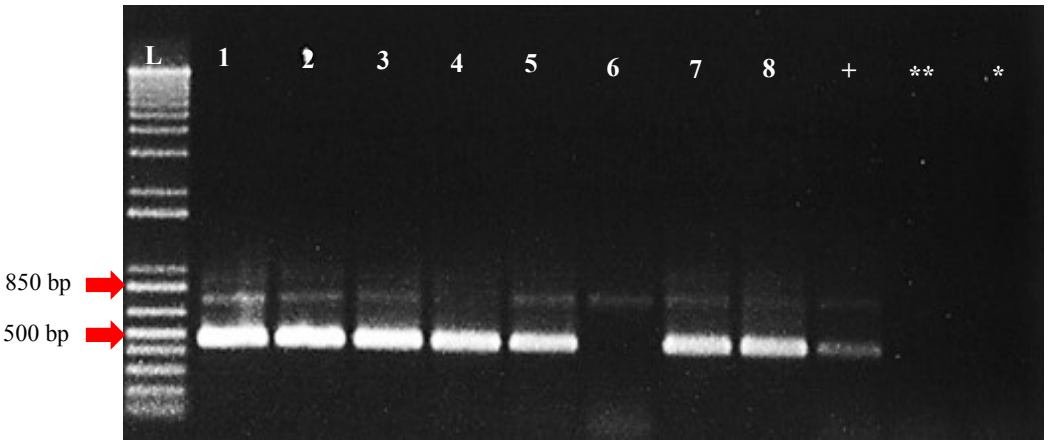

Figure 3.6 1% agarose gel of dissected tissue samples of two symptomatic dryberries amplified using nested PCR with DNA extracted using the modified Plant & Food Research CTAB method. Samples for dryberry 1 and 2, respectively are stems (1, 2), sepals (3, 4), torus (5, 6) and drupelets (7, 8). L=1+KB DNA size marker, *Peronospora sparsa* positive control (+), control from primary PCR step of nested PCR (**), negative control (*).

(C) To determine the inhibitory effect of red berry tissue extract on PCR

Nested PCR produced the expected 820 bp amplimers from the primary PCR and the 660 bp amplimer for all (200-2 pg/µL) concentrations of *P. sparsa* spore genomic DNA, and when the reaction was spiked with the red berry drupelet extracts, amplimers (PCR products) were only produced from 200 and 20 pg spore DNA. A similar result was observed for both drupelet samples indicating that the red berry drupelet extracts caused a tenfold inhibition of the PCR. PCR was not inhibited by the torus extracts from the berries at the red ripe stage. Two of the torus samples (of the three) produced the same level of detection when spiked with spore DNA, however, in the third sample the detection limit was 20 pg suggesting there might be some (≤10 fold) inhibition (Figure 3.7).

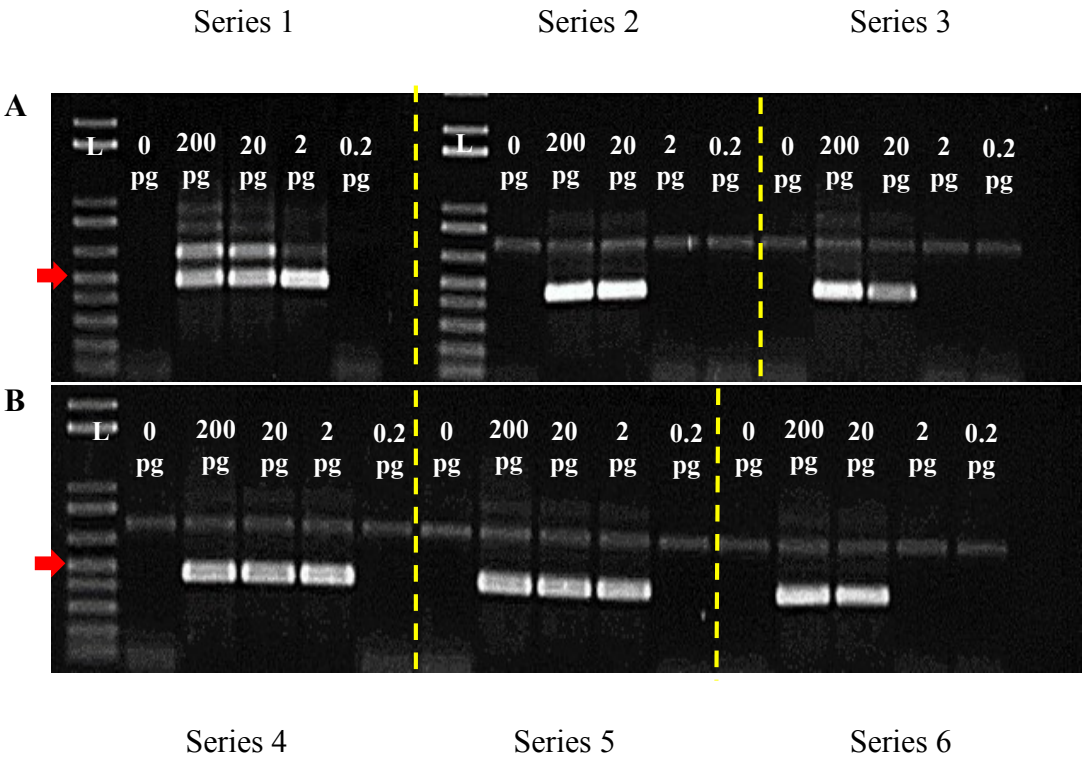

Figure 3.7 1% agarose gel of *Peronospora sparsa* spore genomic DNA (200-0.2 pg) amplified using the nested PCR (series 1, A), spore DNA spiked with drupelet extracts from asymptomatic red berry samples 1 and 2 (series 2 and 3, A), spore DNA spiked with torus extracts from asymptomatic red berry samples 1, 2 and 3 (series 1, 2 and 3, B). L=1+KB plus DNA ladder. The expected band size (660 bp) is indicated by red arrows.

3.3.4.2 To assess detection sensitivity of the nested PCR for *P. sparsa* spores trapped on Petroleum jelly (Vaseline®) coated slides

The selected spore trapping slides (2011) from which DNA was extracted provided only low DNA quantities (1.2 to 5.5 ng/µL) (Figure 3.8). The highest DNA concentration (5.5 ng/µL) was obtained from sample 12, in which the number of spores determined by counting was moderate (468 spores per glass slide). However, sample nine which had the highest spore number (2178 spores per glass slide) only produced a DNA concentration of 2.9 ng/µL, which was less than some of the samples where no *P. sparsa* spores were observed. No visible DNA bands were observed in the gel electrophoresis (Appendix B.4) conducted for 10 samples (6, 10, 12, 8, 15, 2, 4, 9, 1 and 5; Figure 3.8).

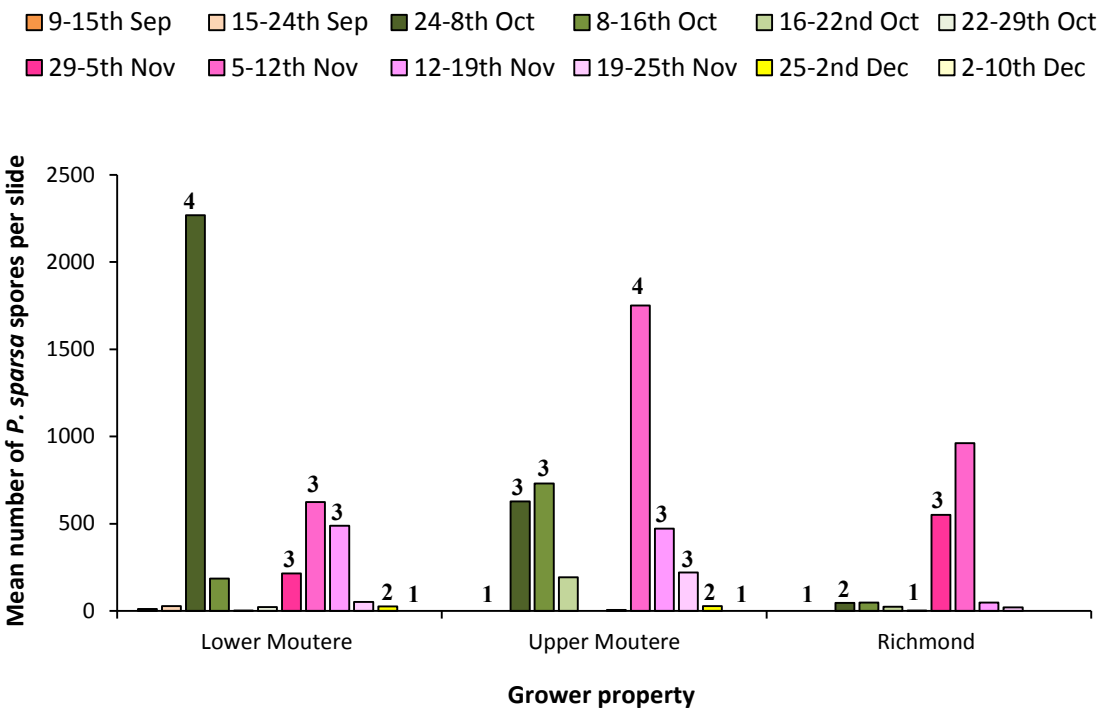

Figure 3.8 Mean numbers of *P. sparsa* spores on representative samples of the Petroleum jelly coated glass slides used to trap spores at three growers' properties [(Upper Moutere (Oct-Dec), Lower Moutere (Sep-Dec) and Richmond (Sep-Nov)] in 2011. Slides selected to represent zero (1), low (2), moderate (3) and high (4) spore counts per glass slide.

Using DNA extracted from the slides and amplified by nested PCR, gave conflicting results. When 1 µL DNA was used in the nested PCR only a few samples (3, 8 and 12) produced positive bands (Figure 3.9A). When 2 µL DNA was used as a template, samples 3 and 12 did not produce a positive band but samples 8, 10, 15 and 16 did (Figure 3.9B). When 3 µL of DNA was used in the PCR (Figure 3.9C) only samples 6 and 8 were positive for *P. sparsa*. At least one sample gave a positive result from each grower property. Seven of the samples which were positive had zero-medium spore counts, whereas none from the high spore counts gave positive results. The brightest bands were produced from samples 6, 8 and 10, in which the purity was 1.50, 1.56, 1.64 and DNA concentration was 2.2, 2.6 and 2.6 ng/µL, respectively. There was no relationship observed between the spore counts and the appearance of the *P. sparsa* specific band by PCR.

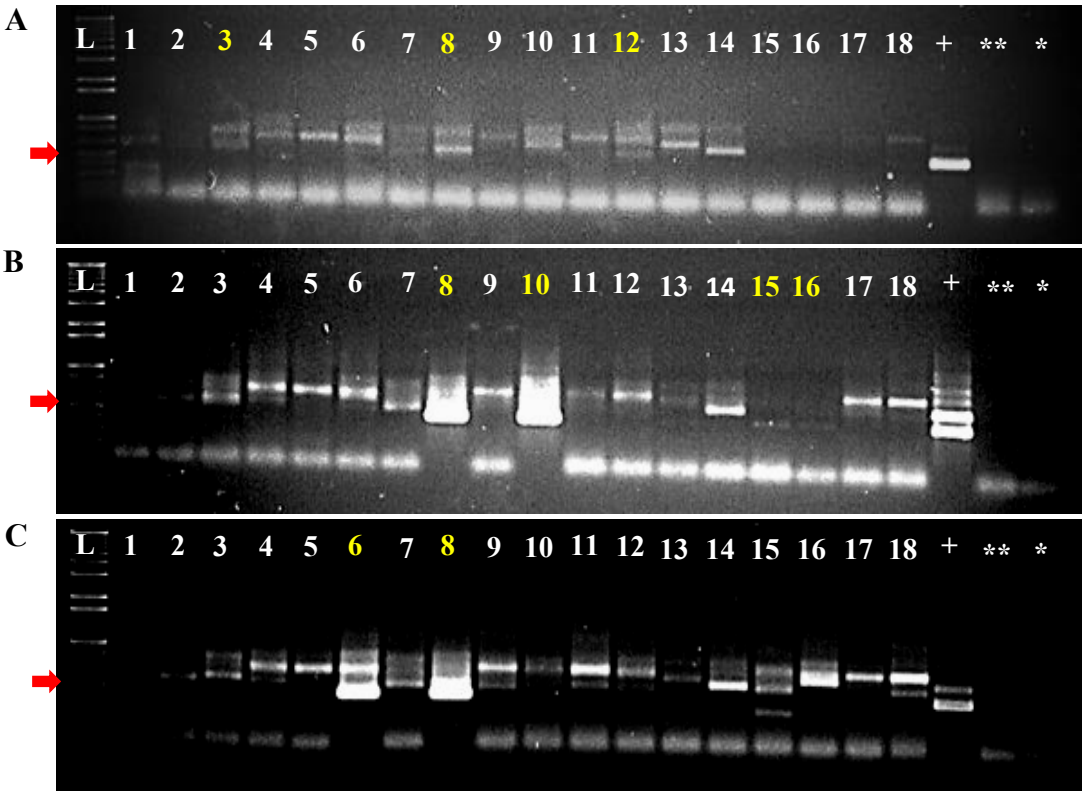

Figure 3.9 1% agarose gel of DNA from representative samples of *Peronospora sparsa* spores trapped on Petroleum jelly coated glass slides in 2011, which had been amplified using nested PCR with A) 1 µL, (B) 2 µL and (C) 3 µL DNA extracted using modified Plant & Food Research CTAB method. Lanes 1-18 are the secondary PCR products, L = 1+KB DNA size marker, control from primary PCR step of nested PCR (**), negative control (*). Samples which gave a positive PCR reaction for *Peronospora sparsa* are indicated in yellow. The expected band size (660 bp) is indicated in red arrows.

3.4 DISCUSSION

This chapter described optimisation and validation of the existing Plant & Food Research (P&FR) protocol for DNA extraction and PCR amplification of *P. sparsa* DNA from asymptomatic plant tissues. The optimised method extracted up to 4 × more *P. sparsa* DNA, with a higher purity, than the original protocol and could detect as little as 0.4 pg of *P. sparsa* DNA. The optimised method demonstrated that *P. sparsa* was present in canes throughout the year in the systemically infected but asymptomatic boysenberry plants.

Replacing the original extraction buffer with the Aegerter buffer (Aegerter *et al*., 2002) improved DNA recovery and purity. Improved DNA extraction may be explained by the chemicals present in the Aegerter buffer. The Aegerter buffer contained 1% polyvinylpyrrolidone (PVP) which binds and inhibits the plant enzyme polyphenol oxidase. Polyphenol oxidase oxidises phenolic compounds, making polyphenol/protein/DNA complexes that degrades DNA (Maliyakal, 1992 cited in Yang *et al*., 2008; Porebski *et al*., 1997; Padmalatha and Prasad, 2006). PVP binds polyphenol oxidase, avoiding formation of this complex and thereby improving removal of phenolic compounds (Möller *et al*., 1992; Kim *et*

al., 1997 cited in Wany *et al*., 2013; Porebski *et al*., 1997). A lower sodium chloride (NaCl) concentration in the P&FR buffer may also have encouraged the co-precipitation of DNA with polysaccharides (Murray and Thompson, 1980). The two methods also had different amounts of the chelation agent ethylenediaminetetraacetic acid (EDTA). However, different extraction protocols use EDTA (Porebski *et al*., 1997; Murray and Thompson, 1980) concentrations ranging from 10 to 20 mM and the threshold concentration for complete inhibition is unknown.

Commercial kits have generally undergone extensive procedural optimisation. In this comparison, the β-mercaptoethanol was excluded from the Aegerter buffer (modified Aegerter's buffer) as it did not make any appreciable difference to PCR. Even though more DNA was extracted by the PowerPlant® DNA isolation kit the quality was poor, indicating that the DNA extract had more impurities such as proteins, polysaccharides and other secondary metabolites (Demeke and Ratnayaka, 2009, Varma *et al.*, 2007 cited in Llongueras *et al*., 2013) which are known to inhibit PCR. The reason for this could be the use of silica-based matrices without exposure to organic solvents such as chloroform (Demeke and Jenkins, 2010). This solvent is known to precipitate impurities; however, it is generally incompatible with high throughput commercial kits. Greater total DNA extracted with the PowerPlant® DNA isolation kit may also be due to more efficient extraction of plant DNA decreasing the relative amount of *P. sparsa* DNA. Correspondingly, more amplification of *P. sparsa* DNA was achieved using DNA extracted by the modified Aegerter's buffer than the PowerPlant® DNA isolation kit.

The optimised nested PCR was sensitive enough to detect as little as 0.4 pg (400 fg) of *P. sparsa* DNA. This is in contrast to the standard PCR (0.4 ng) in the current study (Appendix B.6) and comparable to the qPCR (37 fg) of Hukkanen *et al.* (2006). The result from the current study was more sensitive than the method of Aegerter *et al*. (2002) which could detect 2 pg *P. sparsa* DNA on rose and comparable to the reported work of Lindqvist *et al*. (1998) who could detect 0.25 pg *P. sparsa* DNA in arctic bramble. It was less sensitive than the work of Hukkanen *et al.* (2006) who could detect 0.037 pg by qPCR. The *P. sparsa* genome size (C-value) can be assumed to be similar to that of *P. infestans* (Tooley and Therrein, 1987), which leads to the conclusion that one diploid nucleus weighs 0.52 pg or 0.26 pg per genome copy. Thus, the PCR can detect the equivalent of 1.6 genomes. If 1.6 genomes are added as 1 µL of DNA extract from a 50 µL original DNA extract, then DNA must be extracted from a minimum of 80 genomes. As *P. sparsa* is normally diploid (Latijnhouwers *et al*., 2003) then the DNA extract must be derived from a minimum of 40 diploid spores. The detection limit of the nested PCR in the current study was determined using *P. sparsa* spore DNA and not the asymptomatic infected tissues. Therefore, actual detection threshold of *P. sparsa* latent infection may have been higher if inhibitory compounds were co-purified in the DNA extract.

The significance of this inoculum level in terms of threshold for *P. sparsa* infection has not been widely studied; however in preliminary studies (Chapter 2) it was observed that *P. sparsa*

can infect leaf discs with 20 spores after 12 days of incubation in optimum conditions *in vitro.* A higher numbers of spores are likely to be required for optimum infection of *P. sparsa* in intact boysenberry plants; hence the detection limit in this nested PCR should be sufficient for accurate early diagnosis. Although generally considered to be more sensitive (Hukkanen *et al.,* 2006), the qPCR method was not utilised in this study because the nested PCR was sufficiently sensitive to detect early infection. It was also noted that the pathogen distribution was highly variable (Filion, 2012) even in similar tissue types and that the pathogen was present in different forms (Schena *et al*., 2013) such as mycelium, haustoria, spores, sporangiophores with different proportions of nuclear content. Thus, qPCR data may be inconsistent for a tissue type and relativity to different fungal structures may be difficult to determine.

Validation of the detection sensitivity of the nested PCR using DNA extracts obtained from the *P. sparsa* spores trapped on Vaseline® coated slides in the field did not produce consistent results. The low DNA concentrations (Appendix B.4) recovered from all slides suggested two possibilities i) the degradation of *P. sparsa* spore DNA during long term storage of the spore trapped slides and/or ii) the presence of inhibitory compounds on the slides. When the purified DNA was examined by gel electrophoresis (Appendix B.5) no high molecular weight genomic DNA was observed, indicating that little to no intact DNA had been recovered from the slides. It is therefore likely that degradation of DNA during storage was the cause of inconsistent amplification by PCR. DNA extraction from freshly collected spores adhering to glass slides may circumvent these problems. Some bands for fungal ribosomal rRNA gene were present in the primary PCR, however this is the sum of all fungal DNA extracted from the slide and the relative proportion of amplifiable *P. sparsa* DNA may have been too small for successful nested PCR in many instances. The amplification may have been further compromised by any inhibitory compounds present and co-purified from the slide. Other authors have suggested that dilution of DNA extracts (1: 10) is useful to decrease the effect of PCR inhibitors (Ma *et al*., 2003). Ideally a qPCR system could have been used to quantify spores trapped on the slides; however, the described problems with DNA extraction and amplification meant that this was not pursued.

The consistency with which latent infection was detected by PCR was varied with respect to the tissue type and season of sampling. It was reported that oomycete obligate plant pathogen *Albugo candida*, contains numerous mitochondria, but no nuclei in its young haustoria (Grenville-Briggs and van West, 2005). However, Baka (2008) observed more nuclei in young haustoria than older haustoria for the same pathogen in Brassicaceae plants. This shows that presence of nuclei in haustoria is variable in obligate oomycete plant pathogens. In addition, vesicular arbuscular endophytic fungi, which have coenocytic, large branched hyphae as do *Peronospora* spp., have multinucleate hyphae (Nicolson, 1959). However, the nuclei are not always distributed evenly along hyphae but often congregate in segments where branching takes place. Some lengths of hyphae are devoid of nuclei (Nicolson, 1959). Haustoria were observed

in the samples examined in the current study, but the method did not allow for nuclei to be observed. However, it is possible that inconsistency of the PCR results could be due to absence of the nuclei in mycelia being sampled. This also can be suggested for *P. sparsa* as a potential reason for hyphae being present in some samples, but not others in the current study. In order to avoid the inconsistency in the PCR more replicate samples are required from each tissue type. However, *P. sparsa* was consistently detected in the canes of systemically infected, asymptomatic, two-year old boysenberry plants (floricanes) grown in the shadehouse indicating that cane tissues are the most reliable for detection of latent infection. This may also indicate that the pathogen primarily resides in boysenberry canes. Tate (1981) also observed that the pathogen overwintered in cane/stem tissues in boysenberry in New Zealand. This observation was further extended by the histological studies of Williamson *et al*. (1995) for the same pathogen on other *Rubus* spp.

Peronospora sparsa was detected infrequently in primocane tips even when the canes had latent infection. Dodd *et al*. (2007) also reported that *P. sparsa* was present throughout the boysenberry plant except in primocane tips; however, the evidence was not presented. The apparent absence of the pathogen in these tissues may be due to the inhibition of PCR by the inhibitory compounds contained in the primocane tip or due to *P. sparsa* not colonising the tip tissues.

During active plant growth the pathogen was detected in leaf buds of asymptomatic plants using PCR, but rarely in leaves, flower buds and flowers. This indicated that the pathogen is able to move within the plant systemically, presumably by mycelial growth or as propagules carried in sap/xylem. Tate (1981) reported that *P. sparsa* may overwinter in the canes and buds although; he did not investigate the stage at which the buds become internally infected. As the flower buds and flower samples contained parts of both sepals and calyx it was not clear whether the pathogen was restricted to the calyx. However, Ederli *et al*. (2015) reported that sepal and petal express a distinct set of genes to provide tissue specific host defense responses against biotrophic pathogens such as *Golovinomyces cichoracearum* in Arabidopsis plants. They hypothesized that the giant cells in the sepal produce the biotic defense-associated phytohormone salicylic acid (SA) and observed that it was at significantly higher levels in sepals and petals than leaves. In addition, tobacco and petunia also contained chemically distinct floral defensins (Lay *et al*., 2003 cited in Ederli *et al*., 2015) that also can retard the growth of oomycetes during the early stages of flower development in the outermost cell layers of sepals, petals, anthers, and styles. Further, restriction to the calyx has been described for other pathogens and may be due to the hypersensitive reactions triggered in the flower tissues. Radwan *et al*. (2005) reported a similar model for root infecting biotrophic oomycetes such as *Plasmopara halstedii,* with root infection initiating hypersensitive reactions in the plant's more distal tissues (hypocotyl).

The presence of *P. sparsa* in roots was consistent throughout spring suggesting that roots may become infected by close proximity of the infected leaves that fell in winter or the spores washed/blown off during the rainfall/wind in early spring. The mode of transmission of *P. sparsa* into roots was not clear as oospores were not observed in any of the naturally infected aerial tissues in this study irrespective of season. Hall and Shaw (1982) observed oospores in New Zealand on naturally infected leaves and sepals of boysenberry and other wild *Rubus* spp. collected in spring and early summer, but not in mid-late summer. In previous studies conducted in the UK, oospores were observed abundantly in naturally infected tummelberry leaves (Williamson *et al*., 1995). No tissues were sampled in early and late winter (June and August), mid and late autumn (April and May), and mid and late summer (January and February) in the current study and this may have contributed to the absence of oospore observations. Further work should assess the fallen sporulated leaves and calyx of fallen berries for oospores in spring, early summer and late autumn. In the three-month old dormant young boysenberry plants examined in Chapter 6 no oospores were observed by fluorescent microscopy in roots or crowns. This suggests that oospores are produced rarely in New Zealand and/or that they are not important in the disease cycle of *P. sparsa* in boysenberry (Tate, 1981). In contrast, production of oospores in *in vitro* studies on boysenberry leaf discs (Chapter 2) may be mainly due to the depletion of food as the leaf discs decayed. Collectively these results show that the pathogen can reside within live plants.

Differences in berry maturity may influence infection as only one of the three green unripe were infected compared to infection of all three red partially ripe berries. In agreement with this, researchers have reported that fruit infection is affected by the maturity of strawberries with green fruit more resistant to infection by *Botrytis cinerea* than red fruit (Hennebert and Gilles, 1958; Gilles 1959; Irvine and Fulton, 1959 cited in Jarvis, 1962). However, this is not true for all pathogens as strawberries at the green stage were more susceptible for powdery mildew infection (Carisse and Bouchard, 2010). The resistance of immature fruits to colonisation by *Colletotrichum* species was attributed to one of four possible mechanisms of the host plant; 1. pre-formed toxic compounds 2. lack of nutritional and energy sources in unripe fruit 3. poor enzyme potential of the pathogen to colonise unripe fruit and 4. phytoalexin production in unripe fruit (Verhoeff, 1974) . All or some of those factors could apply to boysenberry however; further research would be needed to define the effect of berry maturity on *P. sparsa* infection.

The PCR investigation into the location of the *P. sparsa* infection in dissected asymptomatic boysenberries showed that *P. sparsa* colonised all tissues of the green unripe berries, whereas it was found in 80% of sepals/calices, 60% of drupelets and none of the stem or torus samples of red, partially ripe berries. Walter *et al.* (2008a and b) used PCR to demonstrate that pedicels (fruit stem), sepals, drupelets and receptacles (white plug which was known as the 'torus' in this study) of dissected asymptomatic green boysenberries detached from the field crops were readily infected by *P. sparsa*. However, they did not report infection of the red stage. Also *P.*

sparsa infection was not affected by the location of the drupelets whether being close to the sepals or not. In addition two (n=5) red ripe berries had only the sepals infected and one berry had only drupelets infected, indicating that some drupelet samples were likely to be infected by spores rather than systemically. More acidity (Vicente *et al*., 2006) in the red ripe (345.40 meq/Kg) boysenberries than green unripe (186.40 meq/Kg) might have retarded systemic *P. sparsa* colonisation as was observed in the blueberries by Wharton and Diéguez-Uribeondo (2004) for *Colletotrichum* spp. However, the pathogen might have possessed the enzymes to successfully infect the skin of the boysenberry fruit via external spore infection; hence spore infection may be the major infection pathway at the red ripe stage.

In the current study, some compounds in the asymptomatic red partially ripe berries were shown to have a ten-fold inhibitory effect on the PCR. In contrast, symptomatic dryberries detached at the same stage of maturity gave brighter bands for stem, sepal, drupelet and torus tissue DNA extracts. This seems to conflict with the studies by Vicente *et al*. (2006) who found the greater concentrations of phenolic compounds in boysenberries at the green unripe stage (3.57 g/kg) than red ripe stage (2.49 g/kg). However, in the current study red, partially ripe berries were used which may have still contained some phenolics. Phenolics were reported to cause the PCR inhibition in plant material (Aegerter, 2001; Hukkanen *et al*., 2006). However, these latter studies did not attempt PCR with green, partially ripe or ripe berries.

Of the two common clearing methods compared for epifluorescence microscopy investigated to detect latent infection of *P. sparsa* in boysenberry, the results showed that the fix specimen/NaOH method was more effective (Williamson *et al*., 1995). This method has been used widely to study host pathogen interaction of *P. sparsa* on *Rubus* (Williamson *et al.,* 1995) and rose (Aegerter, 2001), including observations of oospores of *P. sparsa*/*P. rubi* on sporulated leaf discs of *Rubus* and rose by Breese *et al.* (1994). The heat specimen/KOH method with either 1 M KOH or 10% KOH gave poorer contrast due to poorer clearing of the plant tissue. This was in contrast to the reported results of Hood and Shew (1996). However, they studied *Peronospora tabacina* on *N. tabacum* plants and some other fungi on several other hosts but not *P. sparsa* on boysenberry.

Fluorescence microscopy was useful in detecting latent infection of boysenberry tissues, with large aseptate/ coenocytic, branched hyphae characteristic of *P. sparsa* observed in tissue. However, vesicular-arbuscular mycorrhizal (VAM) endophytes are also known to be aseptate/ coenocytic with slightly constricted, large, branched hyphae similar to the haustoria of Peronosporaceae (Hawker *et al*., 1957). Also the intercellular irregular coenocytic hyphae develop longitudinally through the root, putting haustorium-like processes into the cortical cells in a manner similar to *Peronospora*. Similarly the VAM endophytes never colonise vascular tissues. In addition the irregular diameter of the intercellular hyphae and the morphology and development of vesicles and arbuscules of the VAM endophytes show a close affinity with the

Peronosporales which cause downy mildews (Hawker *et al*., 1957). However, Kemen and Jones (2012) reported that there are significant differences between arbuscular fungi and oomycetes with respect to the haustoria in intracellular growth. According to them, oomycete haustoria do not form secondary haustoria within the host cell comparable to arbuscules. This level of differentiation was beyond the scope of the current study but could be investigated by electron microscopy. Although microscopic observations of host-parasite relationships are important to understand disease progression in asymptomatic tissues, nucleic acid techniques (PCR, nucleic acid hybridization such as Fluorescent In Situ Hybridization (FISH), Restriction Fragment Length Polymorphism (RFLP)) and immunochemical techniques (enzyme linked immunosorbent assay (ELISA) using monoclonal antibodies) (Shcherbakova, 2007) are more applicable for confirmation of the systemic biotroph identity. However, a further study to assess the niche at dormancy and movement upon breaking of dormancy of *P. sparsa* in Chapter 6 will use both fluorescent microscopy and PCR on the same tissues to verify the microscopy observations.

CHAPTER 4

Environmental factors affecting expression and development of the disease, and flower and berry infections

4.1 INTRODUCTION

Peronospora sparsa infection of boysenberry can occur either through systemic or spore initiated infection (Tate, 1981; Shaw, 1982). Tate (1981) suggested that dryberry symptoms on the fruit primarily resulted from secondary infections of flowers and developing berries by spores that had been produced on sporulating lesions during wet warm weather from flowering onwards (Tate, 1981). He also reported that *P. sparsa* spores were observed on the protected pith surfaces in split boysenberries, inner calyx surfaces, and lower drupelets indicating spore initiated infections are likely. However, whether dryberries can also develop through systemic infection into the developing flowers and berry initials has not been determined. Dryberry disease in boysenberry and blackberry can occur even in the absence of foliar symptoms (Tate, 1981; McKeown, 1988; Breese *et al*., 1994; Lindqvist *et al*., 1998; Reboller-Alviter *et al*., 2012) on which the spores would then be produced. This suggests that systemic infection by the pathogen may occur and result in internal infection of berries and the subsequent development of dryberry disease. The variability in PCR results for the presence of *P. sparsa* in asymptomatic berries at two different maturities (green unripe and red partially ripe) in the previous chapter were inconclusive, thus, systemic infection of berry initials and/or spore infection at the red partially ripe stage may cause dryberries. However, this needs to be investigated further by inoculation of both flower and berry stages with the aim to determine the pathway(s) of berry/fruit infection.

Development of downy mildew disease is strongly affected by environmental conditions (Aegerter *et al*., 2003; Restrepo and Lee 2007; Caro, 2014). Restrepo and Lee (2007) observed that the incidence of downy mildew in greenhouse roses decreased as the length of time exposed to low RH (30-44%) increased during day time, whereas, the incidence increased when exposed to increasing time at higher RH (72-86%). However, apart from this study, most other studies have looked at the effect of environmental factors on infection, with limited work on disease expression. No studies have been conducted on boysenberry or other berry species to assess the effect of environmental factors on *P. sparsa* disease expression. Results on detached boysenberry plant material in Chapter 2 demonstrated that *P. sparsa* sporulation was favoured by warm temperatures and high relative humidity (RH). However, since these experiments were conducted on detached tissue verification on whether these conditions promote *P. sparsa* disease expression on systemically infected boysenberry plants is required to inform disease management strategies.

More detailed studies of the pathway/s of infection of flower and fruit which result in dryberry production and the environmental conditions which are conducive to infection and/or disease expression will enable better targeting of disease control strategies in the field. The overall objectives of this research were to determine (i) the environmental factors that affect disease expression of boysenberry, (ii) the effect of spore infection at flower or berry stages to cause dryberry disease, (iii) effect of temperature and RH on sporulation and infection of flowers and berries by *P. sparsa* under controlled conditions, and (iv) the pathway of flower infection and fruit infection to cause dryberry disease. To enable the role of systemic and spore initiated infections to be separated two fungicides, which have been reported in other studies (Walter *et al*., 2004; M. Walter, pers. comm., 2012) to prevent infection, were initially tested for ability to prevent *P. sparsa* spore germination.

4.2 MATERIALS AND METHODS

4.2.1 Effect of environmental conditions on expression and development of disease in systemically infected potted plants

Systemically infected 14 month-old boysenberry (cv. Mapua) plants, which were potted in clean 2.5 L pots containing 12-14 month potting mix (Appendix A. 2) were pruned to leave two or three floricanes in autumn, 2012. In early September, 20 plants were placed in both the shadehouse and the greenhouse, Lincoln University, with half (10) being placed directly in the greenhouse/shadehouse and half in humidity tents within the respective 'houses'. Humidity tents (140 x 80 x 140 cm) consisted of wooden frames, which were covered with polyethylene plastic sheet (Figure 4.1). To maintain high humidity, the bottom of the tents was completely covered with newspaper which was kept wet. In addition, 16 clean, plastic lids (18 cm diameter, 2.5 cm height) filled with water were placed in the bottom of each tent to maintain high RH. The tents were not completely sealed to allow air exchange and some escape of water vapour (Obanor, 2006) and also to allow access by pollinating insects.

Figure 4.1 Systemically infected boysenberry plants arranged in humidity tents at ambient conditions in the (A) greenhouse and (B) shadehouse, Lincoln University.

At weekly intervals, the plants were observed for development of characteristic *P. sparsa* symptoms. As soon as the plants were observed to have well-developed symptoms, one leaf was selected at random from a primocane and a floricane from each of the five plants. The leaves were washed separately by shaking them in 10 mL sterile water followed by centrifuging at 4696 *x g* for 10 min. Each pellet was resuspended in 1 mL water and the numbers of spores were counted using a haemocytometer. Tinytag® relative humidity (0-95%) and temperature (-40 – +75ºC) data loggers (Gemini Data Loggers, UK) were placed inside and outside the humidity tents in both houses to record RH and temperature for the duration of the experiment. This was to provide information on the environmental conditions which triggered disease expression in the systemically infected plants. The plants were allowed to develop fruit and disease development on fruit was also monitored for each treatment (Section 4.2.2.2). A fungicide (Rovral) was sprayed according to manufacturer's (Bayer CropScience, North Carolina) recommendations to close to run off on the greenhouse plants incubated under high humidity to control *B. cinerea* infection of unripe berries.

4.2.2 Investigation into the role of systemic and/or spore infection on dryberry production

4.2.2.1 Evaluation of a suitable protectant fungicide to control spore infection *in vitro*

In vitro studies were conducted to evaluate EC_{50} for the two fungicides mancozeb (Manco 75WG a.i. 750 g/Kg mancozeb) and metalaxyl-M (Apron®XL, a.i. 350 g/L metalaxyl-M) belonging to two different chemical families dithiocarbomate and phenylamide, respectively (Novachem manual, 2015). Selection was based on chemicals shown to be effective in the field according to Walter *et al*. (2004).

4.2.2.1.1. Effect of mancozeb and metalaxyl on spore germination

Experiment 1

Mancozeb and Metalaxyl-M were tested *in vitro* for their ability to inhibit the germination of *P. sparsa* spores. Stock solutions (10 mL) of 1500 µg a.i./mL were prepared in sterile water for each of the two fungicides and used to prepare four different concentrations which were twice the final concentrations required i.e. 150, 75, 15 and 3 µg a.i./mL. The recommended field rates for mancozeb and metalaxyl-M were 200 g (150 g a.i.), and 250 g (10 g a.i.) /100 L water, respectively. The field rate used for pure metalaxyl-M was the rate recommended for Ridomil Gold MZ WG (metalaxyl-M + mancozeb) in which one of the chemicals was metalaxyl-M (Chapter 5). However, that rate (250 g/100 L) contained 10 g a.i. metalaxyl-M + 160 g a.i. mancozeb /100 L. A sterile water control was included. *Peronospora sparsa* inoculum was prepared from spores produced on artificially inoculated boysenberry leaf discs (12 mm), after 14 days incubation at 15ºC on 1.5% water agar as described in Section 2.2.3. A *P. sparsa* spore suspension (10 mL) was prepared in sterile distilled water and adjusted to 2 x 10^4 spores/mL based on haemocytometer counts, and used within 1 h of preparation. The spore suspension (1 mL) was then mixed with 1 mL of each fungicide solution. For the control treatment (no fungicide) the spore suspension was mixed with an equal amount of sterile water. Six separate drops, each containing a 20 µL aliquot of fungicide/spore suspension (200 spores/20 µL) was used to inoculate six surface sterilised boysenberry (cv. Mapua) leaf discs (Section 2.2.3.2) placed on 1.5% WA. The inoculated leaf discs were then incubated at 20ºC under continuous darkness for 24 h. The experiment was arranged in a randomised block design in the incubator. Spore germination was assessed as described in Section 2.2.3.3 whereby a cellotape strip was used to pick up the spores from the droplet on the leaf surface, with this then mounted on a lacto-glycerol cotton blue drop placed on a microscope slide. The total number of spores and the number germinated were counted under a bright field microscope at x10 magnification and germination frequencies assessed. A spore was considered germinated if the length of the germ tube exceeded half the length of the spore. From the mean percent germination, relative to the no fungicide controls, the percent inhibition of germination was calculated for each fungicide/spore suspension. The EC_{50} values and 95% confidence intervals were calculated for each fungicide using probit analysis in the generalised linear model (GenStat Ver. 16).

Experiment 2

The experiment was repeated with the methods described in Experiment 1 modified slightly by making the *P. sparsa* spore suspension directly in each fungicide solution instead of making a spore suspension in sterile water. This was to avoid any potential germination in water prior to exposure to the fungicides. For each of the two fungicides, stock solutions (10 mL) of 750 µg a.i. /mL were prepared in sterile water for each of the two fungicides and used to prepare four different concentrations, 75, 37.5, 7.5 and 1.5 µg a.i. /mL. A sterile water control was included.

Peronospora sparsa inoculum was prepared as described in Experiment 1. To ensure sufficient spores were in each fungicide solution, three sporulating leaf discs selected at random were placed in each fungicide solution and shaken by hand to facilitate release of spores into the fungicide solution. From each suspension, a 20 µL aliquots was used to inoculate six replicate surface sterilised leaf discs (Section 2.2.3.2) placed on 1.5% WA and three clean microscopic slides placed on 1.5% WA, two 20 µL aliquots on each slide. The inoculated leaf discs and slides were incubated at 20ºC under continuous darkness for 24 h and 4 h, respectively. The experiments were arranged in a randomised block design in the incubator. Spore germination on the leaf discs was assessed as described for Experiment 1, whereas on microscope slides the spores in the spore droplets were directly stained on the slide with lactoglycerol cotton blue. The total number and the number of germinated spores were counted under x 10 magnification using a bright field microscope. From the mean percent germination, the percent inhibition of germination relative to the no fungicide controls was calculated for each fungicide concentration. The EC_{50} values and 95% confidence intervals were calculated for each fungicide using probit analysis in the generalised linear model (GenStat Ver. 16). In addition, spore length and width (µm), and the germ tube length (µm) of 10 germinated spores per replicate for each fungicide concentration selected at random were measured using the Olympus image analysis® software (Olympus soft imaging solutions, Germany) which was then statistically analysed by ANOVA in the general linear model (Minitab Ver. 16).

4.2.2.1.2. Effect of mancozeb and metalaxyl on Peronospora sparsa *infection* in vitro

From each of the spore/fungicide solutions prepared in Experiment 1, three replicate boysenberry leaf discs were inoculated with a 20 µL aliquot. These were then incubated on 1.5% WA at 15ºC under 12h/12h light/dark conditions for 14 days. The replicates were arranged in a randomised block design in the incubator. The presence or absence of sporulation (incidence) on each leaf disc was assessed after 14 days.

The experiment was repeated by inoculating six replicate leaf discs with the spore suspension prepared in experiment 2. The sporulation presence/absence data was analysed by generalized linear model with Bernoulli distribution in GenStat Ver.16. The significance of the parameter estimates in the accumulated analysis of deviance was used to analyse sporulation incidence differing in the two fungicides tested and concentrations.

4.2.2.2 Effect of spore infection at flower/berry stage on subsequent dryberry production

By early November 2012, the boysenberry plants used in Section 4.2.1 and incubated in the shadehouse were at the flower stage, whereas, in the greenhouse they were at the berry stage. In order to assess whether dryberry symptoms are caused by systemic or spore infection and whether it is due to infection at the flower or berry stage, the following modifications to the experiment were conducted.

4.2.2.2.1 Effect of spore infection at the flower stage (shadehouse plants)

When there were approximately equal numbers of flowers (around 25) on both the 10 boysenberry (cv. Mapua) plants incubated in the humidity tent and those at ambient RH (9th November, 2012), the plants were removed from the humidity tent. Once the king flower, which was the earliest blooming flower of the three or four flower buds in a bunch (Figure 4.2A and B), had opened, the open king flowers on all of the bunches (four to twelve flower bunches, each containing 1 king and 2 or 3 non-king buds per plant) on each of five plants, for both plants that were previously in the humidity tent and at those at ambient conditions, were sprayed with mancozeb at recommended field rate 200 g /100 L water (150 g a.i. /100 L water) in the late afternoon (Figure 4.2C). Non-king flowers were not sprayed. Spraying was done using a 2 L pressure sprayer bottle (Aqua Systems, New Zealand, Max. pressure 245 KPa, brass nozzle) to run off.

Figure 4.2 King and non-king flower bud stages on a flower lateral with (A) two non-king flower buds and (B) three non-king flower buds. (C) Flower lateral after the king flower was sprayed with mancozeb. King bud or king flower on each lateral are indicated by red arrows.

As the timing of the opening of the king flowers was variable, two, four and six days after the first spray the spraying was repeated to ensure that all king flowers were sprayed at least twice (Walter *et al*., 2004). The flowers were marked with a colour indicator plastic twine to distinguish the different spray dates. The remaining five plants from both the humidity tents and the ambient conditions were sprayed with sterile water as the untreated controls. Different colour identifiers were used to distinguish different plant treatments (high RH or ambient, sprayed or unsprayed) and also to identify the king from the two non-king stages of development on each bunch. The morning after the last fungicide spray, all the plants from both the high humidity and the ambient conditions were randomised and placed in the open air in the shadehouse. All plants were misted with water to facilitate spore landing/adherence for germination and the unsprayed (control) plants shaken to release spores from the sporulating lesions on leaves, stems and flowers. Misting and shaking of plants was repeated the following day. Each treated bunch was examined weekly for dryberry symptoms. The total number of berries and number of dryberries were assessed both at the green unripe stage and the red ripe

stage. Incidence data (proportion of dryberries per plant) was statistically analysed using general linear model in Minitab Ver. 16. The data was arcsine transformed prior to analysis to satisfy the assumption of homogeneity of variance. When factors were significant, means were compared between treatments using Tukeys HSD test at $P \leq 0.05$.

4.2.2.2.2 Effect of spore infection at the berry stage (greenhouse plants)

This experiment was conducted at the same time as the shadehouse experiment described in Section 4.2.2.2.1. The ten plants were removed from the humidity tents and placed with the other plants. For five plants that were previously in the humidity tents and five plants at ambient conditions all of the berry bunches (four to twelve whole berries bunches per plant) were sprayed with mancozeb at the same rate as in Section 4.2.2.2.1 [200 g /100 L water (150 g a.i. /100 L water)] in the afternoon and allowed to dry. Two days later the spraying was repeated. Colour identifiers were used to distinguish the berries that were at the green unripe and red partially ripe stages at the time of spraying (Figure 4.3). The remaining five plants from the high and ambient humidity treatments were sprayed with sterile water (untreated control). The plants were then moved to the shadehouse and placed next to boysenberry plants with sporulating lesions. The following morning, the plants from the different treatments were placed in a completely randomised design and the berries on each plant were misted and the adjacent infected sporulating plants were shaken to facilitate release of spores. This was repeated the following day. Each treated bunch was examined weekly for dryberry berry symptoms. The total number of berries and the number of dryberries on each replicate plant for both the green unripe and red partially ripe stages were assessed after 2-4 weeks. The data was arcsine transformed prior to being analysed by general linear model, with means compared between treatments using Tukeys HSD test at $P \leq 0.05$.

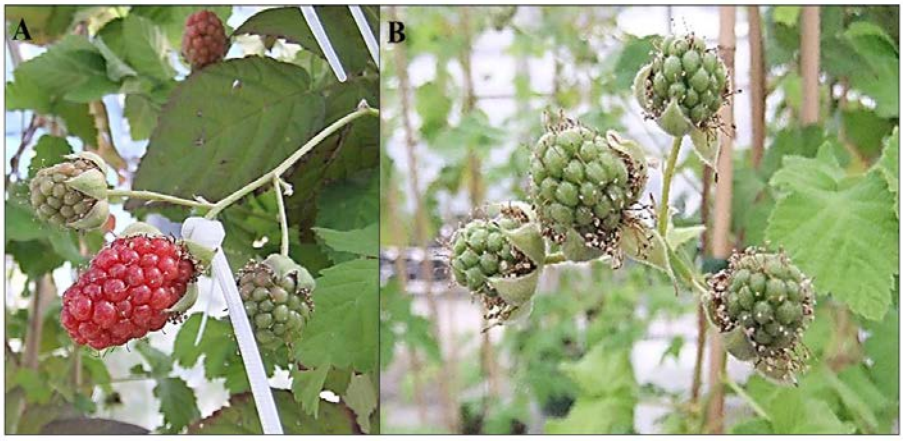

Figure 4.3 The two berry maturity stages (green unripe and partially ripe red) which developed on the systemically infected boysenberry (cv. Mapua) plants in the greenhouse prior to spraying with mancozeb (A) Partially ripe red stage was tagged with a white plastic identifier and (B) the green unripe stages were left untagged.

4.2.3 Effect of temperature and RH on disease expression in systemically infected plants

The experiment was set up to evaluate the effect of temperature and RH on expression of the disease under controlled conditions. Ninety six young (6 weeks old) systemically infected boysenberry (cv. Mapua) plants were propagated and potted in 7 cm diameter pots containing 3-4 month potting mix (Appendix C.1). Twenty four replicate plants were incubated at either 15 or 20ºC and at either 90 or 100% RH under full light conditions, 14 h light and 10 h dark (30 min ramp), in controlled environment growth rooms (PGV 36 with H319UV-B lighting system, floor area 1.37 x 2.45 m, lighting max. 960 μmols/m^2/s) at Lincoln University. The 100% relative humidity was achieved by placing the plants in humidity tents (Section 4.2.1) and the ambient RH by setting each chamber to 90%. The plants were watered daily as required. The experiment was set up in mid-October 2012. Tinytag® relative humidity (0-95%) and temperature (-40 – +75ºC) data loggers (Gemini Data Loggers, UK) were placed in the growth cabinets to record RH and temperature during the experiment. At weekly intervals, the plants were observed for two months for development of characteristic *P. sparsa* leaf and stem lesions. After this time the plants were potted into 2.5 L clean pots containing 12-14 month potting mix (Appendix A.2) and moved into the shadehouse (Lincoln University). The plants at 100% RH (48 plants) were placed in a clean humidity tent (140 x 80 x 140 cm as described in Section 4.2.1) with the remaining 48 plants that were at 90% RH placed at ambient conditions in the shadehouse. These were further monitored for development of characteristic *P. sparsa* leaf and stem lesions at weekly intervals for 2.5 months.

4.2.4 Infection pathway for flowers and berries

4.2.4.1 Development of flower infection on inoculated excised floricane laterals

4.2.4.1.1. Effect of temperature and RH on infection and sporulation

A modified method of Walter *et al*. (2005) was used. Excised floricanes laterals (approximately 60 cm) at tight bud stage were collected (8 October 2012) from asymptomatic but potentially infected boysenberry (cv. Mapua) plants from a growers property (Lower Moutere). The floricanes were collected from an unsprayed row where spore trap slides were placed in 2011 (Section 2.2.1). The floricanes were transported to Lincoln University in buckets containing clean water that had been covered with plastic bags to avoid drying. Once the main king flower had opened on the bunch, any dried and symptomatic leaves and other flower/bud bunches were removed leaving only one flower bunch per lateral. A flower bunch included the king flower and 2-3 non-king buds. The base of each lateral was inserted into an oasis (Magic floral foam, FOAM24, New Zealand) block (7.5 x 7.5 x 10.5 cm) which had been saturated with half strength Hoagland solution (Appendix C.2) and placed on a clean plastic tray (82 x 70 x 6 cm) filled with half strength Hoagland solution to allow the laterals to absorb nutrients as required for further development.

The floricane laterals were then placed in growth rooms at two temperatures (15 and 20ºC) and two relative humidities (90% and 100% RH). Thirty six replicate flower laterals were placed in each temperature and relative humidity treatment. To obtain 100% RH, the laterals were placed in a humidity tent as described in Section 4.2.1, with the main chambers set at 90% RH. Laterals were continuously monitored, and once the king flower had fully opened the open flowers were pollinated using a clean paint brush (Jasart White Taklon Round Brushes, China). Each of the opened flowers was pollinated twice on the same day. After the repeated pollination, the opened king flower and two non-king buds were each inoculated twice, with a 50 µL drop of freshly prepared spore suspension containing approx. 200 spores each time. This was determined to be the optimum spore number for infection in Section 2.3.4.2. The two spore inoculation droplets were applied to different areas of the flower/bud tissue, and each flower or bud was inoculated again the following day to ensure infection. Once inoculated, each lateral was carefully covered with a plastic bag which was supported by a wooden stick to avoid collapse of the plastic bag onto the inoculated lateral. Flowers on the untreated control floricanes (18 replicates per incubation condition) were inoculated with 50 µL sterile water. For each treatment 18 replicates were set up with each replicate having all development stages; similar development stages were tagged with the same colour identifier. The control and inoculated floricanes were arranged in a randomised block design in each of the four incubation conditions (15 or 20ºC at 90% or 100% RH). The initial aim of the experiment was to determine the relative role of spore inoculation of flowers and systemic infection on the production of dryberry, however no berries were produced on the detached floricane laterals and no visible infection of the flower/buds were observed. Therefore, asymptomatic infection of different plant tissues, including buds, petioles and sepals or styles was observed by fluorescent microscopy after 6 weeks.

4.2.4.1.2. Effect of incubation conditions on development of dryberry symptoms from inoculated bud or flower (shadehouse laterals)

i. High RH (100% RH) under ambient temperature

The experiment in Section 4.2.3.1.1 was repeated using excised floricane laterals (approximately 60 cm long) at the tight bud stage collected (mid-November 2012) from 16 months old asymptomatically infected boysenberry (cv. Mapua) plants grown in the shadehouse (Lincoln University). Of the 36 replicate flower laterals, half were inoculated with a *P. sparsa* spore suspension once the king flower had opened. Inoculation of the opened king flower and two non-king buds were conducted as described in Section 4.2.4.1.1. Inoculation was carried out twice on two consecutive days. The remaining flower laterals (18 replicates) were inoculated in the same way but using sterile water. The floricanes were then arranged in a randomised complete design in a humidity tent to maintain high RH and incubated on a bench at ambient temperature.

The experiment was conducted for 6 weeks and the total number of berries and the number of berries with dryberry symptoms were assessed. The number of dryberries (total, king and non-king) was analysed using the generalized linear model in GenStat Ver.16. A binomial distribution was assumed with logit link function to test for the main effects. The significance of the parameter estimates in the accumulated analysis of deviance was used to analyse effect of inoculation on dryberry incidence. Average daily maximum and minimum temperatures were obtained from the closest weather station to Lincoln University (Lincoln, Broadfield Ews NIWA weather station) for the duration of the experiment.

ii. High RH (100% RH) and optimum temperature (at 15ºC) under growth lights

The experiment was repeated using 36 replicate floricane laterals (approximately 60 cm long) collected at the tight bud stage at the same time as for the previous experiment (mid-November 2012) from the shadehouse (Lincoln University) from the same boysenberry plants used in Section 4.2.4.1.2i. Laterals were arranged as described in Section 4.2.4.1.2i with the base inserted in a piece of oases placed in half strength Hoagland solution on a clean plastic tray (82 x 70 x 6 cm). These were then incubated in an incubator at 15ºC under growth lights (cool white fluorescent tubes) set at 14 h light/ 10 h dark and high RH for seven weeks. The number of flowers with *P. sparsa* sporulation from the total number flowers per lateral (incidence) two weeks after inoculation and the development of berry initials after a further five weeks were assessed. The incidence of *P. sparsa* sporulation on the flowers was statistically analysed by generalized linear model in GenStat Ver.16. A binomial distribution was assumed with logit link function to test for the main effects. The significance of the parameter estimates in the accumulated analysis of deviance was used to analyse effect of inoculation on sporulation incidence.

4.2.4.2 Development of berry infection on excised field sourced fruiting laterals

Berries at the green, unripe hard stage

This experiment was set up in early December using fruiting laterals collected from asymptomatically infected boysenberry (cv. Mapua) plants from the same grower property (Lower Moutere) from which the floricane laterals were collected (Section 4.2.3.1). The laterals were transported to Lincoln University in buckets containing clean water and covered with a lid to avoid drying. Each lateral had one king berry and two non-king berries. Each lateral was washed with sterile water for 5 min, allowed to dry and the base inserted into a piece of oases saturated with half strength Hoagland solution in a clean plastic tray (82 x 70 x 6 cm). A *P. sparsa* spore suspension was prepared as described in Section 4.2.4.1.1 and four concentrations (1000, 2500, 10000 and 25000 spores/mL) were prepared based on haemocytometer counts and used within 1 h of preparation. The calyx of all three berries for five replicate laterals were inoculated with a 20 µL aliquot for each of the spore concentrations, to result in a final inoculum concentration of 20, 50, 200 or 500 spores per berry. For an additional four replicate

laterals, the drupelets of all three berries were inoculated with a 20 µL aliquot for each of the spore concentrations. Untreated controls were set up, with these being inoculated with sterile water. Laterals were marked by different colour identifiers to distinguish each treatment and arranged in a randomised block design. After inoculation each fruiting lateral was covered with a clean plastic bag for 24 h to provide high humidity to facilitate infection. The laterals were then incubated at 100% RH at ambient temperature for 24 days after which the development of dryberry symptoms was assessed. The incidence of dryberries was analysed using generalized linear model in GenStat Ver.16. A binomial distribution was assumed with logit link function to test for the main effects. The significance of the parameter estimates in the accumulated analysis of deviance was used to analyse the effect of different spore numbers and region of inoculation on dryberry incidence.

Berries at the red, partially ripe stage

The experiment for the unripe berry stage was repeated using red, partially ripe fruiting laterals (Figure 4.4) collected from the same grower property in late December, 2012.

Figure 4.4 Red, partially ripe boysenberry fruiting laterals arranged on oases saturated with half strength Hoagland solution in a clean plastic tray and incubated at 100% RH at ambient temperature. Fruiting laterals were supported using clean plastic straws.

4.2.4.3 Effect of inoculation of berries at different stages and regions on dryberry production

Detached berries from the shadehouse

Berries were detached with 2 cm stem at two maturity stages, green unripe hard and red partially ripe (48 berries each stage), in late December from 7 weeks old boysenberry (cv. Mapua) plants grown in the shadehouse at Lincoln University. The stem attached to each berry was inserted into a piece (4 x 4 x 2 cm) of OASIS® floral foam which was saturated with sterile water. These were then placed on a clean plastic rack inside a plastic container (32 cm x 26 cm x 8 cm), containing saturated moist paper tissues to ensure high RH and then a plastic bag

placed over the top. Twenty four berries at each maturity stage were inoculated with 20 µL of *P. sparsa* spore suspension (200 spores), half the berries (12 berries) were inoculated on the calyx and the other half on the drupelets. Twenty four berries at the same maturity stage were inoculated in the same way with sterile water as the control treatments. The berries were arranged in a randomised block design in an incubator with four containers, each with three replicates for each treatment. The berries were incubated at 15ºC under growth lights (cool white fluorescent tubes) set at 14 h light/ 10 h dark for 25 days. After 14 days, the incidence (presence or absence) of sporulation and development of dryberry symptoms were assessed and after a further 11 days 10 berries selected at random from each treatment were dissected, samples pressed onto the surface of clean microscope slides and stained with lactoglycerol cotton blue and observed under a bright field microscope for presence of oospores. The sporulation and dryberry incidence data were analysed by generalized linear model with Bernoulli distribution in GenStat Ver.16. A Bernoulli distribution defined as a binary variable was assumed with logit link function to test for the main effects and interactions. The significance of the parameter estimates in the accumulated analysis of deviance was used to analyse effect of inoculation, berry maturity and region of inoculation on sporulation incidence differing in the inoculated/control, unripe green/ripe and calyx/drupelet inoculation.

Detached berries from the field

Berries at both green unripe and red partially ripe stages (24 replicates each) were collected from the same grower property as described in Section 4.2.4.2. The berries were bisected and one half placed on a clean Petri dish lid. These were placed on a plastic rack in a plastic container (32 cm x 26 cm x 8 cm) containing either 300 mL of saturated potassium nitrate (KNO_3; 551.67 g/L) salt solution or water to produce 90-95% or 100% RH, respectively (Section 2.2.2.1). For each berry maturity stage and relative humidity, six berry halves were inoculated with a 20 µL aliquot of a 1 x 10^4 spore/mL freshly prepared *P. sparsa* spore suspension (200 spores/berry), and six berry halves were inoculated with sterile water (20 µL aliquot) as the untreated control. The berries were arranged in a completely randomised design with 24 replicate berries per maturity and inoculation treatment in each container and incubated at 15ºC. After 10 days the incidence (presence/absence) of sporulation and dryberry symptoms were assessed. No statistical analysis was carried out since; all of the berries were contaminated with *Botrytis cinerea*.

4.3 RESULTS

4.3.1 Effect of environmental conditions on expression and development of disease in systemically infected potted plants

Greenhouse plants developed faster than the shadehouse plants; therefore, direct comparison of different tissue types was not possible. Flowers were first observed on the 1 October, 2012 for the greenhouse plants and the 28 October in the shadehouse, with berries first seen on the 20 October, 2012 in the greenhouse and the 21 November, 2012 in the shadehouse.

At ambient RH, characteristic systemic *P. sparsa* lesions were only observed on two leaves on the same floricane shoot of one greenhouse plant (of the 10) on the 5 October. These leaves were removed and incubated at high humidity on 1.5% WA at 15°C to induce sporulation, however, no sporulation developed after 7 or 14 days incubation. No symptoms were observed on any of the other plants incubated under high humidity in the greenhouse.

For the shadehouse plants, a few symptomatic leaves (on floricanes) were observed on three plants incubated in ambient RH conditions on the 10 October, 2012. In contrast, disease expression consisting of both systemic symptoms and sporulation were observed on all of the plants incubated under high humidity. For these plants, symptoms and sporulation was observed on the leaves and young petioles. Symptoms were observed on both floricane and primocane leaves from plants incubated in the shadehouse under high humidity, with sporulation levels being 1.92×10^4 and 0.86×10^4 spores per leaf, respectively. Spore production on floricane leaves from shadehouse plants under ambient conditions was 0.12×10^4 spores per leaf. No sporulation was observed on the leaves detached from primocanes from shadehouse plants incubated under ambient conditions.

Six weeks after the start of the experiment (31 October, 2012) symptoms were observed on primocane leaves on all 10 plants grown under high humidity in the greenhouse. Spore production on primocane leaves from greenhouse plants incubated under high humidity was 0.14×10^4 spores per leaf. No sporulation was observed on the leaves detached from, floricanes from greenhouse plants under high humidity and both floricanes and primocanes from greenhouse plants incubated under ambient condition. The data was not statistically analysed due to the large number of zero values.

For boysenberry plants incubated at high humidity in the shadehouse, symptoms and abundant *P. sparsa* sporulation was observed both on leaves and young petioles. The sporulation on the petioles was mainly on the mid portions (~mid 4 cm of the ~7 cm long petiole). Sporulation remained for around 5 to 7 days and after 7 days the sporulated region turned a light purplish colouration. After 10 days, the sporulated region was a red-purple colouration, similar in colour to the characteristic colour of the leaf lesions (Figure 4.5). The lower side of the petiole remained a normal green colour. Finally the red-purple colouration spread along the entire

petiole. When sporulation was observed on the mid region of the petiole, the edge that connects the petiole to the stem also appeared pale red in colour.

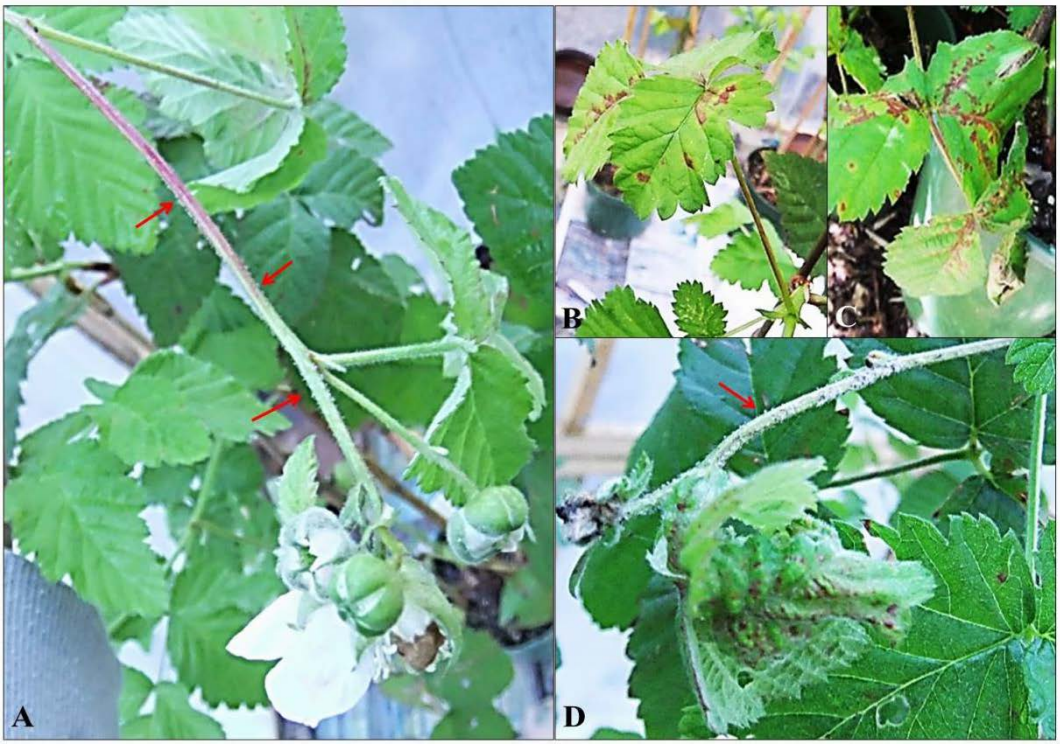

Figure 4.5 Red-purple colouration of stem symptoms characteristic of *Peronospora sparsa* developing after sporulation on (A) a flower stem lateral, (B) leaf petiole, (C) stem in between leaflets and (D) abundant sporulation on a young stem of systemically infected boysenberry plants incubated at high relative humidity in the shadehouse (Lincoln University). Sporulating regions on the stems are indicated by the red arrows.

No wilting of the affected tissue was observed. *Peronospora sparsa* sporulation was observed on both the surfaces of flower petals, stamens and filaments, and on the outside of the calyx by mid-late October for shadehouse plants (Figure 4.6A, D, E, F, G, H, L). In most cases, *P. sparsa* sporulation was seen on flowers where at the bud stage one petal was pale pink in colour indicating infection (Figure 4.6B). Three to five days after sporulation had finished, the flower petals were brown and appeared dead (Figure 4.6I, J and K).

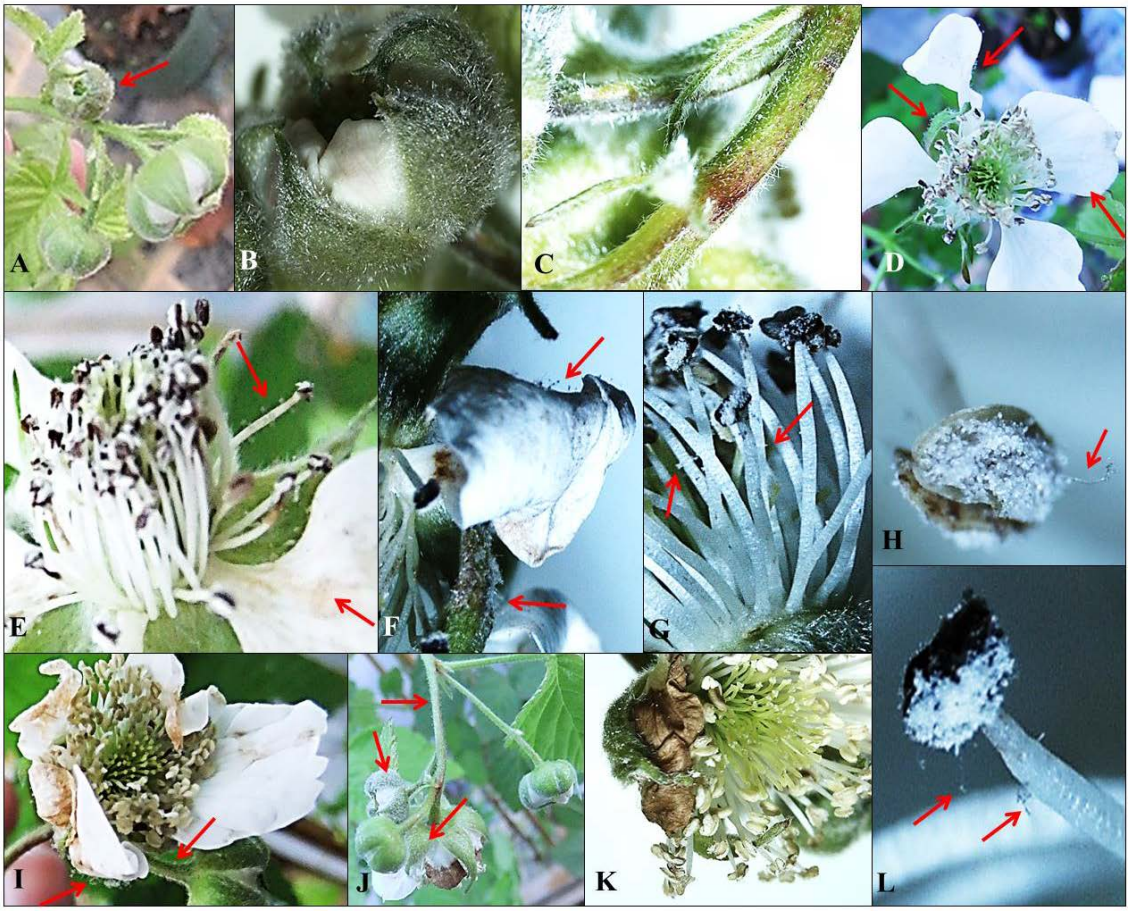

Figure 4.6 *Peronospora sparsa* sporulation on (A) flower calyx at bud stage, (B) petal appearing pale pink in colour on which sporulation observed to develop at the blooming stage, (C) sporulation on flower stem remained red purple in colour, (D, E, F and I) sporulation on petals, (E, G and L) sporulation on stamen filament and (H) pollen grains. (I, J, and K) Sporulated petals starting to brown and appeared dead. The sporulating areas are indicated by the red arrows.

When abundant sporulation was observed on the leaves of the laterals, sporulation was also observed on the adjacent stem lateral (Figure 4.6A and J). Out of the six petioles on each lateral, symptoms and sporulation was observed on the leaves of five of the petioles. Only the top-most, youngest leaves and supporting petiole of each lateral (side shoot) consistently remained without symptoms/sporulation. Expression of symptoms and/or sporulation was not restricted to a particular leaf age as symptoms/sporulation was observed on very young small leaves (around 7 mm leaf length) to larger leaves (50 mm leaf length). A higher proportion of middle aged leaves (35-40 mm leaf length) were symptomatic and/or had *P. sparsa* sporulation compared with the other leaf age/sizes. Lesions typical of both spore infection and systemic infection were observed. Old, hardened leaves or canes did not show symptoms or sporulation, however, old canes were also red in appearance. However, these symptoms and sporulation pattern were only observed from the shadehouse plants at high humidity and none was to be observed in other treatments.

Hyphae with haustoria characteristic of *P. sparsa* were observed on the cleared petals when observed microscopically (Figure 4.7A and B). Spores characteristic of *P. sparsa* were observed on the stamen samples along with pollen grains which were larger in size and stained blue (Figure 4.7C).

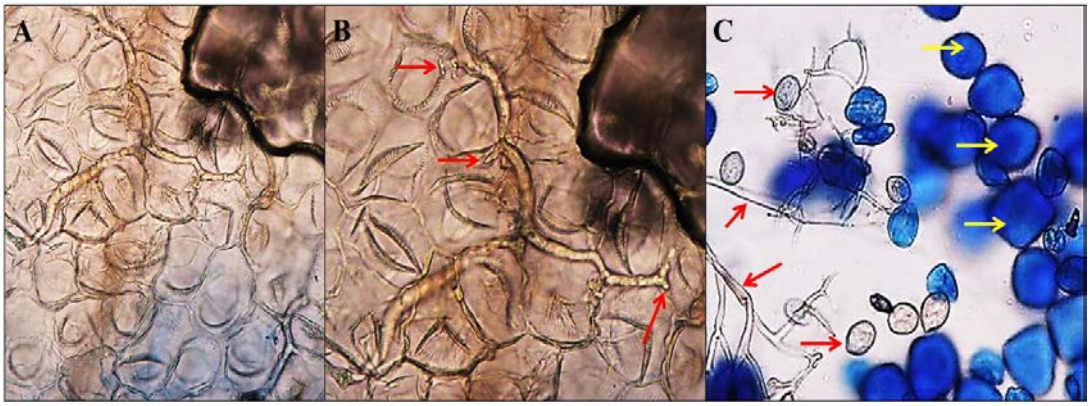

Figure 4.7 *Peronospora sparsa* (A and B) mycelium with haustoria (B, indicated by the red arrows) observed in sporulated boysenberry petal after clearing , (C) spores and sporangiophore (red arrows) with pollen grains (yellow arrows) stained blue and larger than spores.

The mean %RH in the humidity tents in both houses were always above 80%, although the levels were closer to 100% in the shadehouse (Figure 4.8). The mean temperature in the humidity tent in the shadehouse fluctuated between 5 - 15ºC, however in the greenhouse it fluctuated between 15 - 25ºC. Under ambient conditions in both houses temperature fluctuated between of 5 - 15ºC, with the %RH in the shadehouse being 60 - 100% whilst it was between 40 - 80% in the greenhouse (Figure 4.8).

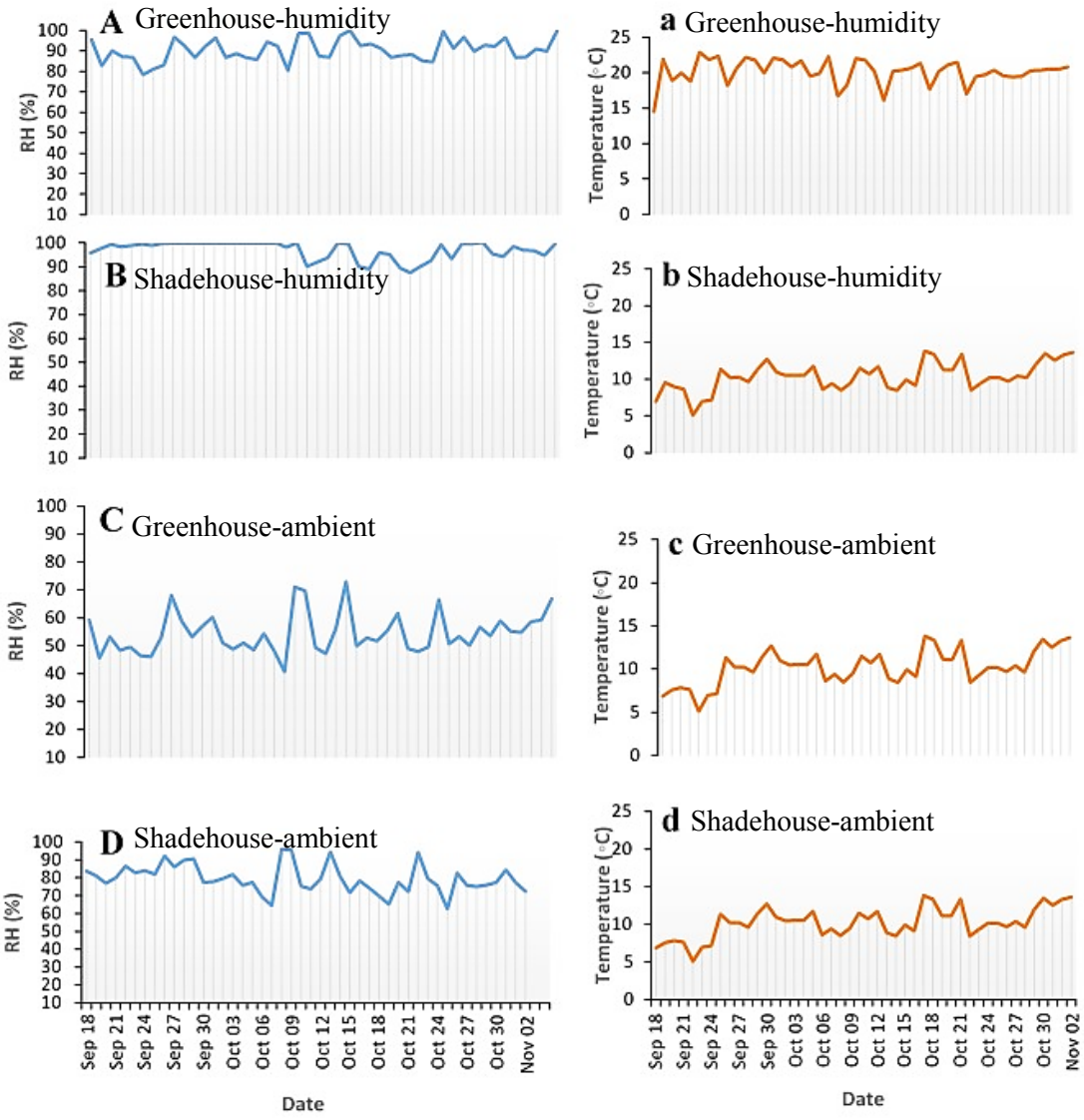

Figure 4.8 Mean % relative humidity (RH) and temperature data recorded by the Tinytag® relative humidity (0-95%) and temperature (-40 – +75ºC) data loggers (Gemini Data Loggers, UK) placed in (A and a) the greenhouse humidity tent, (B and b) shadehouse humidity tent, (C and c) greenhouse ambient conditions and (D and d) shadehouse-ambient conditions at Lincoln University.

4.3.2 Investigation into the role of systemic and/or spore infection on dryberry production

4.3.2.1 Evaluation of a suitable protectant fungicide to control spore infection *in vitro*

4.3.2.1.1. Effect of mancozeb and metalaxyl on spore germination

The EC_{50} values of the two fungicides tested differed in the two experiments (Table 4.1). In experiment 1 where the spore suspensions were prepared in sterile water prior to adding to the fungicide solutions, the EC_{50} values for the effect of mancozeb and metalaxyl on spore germination on leaf discs after 24 h were 29.10 and 24.60 µg a.i./mL, respectively. In contrast, in experiment 2 where the spore suspensions were prepared in the fungicide solutions the EC_{50}

values for spore germination on leaf discs after 24 h was 0.04 and 5.22 µg a.i./mL, respectively for mancozeb and metalaxyl. Spore germination after 4 h on glass slide was extremely low being only 12% for the untreated control (Appendix C.3a) compared with 61% and 60% on leaf discs after 24 h in experiment 1 and 2, respectively.

Table 4.1 The mean EC_{50} (mg a.i./L) values for mancozeb and metalaxyl on spore germination of *Peronospora sparsa* determined either on leaf discs after 24 hours or microscope slides after 4 hours incubation, assessed in two experiments.

Fungicide	Activity	EC_{50} values (µg/mL)		
		Experiment 1	*Experiment 2*	
		On leaf disc (24 h)	On microscopic slide (4 h)[b]	On leaf disc (24 h)
Mancozeb	Protectant	29.10 (17.85-47.07)[a]	4.44×10^{9}	0.04 (0.01-0.11)
Metalaxyl	Systemic	24.60 (15.00-39.73)	2.91×10^{12}	5.22 (3.02-8.31)

[a]The confidence intervals for each fungicide is provided in brackets.
[b]spore germination on glass slide was low, being 12% for the untreated control.

In experiment 2, *P. sparsa* germ tube length after 24 h incubation on leaf discs was significantly affected by fungicide (P=0.006), concentration (P<0.001) and interaction between fungicide and concentration (P=0.034) (Appendix C.4a). The germ tubes were significantly (P<0.05) shorter in the mancozeb treatment (77.8 µm) compared with in the metalaxyl treatment (109.1 µm) (Table 4.2). Across both fungicides, the germ tubes were significantly (P<0.05) shorter for *P. sparsa* spores treated with fungicide concentrations of 1-1000 µg/mL compared with 0 µg/mL (control) (Figure 4.9). The mean germ tube length of the spores of the untreated control after 24 h was 329.6 µm. No germination was recorded for *P. sparsa* spores treated with mancozeb at 50-1000 µg/mL and metalaxyl at 1000 µg/mL (Table 4.2; Appendix C.4a).

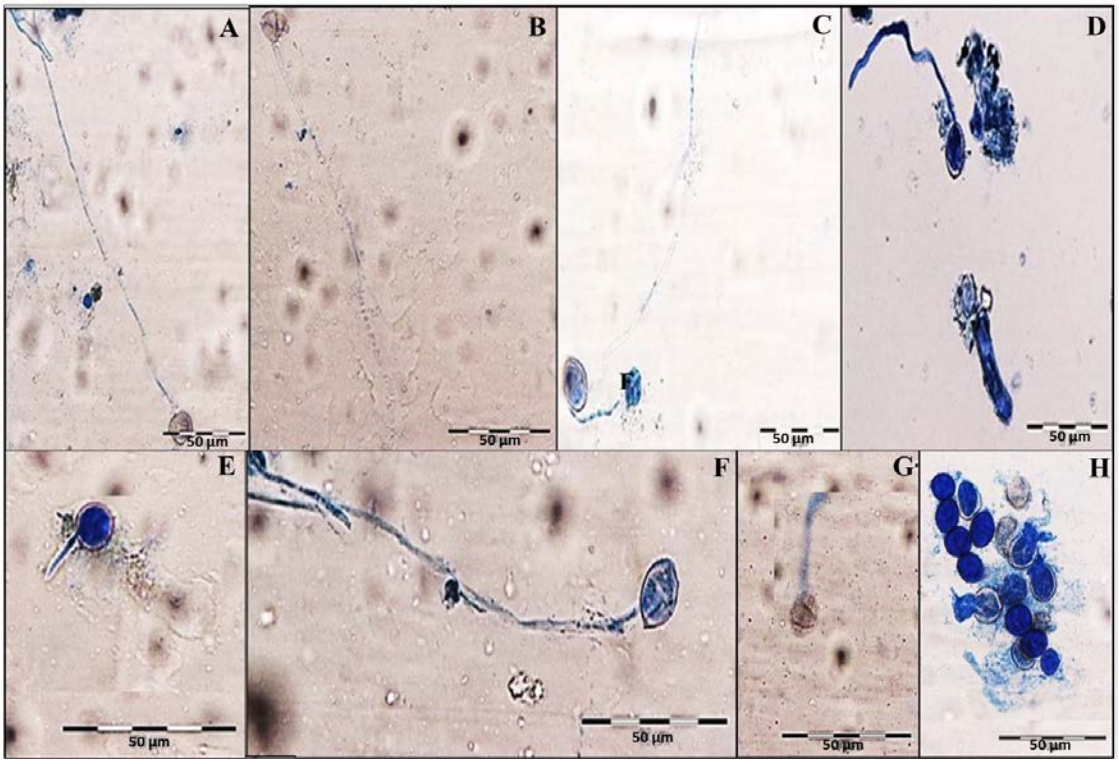

Figure 4.9 Germination and germ tube growth of *Peronospora sparsa* spores exposed to different concentrations of metalaxyl or mancozeb on boysenberry leaf discs incubated at 20ºC total darkness for 24 h. Germinated spores in the (A) untreated control, (B) metalaxyl at 1 µg/mL, (C) 10 µg/mL, (D) 50 µg/mL and (E) 100 µg/mL, and mancozeb at (F) 1 µg/mL and (G) 10 µg/mL. Non-germinated spores exposed to (H) mancozeb at 1000 µg/mL.

Table 4.2 Effect of different concentration of mancozeb and metaxyl on *Peronospora sparsa* spore germ tube length (µm) on boysenberry leaf discs after 24 h under total darkness at 20ºC.

Concentration (µg/mL)	Mean germ tube length (µm) after 24 h on leaf disc		
	Mancozeb	Metalaxyl	Mean concentration effect
0	320.8 A[1]	338.3 A	329.6 a[1]
1	145.9 B	141.7 BC	143.8 b
10	0.3 D	104.6 BC	52.4 c
50	0.0 D	55.3 CD	27.7 c
100	0.0 D	14.5 D	7.2 c
1000	0.0 D	0.0 D	0.0 c
Mean fungicide effect	77.8 x[1]	109.1 y	

[1]Values within the rows and columns followed by the same letter are not significantly different according to Tukey's test at $P = 0.05$. There was a significant effect of fungicide treatment (x-y; P=0.011), concentration (a-c; P<0.001) and interaction between fungicide and concentration (A-C; P=0.050) on mean germ tube length.

There was no significant effect of fungicide (P=0.320), concentration (P=0.938) or the interaction between fungicide and concentration (P=0.932) on the size (length:width ratio) of germinated *P. sparsa* spores after incubation on leaf discs at 20ºC in darkness (Appendix C.4b). In the untreated control the mean spore length was 19.34 µm and mean spore width was 17.42 µm. Across all concentrations of mancozeb the mean spore length was 19.11-19.80 µm, whereas the mean spore width was 17.31-18.06 µm. Similarly, for metalaxyl the spore length was 18.79-19.31 µm and width 16.90-17.38 µm. However, as the spores in most of the mancozeb treatments did not germinate (Figure 4.10H), for these treatments the length and

width of ungerminated spores were measured. The germ tube length or spore size (length and width) of the spores incubated on microscopic slides for 4 h were not assessed due to the low numbers of germinated spores (Appendix C.3).

4.3.2.1.2 Effect of mancozeb and metalaxyl on Peronospora sparsa *infection* in vitro

There was a significant effect of fungicide concentration on the incidence of *P. sparsa* infection in both experiments ($P<0.001$ and $P<0.001$, respectively). In the untreated control for both experiments, lesions developed on all leaf discs inoculated with *P. sparsa*, with incidence being 0.17 and 0.67, and 0.67 and 0.33 for metalaxyl and mancozeb at 1 µg/mL in experiments 1 and 2, respectively. No infection developed on leaf discs inoculated with *P. sparsa* spores and either fungicide at concentrations of 10 to 1000 µg/mL. There was no significant effect of the fungicide ($P=0.067$ and $P=0.111$ for experiment 1 and 2, respectively), or the interaction between fungicide and concentration ($P=0.138$ and $P=0.317$ for experiment 1 and 2, respectively) on incidence of infection in either experiment. Full statistical analysis is presented in the Appendix C.5.

Mancozeb was more effective at reducing spore germination indicated by the lower EC_50 values and at reducing the length of germtubes compared with metalaxyl and was therefore used in the subsequent experiments to protect flowers/berries from spore infections.

4.3.2.2 Effect of spore infection at flower/berry stage on subsequent dryberry production

4.3.2.2.1 Effect of spore infection at the flower stage (shadehouse plants)

There was a significantly higher proportion of berries which subsequently developed dryberry symptoms for both king ($P=0.049$, Appendix C.6a) and non-king ($P=0.020$) flower stages on plants which had previously been incubated at high humidity (0.68 and 0.58, respectively) compared with those which had previously been at ambient relative humidity (0.33 and 0.24, respectively). However, there was no significant effect of the mancozeb spray ($P=0.154$ and $P=0.082$) or interaction between mancozeb spray and incubated condition (high humidity or ambient) ($P=0.437$ and $P=0.509$) on dryberry production from both king and non-king flower stages, respectively.

4.3.2.2.2 Effect of spore infection at the berry stage (greenhouse plants)

The proportion of dryberries was significantly higher for both berries inoculated at the green ($P=0.004$, Appendix C.6b) and red ($P=0.015$) immature berry stages for plants which had previously been incubated under high humidity (0.46 and 0.31, respectively) compared with ambient relative humidity (0.05 and 0.00, respectively). There was no significant effect of the mancozeb spray ($P=0.261$ and $P=0.271$) or interaction between mancozeb spray and incubation condition (high humidity or ambient) ($P=0.61$ and $P=0.271$) on dryberry production for the green or red immature berry stages, respectively.

Overall across both experiments (Section 4.3.2.2.1 and 4.3.2.2.2), the dryberry symptoms observed were non-glossy, dull looking, prematurely reddened, hardened and dried drupelets (Figure 4.10). For dryberries which developed on plants previously incubated in humidity tents in the shadehouse whole berries were dried (Figure 4.10A and D) with abundant sporulation observed at the flower stage. In contrast, for dryberries which developed on plants previously incubated in the humidity tent in the greenhouse (Section 4.3.2.2.2), only a few drupelets were dried (Figure 4.10). There was no or very few drupelets were dried on berries of plants in greenhouse whereas, very few drupelets on berries of plants in shadehouse incubated at ambient conditions.

Figure 4.10 Dryberry symptoms observed on berries produced on boysenberry plants previously incubated in a humidity tent in the (A, B, C and D) shadehouse showing severe dryberry symptoms where all the drupelets per berry were dried and almost all of the berries per lateral showing symptoms, and (E and F) greenhouse showing mild dryberry symptoms where only a few drupelets per berry were dried (indicated by the black arrows) and not all berries per lateral showing dryberry symptoms.

4.3.3 Effect of temperature and RH on disease expression in systemically infected plants

None of the plants incubated at either 15 or 20ºC under 90 or 100% RH expressed characteristic *P. sparsa* leaf symptoms during the 8 weeks incubation in the controlled environment growth rooms. Healthy growth including development of new green leaves and canes was observed on all of the plants in all the treatments (Figure 4.11A). All of the plants re-potted and moved into the shadehouse under high RH expressed characteristic *P. sparsa* systemic leaf lesions in the autumn (10 weeks after moving into the shadehouse) (Figure 4.11B). However, no lesions were observed on the plants incubated under ambient conditions in the shadehouse.

Figure 4.11 Systemically infected boysenberry (cv. Mapua) plants (A) in the growth room after eight weeks with no development of characteristic *Peronospora sparsa* leaf symptoms and (B) the same plants expressing characteristic *P. sparsa* symptoms 10 weeks after moving into a humidity tent in the shadehouse.

4.3.4 Infection pathway for flowers and berries

4.3.4.1 Development of flower infection on inoculated excised floricane laterals

4.3.4.1.1. Effect of temperature and RH on infection and sporulation

The majority of the field laterals became dried and almost all of the laterals incubated at 15 and 20ºC at 90% RH died (Figure 4.12C). At 100% RH, more of the laterals at both temperatures survived (77.8% and 22.2% at 15 ºC and 20ºC, respectively) however, no sporulation or berry initials were produced (Figures 4.12A and B).

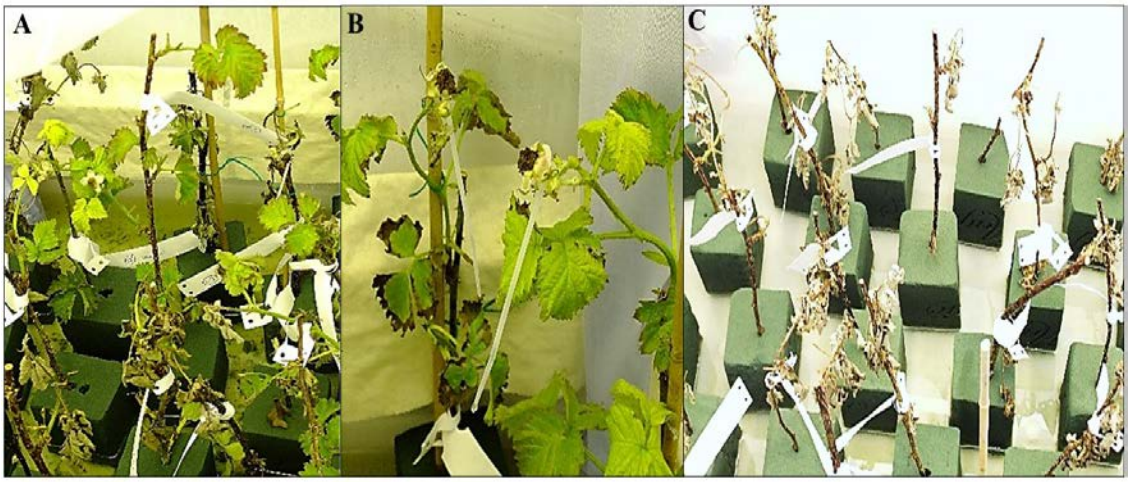

Figure 4.12 Floricane laterals collected from *Peronospora sparsa* systemically infected boysenberry (cv. Mapua) plants, inoculated and incubated in the growth room showing surviving laterals incubated at 100% RH either (A) 15 or (B) 20°C, and (C) dried laterals at 90% RH at 15°C.

Fluorescent microscopic observations of calyx, petal, carpel, stamen and stem samples from flowers sampled at random from the inoculated laterals incubated at 15ºC at 100% RH showed a few germinating spores with hyphae characteristic of *P. sparsa* on calyx (Figure 4.13A) and carpel samples (Figure 4.13B). Hyphae characteristic of *P. sparsa* were observed on all calyx samples (Figure 4.13C) and in some of the trichomes associated with the calyx (Figure 4.13D) in the uninoculated control lateral samples which had been incubated under the same conditions. In addition, hyphae characteristic of *P. sparsa* were only observed on calyx samples from both control (Figure 4.13E) and inoculated (Figure 4.13F) laterals incubated at 20ºC at 100% RH. No hyphae were observed on trichomes of calyx samples from uninoculated control laterals incubated at 20ºC.

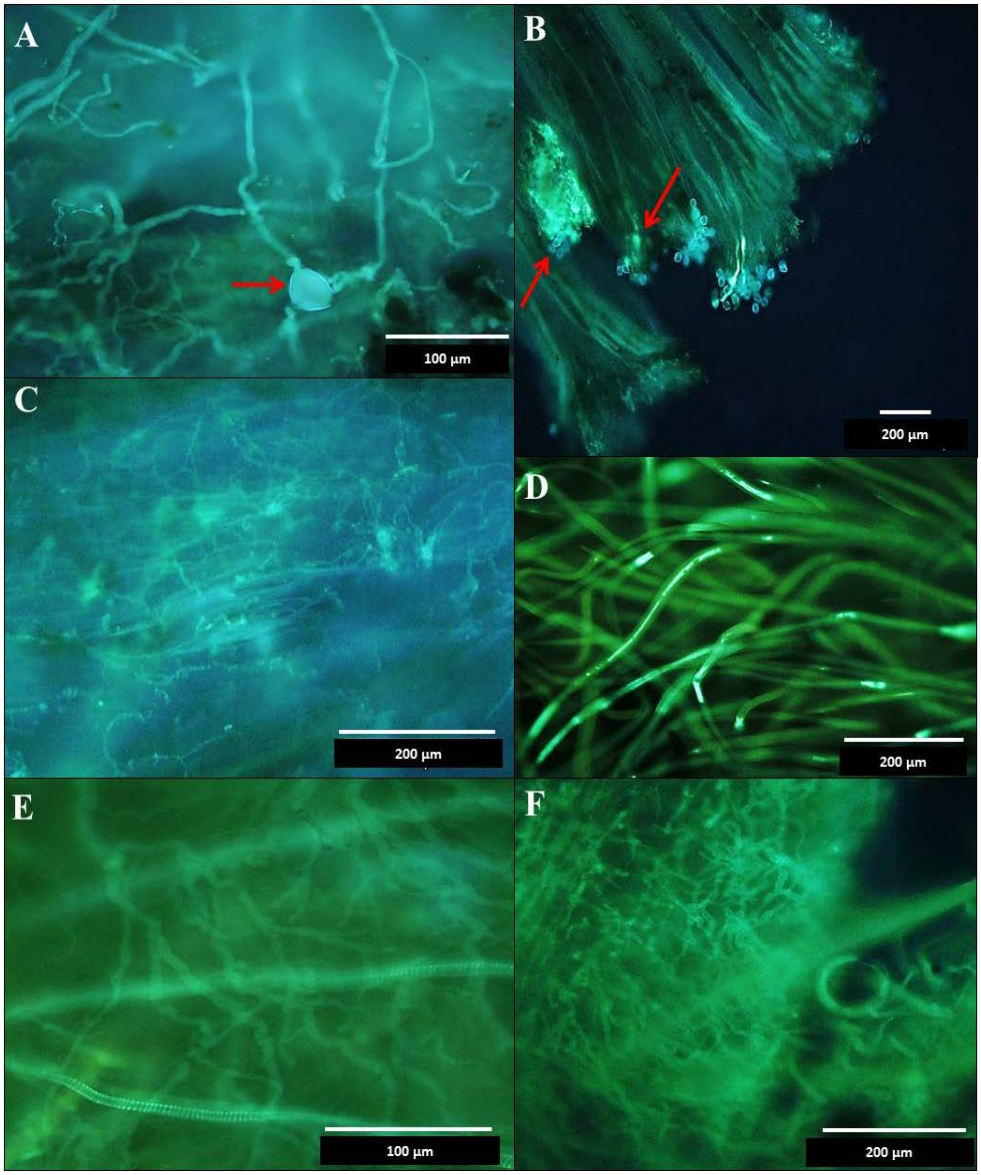

Figure 4.13 Representative fluorescent microscope observations of boysenberry tissue samples from floricane laterals inoculated with *Peronospora sparsa* or uninoculated and incubated under 100% RH. (A) Germinated spore (indicated by arrow) and hyphae characteristic of *P. sparsa* on calyx tissue, and (B) hyphae characteristic of *P. sparsa* on carpel tissue (fluorescing areas indicated by the red arrows, pollen grains are on the top of the stigma and stained blue) from inoculated laterals incubated at 15ºC. Hyphae characteristic of *P. sparsa* or other fungi on (C) calyx and (D) trichomes of calyx (hyphae within the trichomes are fluorescing, whereas the trichomes without hyphae remained non fluorescing) from uninoculated control laterals incubated at 15ºC. Hyphae on calyx of (E) uninoculated control, and (F) inoculated laterals incubated at 20ºC.

Although ungerminated *P. sparsa* spores were observed on the sepals of the inoculated laterals incubated at 90% RH at either 15 or 20ºC (Figure 4.14A and B respectively) some fluorescing areas were also observed indicating germinating spores which had formed hyphae. *Peronospora sparsa* spores and the pollen were distinguished by their relative size, with the latter being larger than the *P. sparsa* spores (Figure 4.14C and D).

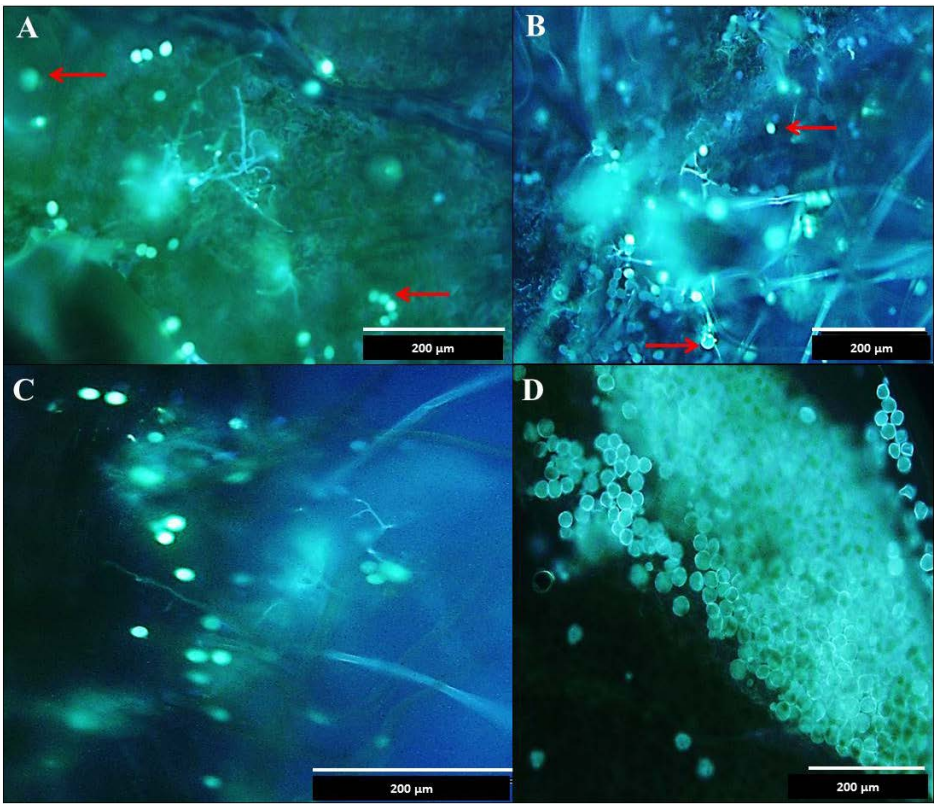

Figure 4.14 Representative fluorescent microscope observations of boysenberry tissue samples from floricane laterals inoculated with *Peronospora sparsa* or uninoculated and incubated under 90% RH. Ungerminated *P. sparsa* spores (indicated by arrow) on the calyx of an inoculated lateral incubated at (A) 15ºC (spores starting to germinate are fluorescing in the middle) and (B) 20ºC. *Peronospora sparsa* spores on (C) an inoculated leaf disc and (D) pollen grains to illustrate the differences between the two structures enabling their discrimination in the fluorescence microscopy observations.

4.3.4.1.2. Effect of incubation conditions on development of dryberry symptoms from inoculated bud or flower (shadehouse laterals)

i. High RH (100% RH) under ambient temperature

Sporulation was observed on the flowers of 95% of laterals for the inoculated treatment 12 days after inoculation and on only the flowers of 11% of the uninoculated control laterals. In addition, the proportion of flowers on each lateral with *P. sparsa* sporulation was significantly higher ($P<0.001$, Appendix C.7a) on the inoculated laterals (0.9) compared with the uninoculated control (0.1). At least one berry developed on 47% of the laterals after one month. The number of laterals which developed at least one berry was almost twice as high in the uninoculated control (11) compared with the inoculated laterals (6).

Of the laterals which produced berries, dryberries were observed on 83% of the inoculated laterals with only 54% observed on uninoculated control laterals. However, there was no significant effect of *P. sparsa* spore inoculation on the proportion of berries (*P*=0.230), proportion of king berries (*P*=0.311) or proportion of non-king berries which produced dryberries (*P*=0.330) (Table 4.3; Appendix C.7b).

Table 4.3 Effect of *Peronospora sparsa* spore inoculation either at the bud (non-king) or flower (king) stage incubated at high RH under ambient temperature on the proportion of dryberries produced per lateral which had been excised from systemically infected boysenberry plants.

Treatment	Proportion of dryberries per lateral		
	Total berries	King-berries	Non-king berries
Inoculated	0.83	0.83	0.33
Control	0.59	0.55	0.09

P. sparsa sporulation was observed on the dryberries but not on asymptomatic berries. Dryberry symptoms observed included premature reddening, hard, dried and dull looking (not glossy) drupelets (Figure 4.15A). Under high humidity *Botrytis cinerea* was also observed to grow on the stems and berries causing the laterals to die and appeared to stop further development of the berries (Figure 4.15B). The average daily maximum and minimum temperatures obtained from the Lincoln, Broadfield Ews NIWA weather station for the duration of the experiment was 9.7 - 20.0ºC.

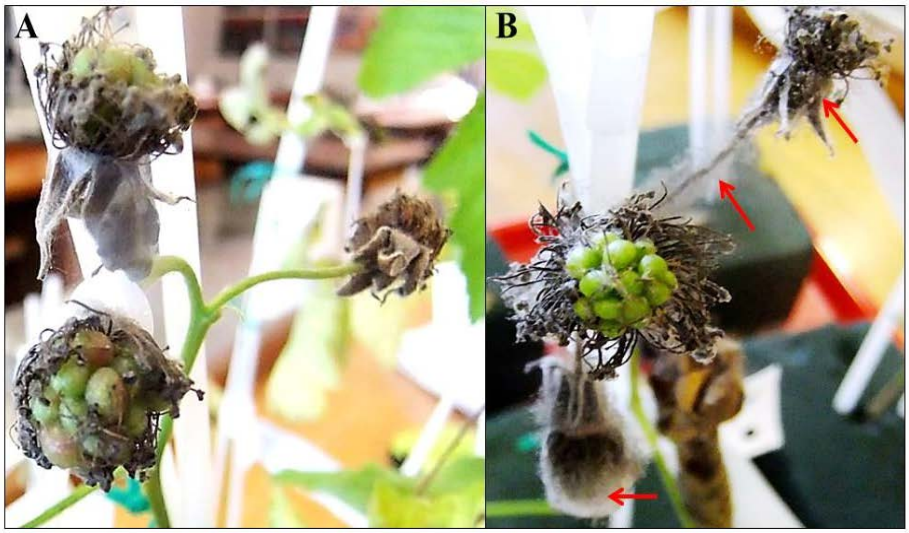

Figure 4.15 Boysenberries with (A) typical dryberry symptoms showing dull, non-glossy, hardened, dried and premature reddening on inoculated, and (B) healthy looking berry from untreated control flower laterals incubated at 100% RH under ambient temperature in the shadehouse. Growth and sporulation of *Botrytis cinerea* observed on some berries and stems indicated by the red arrows.

ii. High RH (100% RH) and optimum temperature (at 15ºC) under growth lights

The floricane flower laterals did not develop berries; however 83% of laterals developed berry initials (3-4 mm diameter) after 45 days (Figure 4.16). There was a significant effect (P=0.023, Appendix C.8) of inoculation on the incidence of *P. sparsa* sporulation on the flowers with the incidence being significantly higher (P<0.05) on the flowers on the inoculated laterals (83%) than on the flowers on the uninoculated control laterals (39%). No dryberry symptoms were observed to develop.

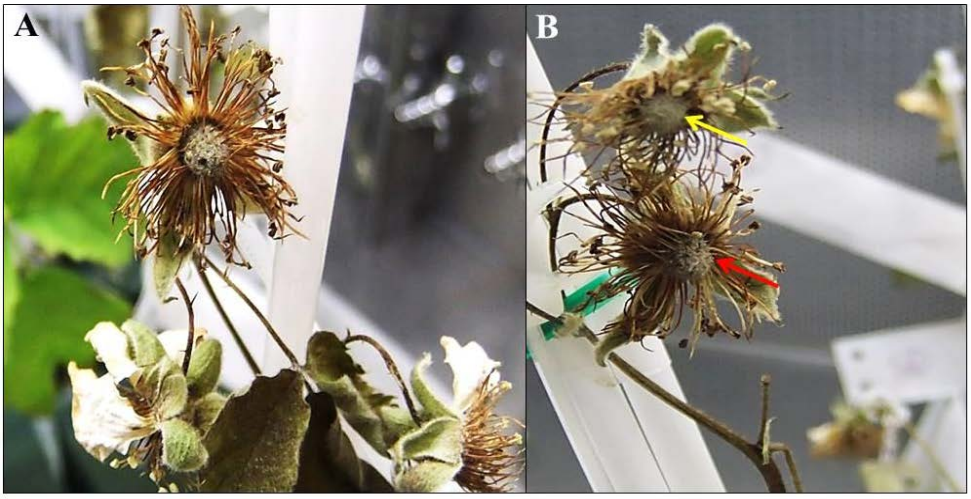

Figure 4.16 (A and B) Berry initials which developed from king and non-king flowers after 45 days on detached flower laterals incubated at 100% RH and 15ºC under growth lights. King berry initials are indicated by the red arrows and the non-king berry initial by the yellow arrow.

4.3.4.2 Development of berry infection on excised field sourced fruiting laterals

Berries at the green, unripe hard stage

The main dryberry symptoms observed on both the *P. sparsa* inoculated and uninoculated control berries were splitting of the berries (Figure 4.17) with dried, non-glossy drupelets. There was no significant effect of *P. sparsa* spore number (P=0.183, 0.110, 0.075), berry region inoculated (calyx or drupelets) (P=0.897, 0.851, 0.842) or interactions between *P. sparsa* spore number and berry region (P=0.757, 0.837, 0.992) on dryberry incidence (numbers of berries with dryberry symptoms) for king berries, non-king berries or total berries, respectively (Table 4.4; Appendix C.9). No *P. sparsa* sporulation was observed on any of the berry tissues in any of the treatments.

Figure 4.17 Dryberry symptoms observed on unripe boysenberries as splitting of the berry into two portions on (A) king berry and (B) non-king berry. The king berry is indicated by the red arrow.

Table 4.4 Effect of inoculation with different numbers of *Peronospora sparsa* spores on the calyx or drupelets of berries at the green unripe hard stage on the excised field sourced fruiting laterals, on the mean percentage of dryberries which developed on each fruiting lateral.

Spore number	% of dryberries[a]						% of dryberries		
	King berries		Non-king berries		Total berries		King berries	Non-king berries	Total berries
	Calyx	Drupelets	Calyx	Drupelets	Calyx	Drupelets			
0	20	50	60	63	47	58	35	62	53
20	0	25	50	100	33	75	13	75	54
50	60	0	90	0	80	0	30	45	40
200	20	25	60	88	47	67	23	74	57
500	60	50	90	88	80	75	55	89	78
Calyx	32		70		57				
Drupelets	30		68		55				

[a]No significant effect of *P. sparsa* spore number, berry region or interaction on the dryberry incidence.

Unfortunately, although the grower had been requested to not spray the boysenberry plants in the bays which where sampled for the experiment, the plants were sprayed with phosphorous acid (three times on the 2, 15 and 22 Nov 2012) as well as other chemicals such as lime sulphur, acephate, N.P.K., sulphur, wetter, paraquat/diquat, carfentrazone, glyphosate, fluroxypyr, glufosinate, ammonium calcium nitrate, methoxyfenozide, mancozeb, 2-4-DB, thiram, pyroclostrobine+boscalid, gibberellic acid, cyprodinil+fludioxonil, terbuthalazine and diuron.

Berries at the red, partially ripe stage

All berries were colonised by *Botrytis cinerea* after 6 days (Figure 4.18) and therefore no assessments of *P. sparsa* infection could be made.

Figure 4.18 (A-B) Berries on detached fruiting laterals incubated at 100% RH and under ambient temperature colonised by *Botrytis cinerea.*

4.3.4.3 Effect of inoculation of berries at different stages and regions on dryberry production

Detached berries from the shadehouse

There was no significant effect (P=0.126; Appendix C.10a; Table 4.5) of berry maturity stage at inoculation on the sporulation incidence, with incidence being 36% for berries inoculated at the unripe stage, compared with 29% for berries at the ripe stage. Sporulation incidence was significantly affected (P<0.001) by inoculation, with significantly higher incidence on inoculated (47%) compared with uninoculated control berries (0%). The region of the berry inoculated also significantly affected (P<0.001) sporulation incidence, with incidence for drupelet (63%) being significantly higher compared with calyx (0%) inoculated berries. For drupelet inoculated berries sporulation initially started on the drupelets which were inoculated and spread to all parts of the berry, with the berry ending up with, hardened and dried, dull immaturely reddened drupelets. For calyx inoculated berries sporulation was not observed and the berries remained without dryberry symptoms. There was no significant interaction between berry maturity and inoculation (P=0.981), berry region and inoculation (P=0.969), berry maturity and berry region (P=0.991), and berry maturity, region and inoculation (P=0.990) on the incidence of sporulation.

Table 4.5 Effect of *Peronospora sparsa* inoculation of the calyx or drupelets of green unripe or partially ripe red berries detached from shadehouse plants on the incidence of *P. sparsa* sporulation and dryberries.

Means of;	Effect of;					
	Berry maturity		Inoculation		Region inoculated	
	Green unripe	Red ripe	Inoculated	Control	Calyx	Drupelets
Sporulation incidence (%)	36	29	47	0	0	63
Dryberry incidence (%)	33	29	47	0	0	63

Dryberry symptoms were only seen to develop on sporulating berries with no dryberries produced on the uninoculated control berries. Further, dryberry symptoms only developed on the specific drupelets where sporulation was observed. Inoculation and berry region significantly affected (P<0.001 and P<0.001, respectively; Appendix C.10b; Table 4.5) dryberry incidence. *Peronospora sparsa* inoculation significantly increased dryberry incidence compared with the uninoculated control (47% and 0%, respectively). There was significantly higher dryberry incidence for berries inoculated on the drupelets (63%) compared with berries inoculated on the calyx (0%). Of the inoculated berries, 100% of the unripe berries which showed sporulation on the drupelets and 87% of the ripe berries which showed sporulation on the drupelets developed into dryberries, with none of the nonsporulating berries developing into dryberries. Berry maturation stage at inoculation did not significantly affect (P=0.126) dryberry incidence, with incidence being 33% for berries inoculated at the unripe stage, compared with 29% for berries at the ripe stage. There was no significant interaction between berry maturity and inoculation (P=0.981; Appendix C.10b), berry region and inoculation (P=0.969), berry maturity and berry region (P=0.990), and berry maturity, region and inoculation (P=0.991) on dryberry incidence.

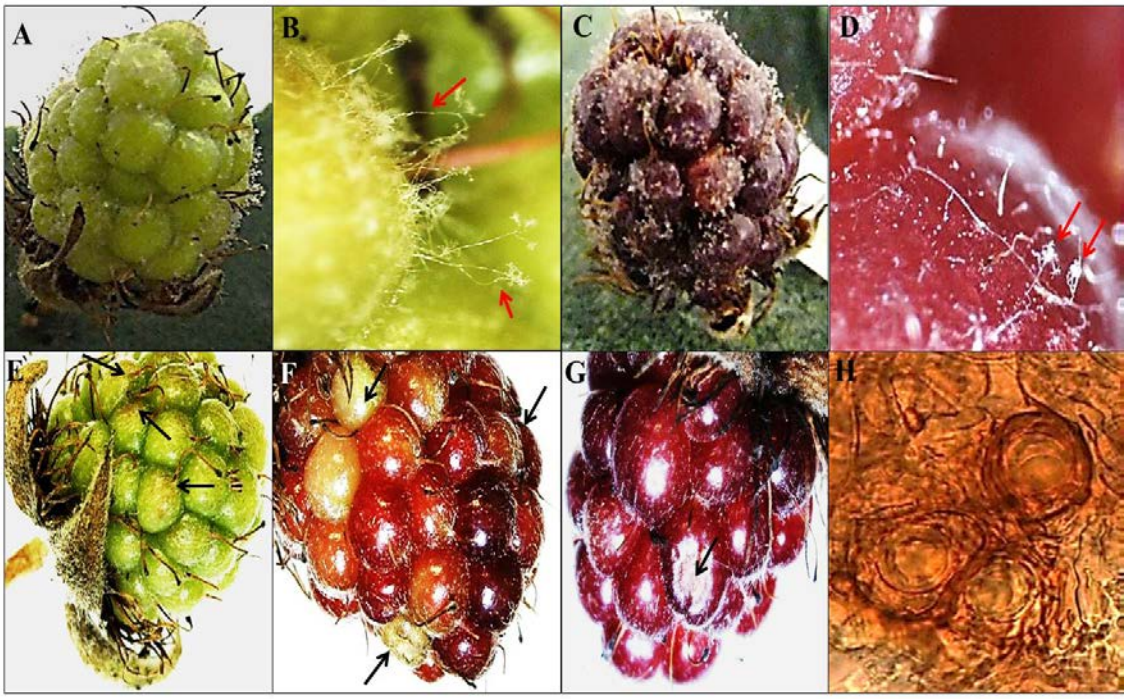

Figure 4.19 Sporulation of *Peronospora sparsa* on drupelets inoculated at the (A and B) unripe and (C and D) red, partially ripe berry stage. Sporulated area is indicated by red arrows. *Peronospora sparsa* sporulation on drupelets of (E) unripe berries becoming light brown to pink shown by the black arrows and (F) with maturity those drupelets remaining dull, non-glossy, dried (shown by the black arrows) resulting in a mosaic appearance of green and red coloured druplets. (G) *Peronospora sparsa* sporulation on the drupelets of ripe berries becoming non-glossy, dried and hardened shown in black arrow. (H) Oospores observed to form in red, partially ripe berry drupelets with *P. sparsa* sporulation.

After a further 11 days incubation (25 days from the start of the experiment) of inoculated sporulating berries, oospores and mycelium were observed microscopically in all berry parts including the sepals, drupelets and torus (Figure 4.19), whereas no oospores or mycelium were seen in the uninoculated control berries.

Detached berries from the field

All of the berry halves incubated at both 90-95 and 100% RH were contaminated by *Botrytis cinerea* within a few days and therefore no assessment of infection by *P. sparsa* could be carried out.

4.4 DISCUSSION

The overall aim of this chapter was to assess temperature and relative humidity as two environmental factors that are suggested triggers for disease expression (sporulation and lesion development) and this was achieved using the systemically infected boysenberry plants (cv. Mapua) placed in the greenhouse or shadehouse to provide the different environmental conditions. This study showed that temperature and relative humidity (RH) play a significant role in expression and development of downy mildew disease in systemically infected boysenberry plants. Temperatures within the range of 5-15ºC and high relative humidity (90-100%) were optimal for disease expression with regards to both symptom expression (lesions) and *P. sparsa* sporulation. Sporulation was observed on a wide range of tissue including flower tissues such as petals, stamen (both anther and filament) and young foliage including stems/canes and calyxes/sepals.

The results indicated that spores of *P. sparsa* can start the germination process as soon as they are suspended in water, since EC_{50} values several orders of magnitude higher, were obtained with spores suspended in water compared with fungicide solutions. Therefore, to accurately determine the EC_{50} values for the fungicides the spores were suspended in the relevant fungicide solution rather than initially suspending in water.

Results indicated that a combination of high RH (90-100%) and temperature of 15ºC or below was required to trigger disease expression. Sporulation was only observed in early spring (Oct) on primocane leaves on plants incubated under high humidity in the greenhouse. Environmental monitoring of the greenhouse showed that in October humidity reached ≥90% and temperature maximum fell to <20ºC. No sporulation was observed when only one of these two environmental factors reached the optima. This is in agreement with the results of Shaw (1982) who reported that a combination of environmental factors (temperature and RH) influenced infection (lesion development) and sporulation of *P. sparsa*. These results were also consistent with the results of Chapter 2 where disease expression of systemically infected boysenberry leaves, stems/canes and calyx tissues sourced from the field were greater at 15ºC than 20ºC

under high humidity. In addition, the spore release data collected from the field indicated that most spores were released in mid-November and thus sporulation would have to have occurred in the early spring (Chapter 2). Although the duration required for high RH and temperature of 15ºC or below to exist for optimum disease expression was not assessed in the current study, it is important to enable decision on the timing of the initial fungicide spray to apply in disease management (Neufeld and Ojiambo, 2012). Thus, additional studies are needed to establish the impact of duration of the optimum environmental conditions required for *P. sparsa* disease expression.

Growth room conditions were not conducive for disease expression of *P. sparsa* by sporulation on systemically infected whole boysenberry plants or flower laterals excised from the field plants despite incubation at 15 or 20ºC and 90 or 100% RH. Cohen *et al*. (2013) reported that as little as 1% light could inhibit sporulation of *Peronospora belbahrii* in infected basil. The inhibition of sporulation by light is temperature-dependent, with light completely inhibiting sporulation at 15-27°C but not at 10°C on other downy mildews and true fungi (Cohen *et al*., 2013). Cohen and co-workers (2013) suggested that reception of light at above 15°C may induce enzyme(s) that produce anti-sporulation compounds and/or cause structural reconformation of the photoreceptor protein thereby inhibiting sporulation. In the current study temperature ≥15°C was used making the pathogen potentially highly light sensitive. It is possible that the growth chambers were leaky and admitted light during the simulated "night time" and thereby supressing sporulation. Similarly, more sporulation on the systemically infected plants incubated at high RH in the shadehouse compared with the greenhouse may also be due to light levels being higher in the greenhouse. Shaw (1982) also reported that temperature was more important than moisture (RH) for sporulation of *P. sparsa* on boysenberry and this may be reflective of the temperature regulation of light reception. Light can also be used to reduce disease under field conditions by pruning and orientation of the plants. This was observed in the field plants with most of the symptoms seen within the boysenberry canopy where not only RH was higher but also lower light levels. Alternatively, it could be that disease expression is favored by fluctuation in temperatures similar to what would be experienced under natural field conditions rather than exposure to a constant temperature of 15 or 20ºC. However, inhindsight it would have been useful to have used light intensity meters to measure the light intensity in growth rooms.

High humidity at both temperatures (15 and 20ºC) in the growth rooms allowed germination, infection and colonisation by *P. sparsa*, but not sporulation. Characteristic germinating spores and hyphae were observed by fluorescence microscopy in the excised flower laterals incubated at 100% RH, whereas most *P. sparsa* spores remained ungerminated after six weeks on the inoculated laterals incubated at 90% RH. This suggested that 90% RH was less favourable for spore germination and/or infection. This result was similar to that observed in the *in vitro* studies conducted on boysenberry leaf discs (Chapter 2) with infection occurring under high RH

at 15 or 20ºC. In addition, hyphae characteristic to *P. sparsa* in calyx and/or trichomes of the inoculated and uninoculated control flowers were only observed at 100% RH indicating either i) systemic colonisation by the hyphae in both treatments (inoculated and control) or ii) both infection and systemic colonisation in the inoculated treatment and only systemic colonisation in the control laterals triggered in the growth rooms. It is possible that plastic sheet used to make the humidity tent (100% RH) may have reduced the light intensity making it more conducive to colonisation/infection. Wolf *et al*. (1934 cited in Fried and Stuteville, 1977) showed that even when suspended in water intense sunlight for 1 h could kill spores suggesting that a reduction in light intensity may have favoured germination. However, separation of the infection originating from inoculated spores and from systemic colonisation was impossible.

Spraying the protectant mancozeb at both the flower and berry stage of systemically infected boysenberry plants did not reduce dryberry incidence, even though it reduced spore germination (EC_{50} 0.04 µg/mL) and germ tube growth *in vitro.* This suggests that either the mancozeb failed to protect the berries or that the dryberry resulted from systemic infection in which the pathogen was protected from surface applications of the fungicide. However, the mancozeb spray will also have increased the humidity around the flowers/berries and thereby may have facilitated spore infection. But, as all unsprayed flowers also produced dryberries it is unlikely that the increased humidity was responsible for dryberry production in sprayed flowers. Also spraying mancozeb to run off may have reduced the amount of active ingredient deposit on leaves (Fourie *et al*., 2009). It cannot be ascertained whether this would have been enough to overcome the inhibitory effect that was observed *in vitro*. Research studies in Michigan have shown that weekly applications of mancozeb protect against onion downy mildew (Hausbeck, 2010). However, in the current study the interval between applications was only two days. It is possible that the inoculum pressure was high enough to overcome the mancozeb spray. Spore release was not measured in the shadehouse and therefore, the inoculum pressure present during the experiment was unknown. Thus, an improvement to the method can be recommended by incorporating spore traps to record aerial spore numbers. It was also reported that protectant fungicides such as mancozeb are subject to weathering; hence its effect against the pathogen is lost once the fungicide has penetrated the host (rose) tissue (Aegerter, 2001). Thus, a combination of metalaxyl and mancozeb has been recommended. However, in the study conducted by Walter *et al*. (2004) a single spray of metalaxyl+mancozeb combination applied with a wetting agent was not able to reduce boysenberry fruit infection. Thus, the flowers and/or developing berries may have been infected by spores after the protective activity of mancozeb had worn off which is typically after 10-14 days from the initial spray (Novachem agrichemical manual, 2015). In addition, is it also likely that the fungicide coverage onto the plant material was not uniform and this associated with any subsequently developing plant tissue not being protected is likely to have resulted in protection not being 100%. The results of this experiment were inconclusive in terms of role of systemic and spore initiated infections in dryberry

production. The use of pathogen free plants would enable the relative role of systemic and spore initiated infections to be assessed. A method for the production of pathogen free boysenberry planting material will be investigated in the next chapter.

Dryberries developed from both inoculated (83% of laterals) and uninoculated (54% of laterals) flower laterals excised from systemically infected boysenberry plants. This indicated a role for both systemic and spore initiated infections. However, it is also possible that the sporadic and random presence of the pathogen in any specific lateral may have contributed to the variation observed between inoculated and uninoculated laterals. A significantly higher proportion of flowers from the inoculated laterals produced spores in both excised flower lateral assays conducted under ambient and growth light conditions (incubator maintained at optimum conditions) at high humidity than the control. In addition, spore infection at both bud and fully opened flower stage resulted in high levels of dryberry production suggesting that spore infection at both stages can cause dryberries, which indicates prevention of infection is necessary at early stages.

The experiment, which aimed to determine whether dryberries are produced by systemic or spore infection at the unripe berry stage, failed to evaluate the effect of spore infection. This was due to the accidental spraying by the grower of the plants from which fruiting laterals were detached. This may have inhibited spore infection of the inoculated berries. It was also observed that green unripe hard berries on the excised fruiting laterals inoculated with a range of spore numbers of *P. sparsa* 20, 50, 200 and 500, or uninoculated all produced dryberries, however with a slight difference in the total dryberry percentage of the control (53%) and inoculated laterals (54-78%). Thus, this experiment was not a test of spore infection. It is also possible that the fungicide sprays were applied after spore infection had already happened in the field or that the chemicals applied were not effective as protectants. Phosphorous acid was applied three times in November to control the systemic spread of *P. sparsa* with mancozeb on 30 October to control spore infection. However, the protectant was applied when most of the flowers were in full bloom. Due to this delay, the infection may have already established due to spore infection of the stamen and/or pistil from the sporulated calyxes of closed buds which would have been protected from the chemical spray. Dashwood and Fox (1988) also reported that without application of the protectant at a time when the airborne inoculum of *B. cinerea* was abundant, early infection could not be prevented. This was studied using dichlofluanid as a protectant on raspberry to control flower and fruit infection by *B. cinerea* and found that dichlofluanid was more effective when applied early. Thus, correct timing of protectant application is crucial before flowers have been infected. However, this will be further investigated using Ridomil Gold® MZ WG which has both systemic and protectant activities in the next chapter (Chapter 5).

Dryberry symptoms can result from spore infection of drupelets of detached green or red immature berries and incubated at optimum conditions (high humidity, 15ºC) under growth lights. None of the uninoculated berries sporulated or developed into dryberries, and also no hyphal structures characteristic of *P. sparsa* were observed microscopically suggesting the pathogen was not present in the asymptomatic berries detached from the systemically infected plants. However, in the *in vitro* assay conducted in Chapter 2, sporulation was observed on the calyx of all of the green unripe berry halves which were incubated in 100% RH at 15ºC. This indicated that either the pathogen was not presented inside in the berry tissue or that detached berries do not express disease on the drupelets. The detached berries in the current experiment also did not sporulate. Those berries were detached in the generally hotter period in late December, whereas it was in November (cooler) for the Chapter 2 experiment. In the summer, the pathogen was detected by PCR (Chapter 3) predominantly in the canes and roots rather than in new tissues (Chapter 3). Therefore, under the higher temperature conditions in which berries were detached, the pathogen may not have moved into the berry stems or calyx; hence the detached berries remained free of *P. sparsa*.

In this study, dryberries resulted from spore infection of the drupelets. Also, sporulation was greater on berries inoculated on drupelets than calyxes, and more dryberries from the berries where drupelets sporulated than calyxes. This further strengthens the hypothesis that spore infection by the pathogen may cause dryberry symptoms. This was likely due to *P. sparsa* not being able to move systemically from calyx infections into berry tissues such as the torus or drupelets. The chemical compounds at the proximal end of the berries may act as a barrier at both maturities preventing mycelial growth into the above berry tissues. Dryberry symptoms observed at both green unripe and ripe stages by sporulation on drupelets at both maturities indicated that *P. sparsa* can infect through the drupelet epidermis; thus it may not be solely a consequence of latent infection as for *Botrytis cinerea* infection of boysenberries (Walter *et al*., 1997), strawberries and raspberries (Jarvis, 1962), and red raspberries (Dashwood and Fox, 1988). However, those were detached berries and the effect of systemic disease expression is unknown.

In conclusion, spore infection at the flower bud, flower, green or red immature berry stages can all result in the development of dryberry disease. Disease expression was seen on systemically infected plants as sporulation on young leaves, canes, petioles, calyx and petals during spring and at high humidity. When the natural conditions were favourable for disease expression this ultimately resulted in dryberries. Depending on the environmental conditions in a particular season being suitable, the developed berries can also be directly infected by spores, initially with a few drupelets being infected which go on to infect the whole berry resulting in dryberries. This might be the reason for the higher number of dryberries developing on plants incubated at high humidity, due to more abundant spores compared with plants incubated at ambient conditions. Therefore, disease management needs to be considered with an appropriate

spray diary which includes both systemic and protectants applications at appropriate flower/berry physiological stages which will be the focus of the next chapter.

CHAPTER 5

To investigate use of control products to limit infection and systemic disease

5.1 INTRODUCTION

Control of *Peronospora sparsa* infection in New Zealand boysenberry gardens is mainly through effective cultural practices and fungicide application (Walter *et al*., 2004). Sourcing pathogen free planting material is also important to control the disease. Cuttings taken from systemically infected plants are likely to be infected, and tissue-cultured plants obtained from a commercial nursery in this study (Chapter 2) were found to be infected. Similarly, Wallis *et al.* (1989) reported *P. sparsa* infection of micropropagated *Rubus* spp. At present there is no effective method to produce pathogen free plants through tissue culture. Heat treatment (thermotherapy) and/or fungicides (chemotherapy) has not been tried but can limit systemic infection commonly from viruses (Baumann, 1982; Trigiano and Gray, 1999) prior to tissue culture propagation for other crops.

Agrochemical applications are used to both reduce systemic infections and hence primary inoculum sources, and to protect leaves, flowers and developing berries from spore infection. Foliar application of fungicides is the most common treatment to control downy mildew, and multiple spray applications per crop cycle are often required due to the development of progressive epidemics (Gisi, 2003 cited in Caro, 2014). At present chlorothalonil, copper oxychloride, mancozeb and metalaxyl-M based agrochemicals are the only fungicides registered for control of *P. sparsa* in boysenberries in New Zealand (Novachem agrichemical manual, 2015). Although the acceptable rates of copper for organically grown crops is 3 kg of copper/ha (Bio Gro standard), effective control of the disease was not achieved with this rate (Richards, 2002), and copper oxychloride was reported as being phytotoxic to boysenberry (Tate and Van Der Mespel, 1983). Phosphorous acid (PA) as an aqueous solution of mono- and di-potassium phosphite is an agrochemical which is currently being used and has been reported to be effective against dryberry in boysenberries (Richards, 2002; Walter *et al*., 2004). Phosphorus acid products are not currently registered for use as a fungicide on boysenberry, but are used as liquid fertilisers (Novachem agrichemical manual, 2015). Effective control at low disease pressure (2000/01 season) has been achieved with two applications of metalaxyl-M, three applications of phosphorous acid or three applications of azoxystrobin plus a single application of dichlofluanid (Walter *et al.*, 2004). However, only the phosphorous acid treatment (three applications at pre-flowering stages) gave acceptable downy mildew control (less than 20% fruit loss) at high disease pressure in the 2001/02 season. Agrochemicals were applied during pre-flowering and flowering with the aim to protect the flowers and developing berries and therefore reduce downy mildew infections of fruit. Dryberry disease was shown in

Chapter 4 to be caused by both systemic and spore infection, whether these chemicals acted to limit dryberry disease due to systemic, spore initiated, or both was not investigated in these studies.

Agrochemicals to control *P. sparsa* on other hosts have also been evaluated (Aegerter, 2001; O'Neill *et al*., 2002; Hukkanen *et al*., 2008; Angel Rebollar, Universidad Autónoma Chapingo, Mexico unpublished). Hukkanen *et al.* (2008) reported that tolylfluanid and Bion (acibenzolar-s-methyl, syn Actigard) gave the best control of *P. sparsa* in artic brambles, whereas fosetyl-aluminium (Aliette), and two phosphorous acid products (phosphites) i.e. salts of PA (Phosfik) and potassium, sodium and ammonium salts of PA (Phostrol) gave moderate protection against the disease. Three or four potassium phosphite applications beginning after bud break (45 days before blooming) with single spraying of azoxystrobin or pyraclostrobin after the potassium phosphite applications were reported as being the best treatment to control dryberry disease of blackberry in Mexico. However, the problem with azoxystrobin and pyraclostrobin is that the number of applications allowed per season was not effective to control the disease. Nevertheless, due to their short preharvest interval it was suggested they could be used to prevent secondary infections, with azoxystrobin application recommended around 7 days before harvest (A. Rebollar, pers. comm., 2015). In addition, two applications of mefenoxam applied as a drench after bud break followed by two to four potassium phosphite sprays was also shown by this researcher to provide good control. However, these fungicides were targeted for control of systemic infections since under Mexican conditions the incidence of sporulation (number of spores) is low. In addition, the efficacy of these fungicides as individual sprays was not evaluated. O'Neill *et al*. (2002) reported a mixture of cymoxanil, mancozeb, and oxadixyl, or a fluazinam application gave good control of *P. sparsa* on both rose and blackberry. Further, fortnightly sprays of chlorothalonil, fluazinam, metalaxyl mixed with either thiram or mancozeb, mixture of cymoxanil, mancozeb and oxadixyl, or a monthly drench of fosetyl-aluminium were also successful in reducing the disease severity in blackberry. In California, pre-planting treatment with the systemic fungicides metalaxyl or mefenoxam were highly effective at reducing *P. sparsa* disease in rose when outbreaks occurred at bud-break (Aegerter, 2001).

In New Zealand, there have been few studies to determine the efficacy of fungicides to provide control of *P. sparsa* systemic and spore infection in boysenberry, and there have been no studies to determine effective methods for developing *P. sparsa* disease-free propagation material. In the work presented in this chapter, protective and systemic chemicals previously shown to control *P. sparsa* on boysenberry or other plant hosts were selected. Initially the chemicals were evaluated for their effect on *in vitro* spore germination and infection. Selected chemicals were then evaluated for their ability to prevent spore initiated infection on disease-free boysenberry plants, and dryberry symptom expression in systemically infected plants. Heat treatment with or without fungicide treatments, were also evaluated for their ability to limit systemic infection of

propagation material prior to tissue culture, so as to enable the production of *P. sparsa* disease-free boysenberry plants.

5.2 MATERIALS AND METHODS

5.2.1 Production of pathogen free boysenberry plants

5.2.1.1 Evaluation of control strategies for production of pathogen free boysenberry plants

Pre-treatments:

Fifteen *P. sparsa* systemically infected boysenberry plants (cv. Mapua) grown in the outdoor cage area at Riwaka Plant and Food research station were repotted and cold stored (4ºC) for six weeks (early-April to mid-May, 2012) to induce dormancy. The plants were then placed in a greenhouse (15-30°C) under lights to induce new growth. Three treatments were applied to each of five replicate plants; these were heat, heat+fungicide (mancozeb and phosphorous acid) (Walter *et al.,* 2004), or untreated control. The heat treatment was based on that used by Baumann (1982) to restrict the growth of heat stable raspberry vein chlorosis virus (RVCV) in red raspberry (*Rubus idaeus* L.) whilst allowing plant growth (38-40°C). A slightly lower temperature of 34°C was used since boysenberry has not been studied for its heat sensitivity compared with raspberry. For the heat treatment, the five plants were grown for four weeks (mid-May to mid-June, 2012) in the greenhouse to allow new primocane growth before being transferred to the growth cabinet at 34°C for a further four weeks. For the heat + fungicide treatment another five plants were sprayed with phosphorous acid (PA) after two weeks in the greenhouse. Two days later the plants were sprayed with mancozeb. Two weeks later (11 June, 2012), the second spray of both chemicals as applied as previously described, that being PA first and two days later mancozeb. Then, immediately after the mancozeb spray, the plants were transferred to the growth cabinet at 34°C for a further four weeks. For the untreated control treatment, the five plants were incubated in the greenhouse for the whole 8 week period without any treatment. The plants for each treatment were then used for initiating tissue culture in July, 2012.

Explant preparation and sterilisation for tissue culture:

Lateral buds were cut from the top most newly developed side-shoot of each plant, and used as the source of explant material for tissue culture. These single-bud stem cuttings were approximately 1-2 cm in length and included a lateral leaf bud. Only stem material which had been produced on the plants during the treatment period was used, and then the top-most material in preference. Initial decontamination of the stem cuttings was done by washing in antimicrobial soap mixed in warm water for 5-10 min. Surface sterilisation of the stem cuttings was done by placing in 10% bleach (0.6% sodium hypochlorite solution) in a beaker (sealed with aluminium foil) on a shaker for 10-20 min, after which the stem cuttings were washed in

three rinses of sterile distilled water in a laminar flow hood. The numbers of lateral buds available for tissue culture on each stem were variable and depended on the stem length.

Production of tissue culture:

Production of tissue culture was based on the method developed by the researchers at Plant and Food Research, Riwaka, using the protocol for initiation of raspberry into tissue culture (adapted from Wu *et al.*, 2009) which had been shown to be a protocol suitable for hybridberry, boysenberry, blackberry and raspberry tissue culture initiation (B. Shunfenthal, pers. comm., 2013).

Initiation

Each sterilised stem cutting (explant) was observed for any bleach damage causing the two ends to become black, if this was observed then those ends were removed aseptically prior to placing the stem cuttings vertically into a plastic tub (8 cm diameter and 9 cm height) containing initiation culture media (Figure 5.1A). The initiation media comprised 1/3 strength MT basal medium (Murashige and Tucker medium) (Wu *et al.*, 2009) (Appendix D.1) supplemented with 1 g L^{-1} 6-benzyladenine (BA), 30 g L^{-1} sucrose, 0.5 g L^{-1} Indole-3-butyric acid (IBA) and medium was solidified with 7.2 g L^{-1} agar (Sigma) (pH 5.8). The explant cultures were incubated (Figure 5.1 C) at 22ºC under cool-white fluorescent light of reduced light intensity and checked daily for contamination, with clean non-infected explants being transferred to fresh media every 4-6 weeks.

Multiplication

For multiplication of the explants into numerous shoots (Figure 5.1B), the cultures were transferred into initiation media supplemented with 0.67 g L^{-1} active charcoal (multiplication medium). The rooting step described by Wu *et al.* (2009) was not necessary as the plants rooted well in the multiplication medium. The plants were grown for a further 7 to 8 weeks in the multiplication medium and incubated under the same conditions used for the initiation step.

Acclimation of tissue culture plants

The rooted plants were transplanted into 7 cm diameter small clean pots containing potting mix [20 L sterile pumice, 20 L Southland peat, 40 g Osmocote (Everris International B.V., Netherlands) exact mini and 160 g Dolomite mixture] and placed in the greenhouse (14-21°C) under a clean plastic cover and sprayed with a fine mist twice a day to create mist house conditions for a further 2 weeks acclimation. Hereafter, this phase is referred to as the "misting phase".

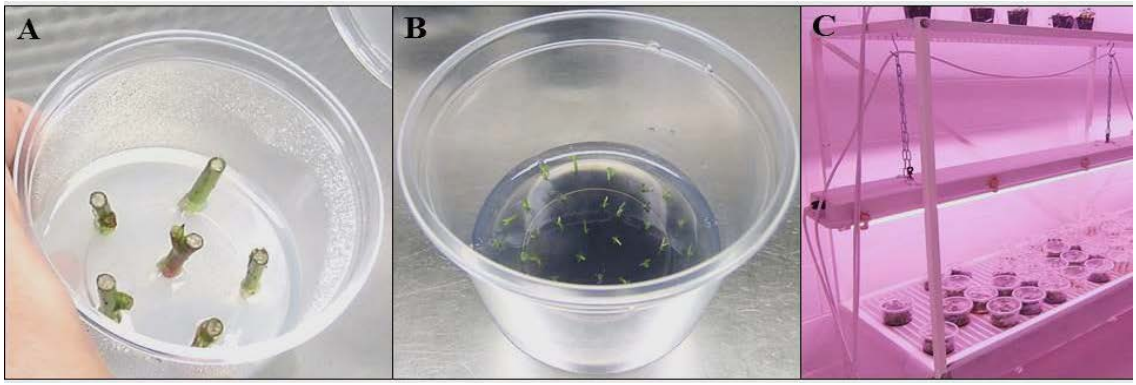

Figure 5.1 (A) Surface sterilised boysenberry (cv. Mapua) explants in the initiation culture medium, (B) buds excised from the explants after four weeks in the initiation medium planted in the multiplication medium, and (C) the plastic tubs containing the boysenberry explants incubated at 22ºC under cool-white fluorescent tubes of reduced light intensity.

5.2.1.2 Verification of the pathogen free status of the tissue culture plants

The tissue culture derived plants produced from the different treatments were potted into clean 2.5 L pots containing 12-14 month potting mix (Appendix A.2) and placed in the same greenhouse (Lincoln University) for 1 week prior to their transfer to a clean shadehouse. The plants were then grown under conditions conductive for expression of systemic symptoms for 3 months. During this time the plants were observed weekly for expression of characteristic *P. sparsa* systemic lesions, and plants showing symptoms were separated as soon as they expressed disease symptoms from those not expressing systemic symptoms. At the end of the 3 month period the infection status of the asymptomatic plants was verified using nested PCR (Chapter 3) on leaf samples. For plants which developed one cane, a disc (12 mm diam.) was taken from a leaf sampled from the bottom of the cane, whereas, for plants with more than one cane composite sampling was conducted where half a 12 mm diameter leaf disc from a leaf from each cane was used for DNA extraction.

Plants diagnosed as free of *P. sparsa* were used for propagation of pathogen free plants to be used for infection studies throughout this chapter. To further ensure that the plants used were free of *P. sparsa* infection, random sampling of the propagated plants was conducted during growth to allow potential increase of pathogen inoculum in the plants and tested with the nested PCR, since the possibility remained that systemic infection was present but below the detection threshold of the nested PCR (Chapter 3).

5.2.2 Effect of fungicides on spore germination and infection *in vitro*

Ten fungicides belonging to different chemical families (Table 5.1) were tested *in vitro* to evaluate their effects to inhibit the germination of *P. sparsa* spores. Selection of fungicides were based on chemicals reported to control *P. sparsa* on arctic bramble (Hukkanen, 2008), and boysenberry (Walter *et al*., 2004; Tate and Van Der Mespel, 1983) and fungicides recommended by Mr Geoff Langford (Berryworld Ltd, Tai Tapu, New Zealand, 2013). Stock

solutions (10 mL) of 1000 µg active ingredient (a.i.) /mL (Table 5.1) were prepared in sterile water for each of the 10 fungicides and used to prepare four different concentrations, 100, 50, 10 and 1 µg/mL. Sterile water controls were included one across all of the fungicides. *Peronospora sparsa* inoculum was prepared from spores produced on artificially inoculated boysenberry leaf discs (12 mm), after 14 days at 15ºC on 1.5% water agar as described in Section 2.2.3. Separate leaf discs were set up for each replicate for each fungicide concentration, and these were randomly selected for each replicate treatment.

To ensure sufficient spores, three randomly selected sporulating leaf discs were placed in each fungicide solution and shaken by hand to facilitate release of spores into the fungicide solution. One replicate solution from each fungicide concentration was made. From this suspension, a 20 µL aliquot was used to inoculate six surface sterilised leaf discs (Section 2.2.3.2) placed in separate Petri dishes (35 mm) containing 1.5% WA. Three of the inoculated leaf discs were used for the germination assay and the other three for the infection assay. For the germination assay the inoculated leaf discs were incubated at 20ºC under continuous darkness for 24 h and for the infection assay at 15ºC under 12h/12h light/dark conditions for 14 days. Both experiments were arranged in a randomised block design in the two incubators.

Table 5.1 Fungicides tested for inhibition of spore germination and infection of *P. sparsa in vitro* and pot experiments.

Active ingredient	Trade name	Chemical class	Conc. (a.i.)	Manufacturer	Activity	Field application rate in New Zealand
Chlorothalonil	Bravo® 750SC	Chloronitrile	720 g/L	Syngenta Crop Protection Ltd	Protectant	245 mL/100 L water
Mandipropamid[1]	Revus	Mandelamide	250 g/L	Syngenta Crop Protection Ltd	Protectant	40 mL/100 L water
Dichlofluanid[2]	Dichlofluanid	Phenylsulfamide	500 g/Kg	SigmaAldrich, Germany	Protectant	250 g/100 L water
Fluazinam[1]	Nando	Phenylpyridinamine	500 g/L	Nufarm Ltd	Protectant	100 mL/100 L water
Cymoxanil[1,3]	Curfew	Cyanoacetamide-oximes	450 g/L	Etec Crop solution Ltd, NZ	Systemic	35 g/100 Lwater
Phosphorous acid[2,4]	Foli-R-Fos400	Inorganics	400 g/L	U/M Agrochemicals Ltd, Australia	Systemic	350 mL/100 L water
Potassium phosphite[5]	PerkSupa	Inorganics	600 g/L	Key industries Ltd, NZ	Systemic	350 mL/100 L water
Acibenzolar-s-methyl[1,6*]	Actigard	Benzothiadiazole	500 g/Kg	Syngenta Crop Protection Ltd	Systemic	5 g/100 L water
Azoxystrobin[1]	Amistar WG	Strobilurin	250 g/L	Syngenta Crop Protection Ltd	Both	250 g/400 L water
Dimethomorph[1]	Sovrin® Flo	Morpholine	500 g/L	Adria Crop Protection Ltd	Both	360 mL/750 L water
Metalaxyl-M + Mancozeb[7]	Ridomil Gold MZ WG	Phenylamide & Dithiocarbamate	40 g + 640 g/Kg	Syngenta Crop Protection Ltd	Both	250 g/100 L water

[1] Not registered for boysenberry in New Zealand, [2]Monika Walter *et al*. (2004) on boysenberry; [3] Brian Smith at Etec Crop solution Ltd, New Zealand (pers. comm.); rates of other chemicals based on information in the Novachem agrichemical manual 2015; [4] Of the phosphorus acid products Foscheck only is registered for use in berryfruit but as a liquid fertiliser and not a fungicide; [5] Rodney French at Fruitfed Supplies/Nelson New Zealand (pers. comm.); [6] Bion 50 WG rate which was efficient against *P. sparsa* on arctic bramble (Hukkanen 2008); [7] Not used for the *in vitro* studies, only in *in vivo* experiments

*also referred to as Bion; a.i.=active ingredient

Germination assay:

Spore germination was assessed as described in Section 2.2.3.3 whereby a cellotape strip was used to pick up the spores from the droplet on the leaf surface, with this then mounted on a lacto-glycerol cotton blue drop placed on a microscope slide. The germination of 100 randomly selected spores for each replicate was observed under a bright field microscope at x10 magnification and percentage germination determined. A spore was considered germinated if the length of the germ tube exceeded half the length of the spore. From the mean percent germination, relative to the no fungicide controls, the percent inhibition of germination was calculated for each fungicide/spore suspension. The EC_{50} values and 95% confidence intervals were calculated for each fungicide using the probit analysis available within the generalised linear model (GenStat Ver. 16).

Infection assay:

Incidence of infection of leaf discs (out of the three replicates) as assessed by counting the number of leaf discs where lesions had developed, *P. sparsa* sporulation on the leaf discs was also assessed. Each replicate leaf disc was washed separately in 2 mL sterile water and the spore concentration determined using a haemocytometer to estimate the number of spores per lesion. Incidence of lesion development was statistically analysed by using generalised linear modelling procedure in GenStat Ver.16. A binomial distribution was assumed with logit link function to test for the main effects. The significance of the parameter estimates in the accumulated analysis of deviance was used to analyse if the infection incidence differed among treatments and concentrations with the means presented with the 95% confidence intervals.

P. sparsa sporulation was assessed using a sporulation scale (0=no spores, 1=1-5 spores, 2=6-12 spores, 3=13-50 spores, 4=51-100 spores, 5=101-1000 spores and 6=>1000 spores). The scale data were analysed by the non-parametric Kruskal-Wallis test in the Minitab Ver.16. No lesions developed on the water inoculated controls and therefore these were excluded from the statistical analysis.

5.2.3 Evaluation of fungicides to prevent *P. sparsa* spore infection

5.2.3.1 Prevention of *P. sparsa* spore infection of pathogen free boysenberry plants

Based on the ability of the 10 fungicides to inhibit the germination (EC_{50} values) of *P. sparsa* spores and infection of leaf discs (Section 5.2.2), seven were selected to evaluate their efficacy at preventing *P. sparsa* spore infection on leaves of potted boysenberry plants. Sixty pathogen free boysenberry plants (cv. Mapua) were propagated from the clean plants produced in Section 5.2.1 and grown for 5 months. The plants, which each had developed one primocane, were potted in 2.5 L pots containing 12-14 month potting mix (Appendix A.2) and moved into a clean shadehouse at Lincoln University and acclimatised for 1 week. No boysenberry plants had been placed in this shadehouse and there were no rose plants which could also be host of *P.*

sparsa growing close by. The fungicides included in the study were two protectants (mandipropamid and chlorothalonil), three systemic chemicals (acibenzolar-s-methyl, phosphorous acid and potassium phosphite) and two protectant + systemic chemicals (azoxystrobin and dimethomorph) (Table 5.1). In addition metalaxyl-M + mancozeb (protectant + systemic) was also evaluated based on the recommendation of Mr Julian Raine (New Zealand Boysenberry Council Ltd). This combination had not been included in the *in vitro* studies in this Chapter but the two component chemicals, mancozeb and metalaxyl, had been evaluated for ability to inhibit spore germination and infection (Section 4.2.1b) in the previous chapter (Chapter 4). Li700® (Loveland Products, Inc., USA) was used as the wetting agent with metalaxyl-M + mancozeb, azoxystrobin, mandipropamid and dimethomorph at a rate of 250 mL/100 L whereas no wetting agents were used with the other fungicides based on the recommendations of R. French (Fruitfed Supplies, 6 Gladstone Road, Richmond, Nelson, pers. comm., 2013).

For each of the fungicides, six replicate plants were sprayed once to complete coverage (approx. 70 mL per plant) but not to run-off (Fourie, 2009) with the selected fungicide applied at the recommended field rate (Table 5.1), and plant then left to dry. Spraying was done using 2 L pressure sprayer bottles (Aqua Systems, New Zealand, Max. pressure 245 KPa, brass nozzle) on a sunny day in late January 2014. The stems were marked with a colour identifier to identify the leaves which were fully open at spraying and those which developed subsequently. The plants were arranged in a randomised block design in the shadehouse. On the following day, the abaxial side of the central leaflet of young leaves (three replicates per plant) of approximately the same size were inoculated each with a 10 µL drop (200 spores) of freshly prepared *P. sparsa* spore suspension in the late afternoon. The *P. sparsa* spore suspension was prepared as described in Section 2.2.3.2. The inoculation point on each leaf was marked with a tiny dot using a permanent marker on the top of the leaf. Each inoculated leaf was carefully covered with a clean plastic bag in which a moistened filter paper disc (90 mm) was placed to ensure high relative humidity for spore germination. The plastic bags were removed carefully after 24 h. Two control treatments were set up, whereby the plants were sprayed with sterile water with the positive control inoculated with a *P. sparsa* spore suspension drop and the negative control inoculated with a drop of sterile water. Tinytag® relative humidity (0-95%) and temperature (-40 – +75ºC) data loggers (Gemini Data Loggers, UK) were placed in the shadehouse to record RH and temperature for the duration of the experiment.

The number of inoculated leaves (out of 3) per plant which developed characteristic *P. sparsa* leaf lesions (incidence) and the lesion size were assessed for each treatment after 18 and 44 days post inoculation (dpi). Further, the development of lesions on the newly developed but not inoculated leaves was also assessed after 44 dpi. Lesion area (mm^2) was assessed using the Digimizer® (Version 4) image analysis software from digital photographs of each lesion. To enable calibration of the digital images, actual measurements of the length, along the midrib of

the leaves were carried out. Incidence data (the proportion of leaves which developed *P. sparsa* lesions per plant) were statistically analysed by General Linear Model (GLM) in Minitab Ver. 16. The data was arcsine transformed prior to analysis to satisfy the assumption of homogeneity of variance. Data for lesion size were analysed by Analysis of Variance (ANOVA) using GenStat Ver.16. When factors were significant, means were compared between treatments using Tukeys test at $P \leq 0.05$ for incidence data and Fisher's protected LSD at $P \leq 0.05$ for data for the lesion size. No lesions were observed in the negative control and this was excluded from the analysis.

To confirm whether the fungicide treatments had prevented infection as well as lesion development tissue samples were taken 44 dpi from three randomly selected replicate plants from each treatment in which symptoms did not develop and assessed for latent infection using nested PCR (Chapter 3). The top most inoculated leaf for each of the plants was selected for sampling with the samples collected from the petiole of that leaf, the stem just above and just below the point of attachment of the petiole with the main stem, and a sample from the crown of that plant (Figure 5.2). DNA extraction and PCR was conducted as described in Sections 3.2.1.2.Bi and 3.2.1.3.

Figure 5.2 Photograph indicating the tissues collected for PCR verification of latent *Peronospora sparsa* infection of asymptomatic boysenberry plants inoculated with *P. sparsa* following treatment with different fungicides. The three inoculated leaves are indicated by the red arrows, the petiole of the top most inoculated leaf sampled indicated by the black circle, with the stem pieces sampled from above and below the inoculated leaf petiole indicated by the black arrows.

5.2.3.2 Evaluation of systemic fungicides for prolonged protection against *P. sparsa* spore infection

Three systemic fungicides phosphorous acid, potassium phosphite and acibenzolar-s-methyl were selected to evaluate their efficacy to protect boysenberry plants from *P. sparsa* spore infection after different times of application. Forty, five months old pathogen free boysenberry plants (cv. Mapua) were potted in 2.5 L pots in 12-14 month potting mix (Appendix A.2) and moved into a clean shadehouse at Lincoln University for 1 week to acclimatise.

Eight replicate plants were sprayed with one of the three fungicides as described in Section 5.2.3.1 in late January, 2014. Plants were arranged in a randomised block design in the shadehouse. On the following day (25 January, 2014), the abaxial side of the central leaflet of three young leaves per plants were inoculated with a 10 µL drop (200 spores) of freshly prepared *P. sparsa* spore suspension in the late afternoon as described in Section 5.2.3.1. Two control treatments were set up, whereby the plants were sprayed with sterile water, with the positive control inoculated with *P. sparsa* spore suspension drop and the negative control with a drop of sterile water. Relative humidity and temperature were recorded as described in Section 5.2.3.1.

After each inoculation time, the number of leaves (out of three) per plant which developed characteristic *P. sparsa* leaf lesions (incidence) and the lesion size for each treatment was assessed 18 days post inoculation (dpi) as described in Section 5.2.3.1, and the inoculated leaves were removed from the petiole to avoid systemic infection of the plant. The second inoculation was conducted four weeks after the initial fungicide application (19 February, 2014) in the same way using another three separate leaves on each plant. Assessments were conducted in the same way. The third inoculation was carried out seven weeks after the initial fungicide application (18 March, 2014). At each inoculation time, the stems were marked with different colour identifiers to separate the leaves which were fully opened at that inoculation time and those which developed subsequently. Further, the development of lesions on the newly developed but not inoculated leaves was also assessed 45 days after the first inoculation.

Incidence data (proportions of leaves with *P. sparsa* lesions per plant) were arcsine transformed to satisfy the assumption of homogeneity of variance prior to analysis using the GLM in Minitab Ver. 16. Data obtained for lesion size were analysed by ANOVA using GenStat Ver.16. When factors were significant, means were compared among treatments using Tukeys test at $P \leq 0.05$ for the incidence data and Fisher's protected LSD at $P \leq 0.05$ for the data for lesion size. No lesions were observed in the negative control and this was excluded from the analysis.

To confirm whether the fungicide treatments had prevented infection as well as lesion development, tissue samples were taken 63 dpi after the first inoculation from three randomly selected plants from each treatment in which symptoms did not develop and assessed for latent infection using nested PCR. A stem piece just above the top most inoculated leaf at the first

inoculation (January) and the third inoculation (March) was sampled. DNA extraction and nested PCR was conducted as described in Section 3.2.1.3.

5.2.4 Evaluation of fungicides to prevent dryberry production on systemically infected plants

Three systemic fungicides, phosphorous acid, potassium phosphite and acibenzolar-s-methyl, were evaluated for their efficacy in preventing systemic infection developing from canes to flower buds and developing berries in systemically infected boysenberry plants. Sixty, 2 year old systemically infected potted (12-14 month potting mix; Appendix A.2) boysenberry (cv. Mapua) plants which had 1-2 floricanes were used. The plants were moved (28 October, 2013) to the Horticulture amenity block area at Lincoln University to a well shaded area similar to the conditions in the shadehouse and covered with a bird-proof net and allowed to acclimatise for 1 week prior to the start of the experiment. Fifteen plants per treatment were arranged in a randomised block design. Fungicide sprays were applied three times corresponding to different plant physiological growth periods, that is, at pre-flowering (5 November), flowering (19 November) and green, hard unripe berry stage (2 December) in 2013. Whole plants were sprayed (130 mL per plant) to ensure complete coverage of the plants at the recommended field rate (Table 5.1) as described in Section 5.2.3.1, and then left to dry. The control treatment plants were sprayed with an equal volume of sterile water.

To evaluate the effect of a metalaxyl-M + mancozeb spray to prevent spore infection of young berries, five randomly selected plants from each of the treatments were sprayed when the berries were still green and hard and half the size of mature berries (6 December, 2014) (Figure 5.3A). The whole plants were sprayed to ensure that all of the berries were treated. Li700 was used as the wetting agent with metalaxyl-M + mancozeb. The remaining 10 plants for each treatment were left untreated. On the day after application of the metalaxyl-M + mancozeb, boysenberry plants with numerous sporulating *P. sparsa* lesions were transferred to the experimental area and shaken vigorously to disperse spores and provide an inoculum source. After 2 h, these sporulating plants were removed.

Dryberry incidence, determined as the number of berries with symptoms (dryberries) out of the total number of berries per plant (dryberry proportion) was assessed on the 26 December and 31 December, 2014. Dryberry disease was assessed using a scale based on the number of drupelets which were dried, with 0 dried drupelets = 0, 1-10 dried drupelets= 1, 11-30 dried drupelets = 2, 31-50 = 3 and >50 dried drupelets = 4. Each berry consisted of 37-54 drupelets. Dryberry incidence data were arcsine transformed to satisfy the assumption of homogeneity of variance prior to analysis by GLM using Minitab Ver. 16. Data obtained for mean score per plant was analysed using GLM in Minitab Ver. 16.

In addition, asymptomatic berries at the red partially ripe stage (Figure 5.3B) (1 replicate from each plant) were detached on the final assessment day (31 December) to evaluate whether the

berries were systemically infected. This resulted in 10 replicates for each of the phosphorous acid, potassium phosphite, acibenzolar-s-methyl and untreated control treatments and five replicates for the each of the metalaxyl-M + mancozeb in combination with phosphorous acid, potassium phosphite, acibenzolar-s-methyl or untreated control treatments. DNA extraction was carried out from the berry tissue and nested PCR conducted as described in Section 3.2.1.2.Bi. Berry stem and calyx/sepals were not used for DNA extraction.

Figure 5.3 (A) The green, hard, unripe berry stage at which the metalaxyl-M+mancozeb protectant spray was applied to assess ability to reduce *Peronospora sparsa* spore infection in 2 years old boysenberry plants, (B) asymptomatic berries at the red, partially ripe stage used to assess for *P. sparsa* latent infection using nested PCR.

5.3 RESULTS

5.3.1 Production of pathogen free boysenberry plants

5.3.1.1 Evaluation of control strategies for production of pathogen free boysenberry plants

Survival of tissue culture plants at the multiplication step was highest in the untreated control (20 out of the 27 initiated), with survival for the heat and heat + fungicides treatments (17 of the 41 initiated and 16 of the 33 initiated, respectively) being lower and similar to each other. The tissue culture plants derived from the untreated control boysenberry plants had longer shoots after eight weeks ranging from 10 - 55 cm, whilst the shoots of tissue culture plants derived from both heat and heat + fungicide treatments were between 10 - 35 cm. Fungal contamination accounted for some of the loss of plants at both the initiation and multiplication stages, with the fungi seen to grow around the plantlet in the medium (Figure 5.4). The highest number of tissue culture plants were produced from the heat only treated plants (Table 5.2). All 125 plants across the treatments that were potted survived.

Table 5.2 Survival rate of boysenberry tissue culture (TC) plants derived from boysenberry plants treated with heat (34ºC) or heat + fungicide compared with untreated control treatment. (n = numbers of TC plants set up in the initiation step for each treatment).

Treatment	Total no. of TC plants surviving for multiplication*	Total no. of TC plants potted
Heat only	17 (n=41)	47
Heat + Fungicides	16 (n=33)	42
Untreated Control	20 (n=27)	36

*Some tissue culture plants did not develop from the initiation step (no budding) while others were contaminated.

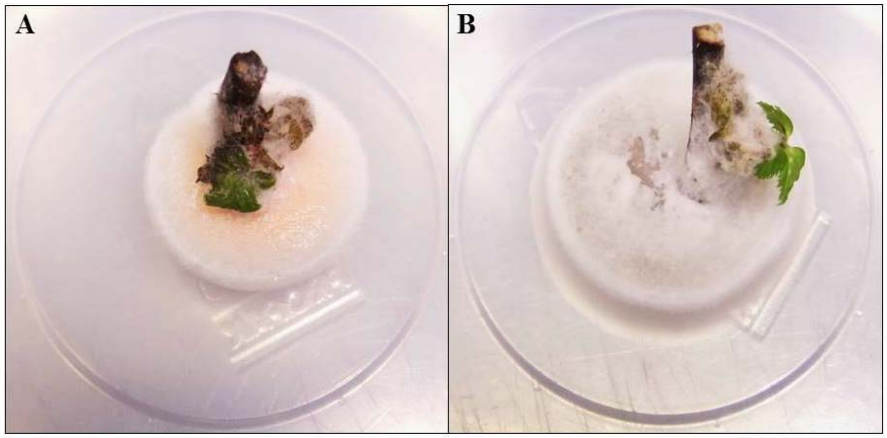

Figure 5.4 Fungal contamination of boysenberry tissue culture plants growing in initiation culture media which were produced from the (A) heat, and (B) heat + fungicides treated plants.

None of the plants in the initiation or the multiplication medium produced *P. sparsa* symptoms. The plants did not produce roots in the initiation medium (Figure 5.5A-C), however all produced roots abundantly in the multiplication medium (Figure 5.5D-F).

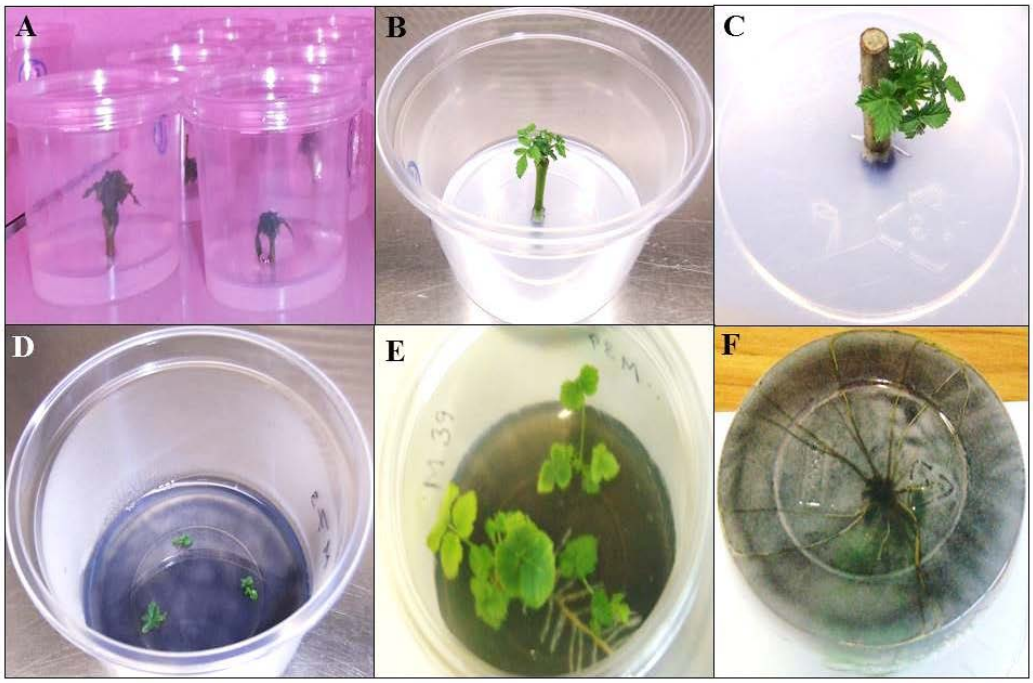

Figure 5.5 The tissue culture boysenberry (cv. Mapua) plants (A, B, C) 2-3 weeks after initiation in the MT initiation medium and (D, E, F) 5 weeks after multiplication in the MT multiplication medium.

No *P. sparsa* symptom expression was seen in any of the 125 potted plants during the misting phase (Figure 5.6A and B). However, in March 2013, 22 weeks after potting up (2 weeks in little pots and a further 20 weeks in the 2.5 L pots) plants in the shadehouse developed systemic leaf lesions characteristic of *P. sparsa* (Figure 5.6C). These were observed in 100% of the untreated control plants, 13% of the heat only and 17% of the heat + fungicide treated plants.

Figure 5.6 Boysenberry (cv. Mapua) plants (A and B) transplanted into 7 cm diameter pots and growing asymptomatically at the misting phase (inside a clean plastic cover and sprayed with a fine mist) after two weeks, and (C) characteristic *Peronospora sparsa* systemic leaf symptoms expressed under shadehouse conditions after planting into 2.5 L pots in the autumn.

5.3.1.2 Verification of the pathogen free status of the tissue culture plants

Seventy six (of the 125) plants both from the heat only and heat + fungicides treatment remained asymptomatic for *P. sparsa* 22 weeks (5.5 months after potting), with each producing 2-3 canes. Testing of composite leaf samples (1/2 disc from each cane per plant) showed that only 2% (n=2) of the asymptomatic plants were positive for *P. sparsa* using nested PCR, with

all being from the heat only treated plants. A further five random PCR tests of asymptomatic plants over the 12 month growing season did not detect *P. sparsa* infection in any of the plants.

5.3.2 Effect of fungicides on spore germination and infection *in vitro*

Germination assay:

The EC_{50} values for the fungicides tested differed, with EC_{50} values ranging from 0.3-736 mg a.i./L (Table 5.3). The most effective fungicides at inhibiting *P. sparsa* spore germination were mandipropamid, azoxystrobin and dimethomorph with EC_{50} values of 0.3 mg a.i./L. Fluazinam and chlorothalonil were the next most effective fungicides at inhibiting spore germination (EC_{50} of 1.0 and 1.8 mg a.i./L, respectively). The least effective fungicides at reducing spore germination were phosphorus acid and potassium phosphite with EC_{50} values of 181 and 736 mg a.i./L, respectively.

Table 5.3 The mean EC_{50} (mg a.i./L) values for different fungicides for *in vitro* inhibition of *Peronospora sparsa* spore germination.

Fungicides	Activity	EC_{50} values
Mandipropamid	Protectant	0.3 (0.07-0.8)*
Azoxystrobin	Protectant+Systemic	0.3 (0.08-0.8)
Dimethomorph	Protectant+Systemic	0.3 (0.08-0.8)
Fluazinam	Protectant	1.0 (0.32-2.7)
Chlorothalonil	Protectant	1.8 (0.62-4.9)
Cymoxanil	Systemic	10.1 (3.74-26.1)
Acibenzolar-s-methyl	Systemic	32.2 (12.37-83.8)
Dichlofluanid	Protectant	65.4 (25.32-172.9)
Phosphorous acid	Systemic	181 (69.68-497.5)
Potassium phosphite	Systemic	736 (273.15-2203.9)

*The confidence intervals for each fungicide are provided in parentheses.

Infection assay:

There was a highly significant effect of fungicide ($P<0.001$) and interaction between fungicide and concentration ($P<0.001$) on the incidence of *P. sparsa* infection of boysenberry leaf discs (Table 5.4; Appendix D.2). The most effective fungicides were mandipropamid, azoxystrobin and dimethomorph which inhibited *P. sparsa* infection of the leaf discs across all the concentrations tested. This was followed by fluazinam and chlorothalonil which inhibited *P. sparsa* infection at all but the lowest (1 µg/mL) concentration. The least effective fungicides were acibenzolar-s-methyl, dichlofluanid, phosphorus acid and potassium phosphite with lesions developing on all leaf discs treated with 100 µg/mL, and for acibenzolar-s-methyl even at the highest concentration (1000 µg/mL) tested.

A similar highly significant effect of the fungicide ($P<0.001$) and the interaction between fungicide and concentration ($P<0.001$) on the disease sporulation score was shown. The most effective fungicides, which showed a dose-related response resulting in an overall disease sporulation score of 0, were mandipropamid, azoxystrobin, dimethomorph, fluazinam and

chlorothalonil (Table 5.5). This was followed by cymoxanil and dichlofluanid with disease sporulation scores of 3 and 4, respectively. The least effective fungicides were acibenzolar-s-methyl, phosphorus acid and potassium phosphite, all with the maximum sporulation score of 6. Full statistical analyses are presented in Appendix D.2.

Table 5.4 Effect of different concentrations of 10 fungicides on *Peronospora sparsa* disease incidence on boysenberry leaf discs. Incidence was assessed as number of leaf discs (out of 3) which developed lesions.

Fungicides	Incidence at different fungicide concentrations (µg/mL)[1]					Fungicide effect on incidence
	1	10	50	100	1000	
Mandipropamid	0	0	0	0	0	0
Azoxystrobin	0	0	0	0	0	0
Dimethomorph	0	0	0	0	0	0
Fluazinam	3	0	0	0	0	3
Chlorothalonil	3	0	0	0	0	3
Cymoxanil	3	3	3	0	0	9
Acibenzolar-s-methyl	3	3	3	3	3	15
Dichlofluanid	3	3	3	3	0	12
Phosphorous acid	3	3	3	3	0	12
Potassium phosphite	3	3	3	3	0	12

[1]Confidence intervals (95%) at incidence 1.00 = (0.292-0.000), 0.00 = (0.000-0.708).

Table 5.5 Effect of different concentrations of 10 fungicides on *Peronospora sparsa* sporulation score (0=no spores, 1=1-5 spores, 2=6-12 spores, 3=13-50 spores, 4=51-100 spores, 5=101-1000 spores and 6=>1000 spores) on boysenberry leaf discs.

Fungicides	Sporulation score at different fungicide concentrations (µg/mL)[1]					Fungicide effect on Sporulation score[1]
	1	10	50	100	1000	
Mandipropamid	0.0	0.0	0.0	0.0	0.0	0.0
Azoxystrobin	0.0	0.0	0.0	0.0	0.0	0.0
Dimethomorph	0.0	0.0	0.0	0.0	0.0	0.0
Fluazinam	2.0	0.0	0.0	0.0	0.0	0.0
Chlorothalonil	4.0	0.0	0.0	0.0	0.0	0.0
Cymoxanil	5.0	4.0	3.0	0.0	0.0	3.0
Acibenzolar-s-methyl	6.0	6.0	6.0	6.0	5.0	6.0
Dichlofluanid	5.0	5.0	4.0	2.0	0.0	4.0
Phosphorous acid	6.0	6.0	6.0	5.0	0.0	6.0
Potassium phosphite	6.0	6.0	6.0	5.0	0.0	6.0

[1]Medians obtained from the Kruskal-Wallis non-parametric test are indicated in the table.

5.3.3 Evaluation of fungicides to prevent *P. sparsa* spore infection

5.3.3.1 Prevention of *P. sparsa* spore infection of pathogen free boysenberry plants

No disease was observed in any of the uninoculated negative control plants and therefore these were not included in the analysis. There was a significant (P=0.005) fungicide effect on *P. sparsa* disease incidence on young pathogen free boysenberry plants. However, there was no significant effect of assessment time (P=0.600). Azoxystrobin, metalaxyl-M + mancozeb and dimethomorph significantly (P<0.005) reduced the incidence of *P. sparsa* infection compared with the untreated positive control (Table 5.6).

Leaf lesion size (mm^2) was not significantly affected (P=0.566) by the fungicides, with lesion size ranging from 0 for azoxystrobin, metalaxyl-M + mancozeb, and dimethomorph to 23.3 mm^2 for the untreated positive control (Table 5.6). Lesions which developed on acibenzolar-s-methyl, potassium phosphite, chlorothalonil and mandipropamid ranged from 4.5-8.4 mm^2 with lesions on phosphorous acid treated plants being 15.6 mm^2. The full statistical analysis is presented in Appendix D.3.

Table 5.6 Mean effects of fungicides on the incidence (proportion of leaves) and lesion size (mm^2) of *P. sparsa* lesions after inoculation of young boysenberry (cv. Mapua) plants.

Fungicides	Incidence (proportion of leaves with *P. sparsa* lesions/plant)	Lesion size $(mm^2)^2$
Negative control*	0	NA
Positive control	0.33 a[1]	23.3
Azoxystrobin	0.00 b	0.0
Metalaxyl-M + Mancozeb	0.00 b	0.0
Dimethomorph	0.00 b	0.0
Mandipropamid	0.06 ab	5.8
Chlorothalonil	0.06 ab	4.6
Potassium phosphite	0.14 ab	8.4
Acibenzolar-s-methyl	0.17 ab	4.5
Phosphorous acid	0.19 ab	15.6

[1]Values within a column followed by the same letter are not significantly different according to Tukey's HSD test (P=0.05).
[2]The effect of fungicide on lesion size was not significant (P=0.566). * No disease lesions observed in the negative control and therefore not included in the analysis.

In addition, none of the plants from the azoxystrobin, metalaxyl-M + mancozeb, dimethomorph, chlorothalonil and acibenzolar-s-methyl treatments developed *P. sparsa* leaf lesions on leaves which developed after the spray application. However, for the potassium phosphite, phosphorous acid and mandipropamid treatments 17% of plants and for the untreated control plants 50% of plants developed leaf lesions on leaves which developed after spray application.

The relative humidity (RH) in the shadehouse during the inoculation period was 79%, and ranged between 30-100 % over the day (25 January, 2014). The mean temperature during the inoculation period was 19ºC and daily range between 11.5-25ºC (Appendix D.7).

The effect of the different fungicide treatments on the incidence of *P. sparsa* latent infection in tissue sampled from the petiole of the top most inoculated leaf, stem above and below the point of attachment of the petiole of the inoculated leaf and crown of three replicate asymptomatic boysenberry plants from each treatment determined using *P. sparsa* specific nested PCR, is presented in Table 5.7 and Figure 5.7. There was significant effect (P=0.008) of fungicide spray on the incidence of *P. sparsa* latent infection across all the tissues sampled. Dimethomorph significantly reduced (Table 5.7; Appendix D.3) the incidence of latent infection of *P. sparsa* compared to the positive control, with no other fungicide treatment having a significant effect. None of the samples from the negative control were positive with the nested PCR.

Table 5.7 Mean effect of fungicides on the incidence of *P. sparsa* latent infection in inoculated asymptomatic boysenberry (cv. Mapua) plants assessed using nested PCR. Incidence determined from four tissue samples in each of three plants (n=12).

Fungicides	Incidence (proportion of tissues +ve PCR per plant)
Negative control	0
Positive Control	0.83 a[1]
Dimethomorph	0.17 b
Acibenzolar-s-methyl	0.25 ab
Chlorothalonil	0.25 ab
Mandipropamid	0.33 ab
Metalaxyl-M + Mancozeb	0.42 ab
Phosphorous acid	0.58 ab
Azoxystrobin	0.58 ab
Potassium phosphite	0.75 a

[1]Values followed by the same letter are not significantly different according to Tukey's HSD test at P=0.05.

In all treatments, except dimethomorph and chlorothalonil, latent infection was detected in the petiole and the stem above and/or below in at least one of the plant sampled (Figure 5.7). Although none of the petiole samples or the stem above the inoculated leaf from dimethomorph treated plants were positive for *P. sparsa* latent infection, the crown and lower stem sample for one plant was positive (Figure 5.7).

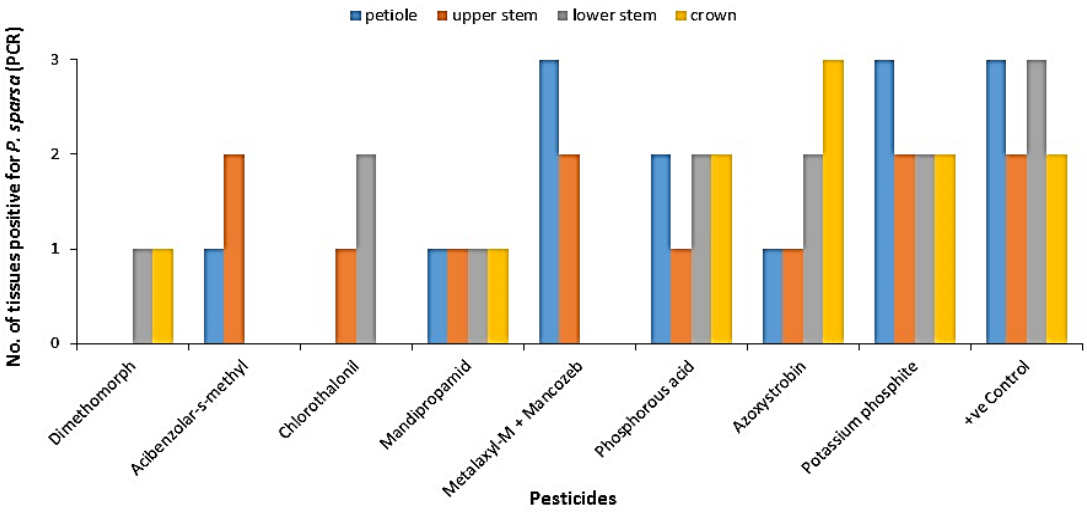

Figure 5.7 Effect of different fungicide applications on the percentage of each tissue type sampled from asymptomatic boysenberry plants giving a positive PCR reaction for *Peronospora sparsa* to evaluate the spread of the latent *P. sparsa* infection. Tissue types sampled were petiole from the top most inoculated leaf, stem above and below the point of attachment of the petiole of inoculated leaf and crown from three replicate asymptomatic plants.

For chlorothalonil, although none of the petioles were positive for *P. sparsa*, latent infection was detected in both the upper (1 of the 3) and lower (2 of the 3) stems samples. For the inoculated control plants and the plants treated with potassium phosphite, most of the samples from all tissue types were positive for *P. sparsa*.

5.3.3.2 Evaluation of systemic fungicides for prolonged protection against *P. sparsa* spore infection

No disease was observed in any of the uninoculated negative control plants and these were not included in the analysis. Incidence was significantly affected by inoculation time (P=0.003), with significantly higher disease incidence after the third inoculation conducted 2 months after fungicide application compared with that observed for the previous inoculations conducted at the time of fungicide application and 1 month later (Table 5.8). There was no significant effect of fungicide or interactions between fungicide and inoculation period (P=0.230, P=0.923, respectively; Appendix D.4) on disease incidence.

Lesion size was not significantly affected by fungicide treatment (P=0.205; Table 5.8), inoculation time (P=0.253) or interactions between them (P=0.782). Full statistical analyses are shown in Appendix D.4.

Table 5.8 Mean effects of systemic fungicides on the incidence (number of leaves) and lesion size (mm^2) of *P. sparsa* infection on young boysenberry (cv. Mapua) plants inoculated 0, 1 or 2 months (mth) after application of fungicides.

Systemic fungicides	Incidence (proportion of leaves with *P. sparsa* lesions/plant) at each inoculation time				Lesion size (mm^2)[2]			
	0 mth	1 mth	2 mth	Fungicide effect	0 mth	1 mth	2 mth	Fungicide effect
Positive control	0.42	0.21	0.63	0.42	17.5	5.7	8.6	10.6
Potassium phosphite	0.17	0.17	0.42	0.25	5.5	0.2	4.2	3.3
Acibenzolar-s-methyl	0.25	0.38	0.58	0.40	6.4	7.1	5.4	6.3
Phosphorous acid	0.38	0.29	0.58	0.42	11.3	9.6	5.0	8.6
Inoculation time effect	0.30 b[1]	0.26 b	0.55 a		10.2	5.8	5.2	

[1]Values within the rows followed by the same letter are not significantly different according to Tukey's HSD test (P= 0.05). The main effect of inoculation time was significant (P=0.003) and no effect of fungicide or interactions (P=0.230 and P=0.923, respectively) on incidence.
[2]No significant effect of fungicide (P=0.205) inoculation time (P=0.253) or interactions (P=0.782) on lesion size.

Leaf lesions characteristic of *P. sparsa* were observed to develop on leaves which developed after the last inoculation on 38% of plants sprayed with the acibenzolar-s-methyl compare with 25% of plants from the potassium phosphite, phosphorus acid and the untreated positive control treatments.

The mean relative humidity (RH) in the shadehouse during the inoculation period for the first (25 January, 2014), second (19 February, 2014) and third (18 March, 2014) inoculations was 79, 51 and 90%, respectively and ranged between 30-100, 0.2-100 and 90-100% respectively over the day. The mean temperature during the inoculation period for the 3 days was 19, 27 and 24ºC, and ranged between 11.5-25, 19-30 and 19-28ºC over the day for the first, second and third inoculations, respectively (Appendix D.7).

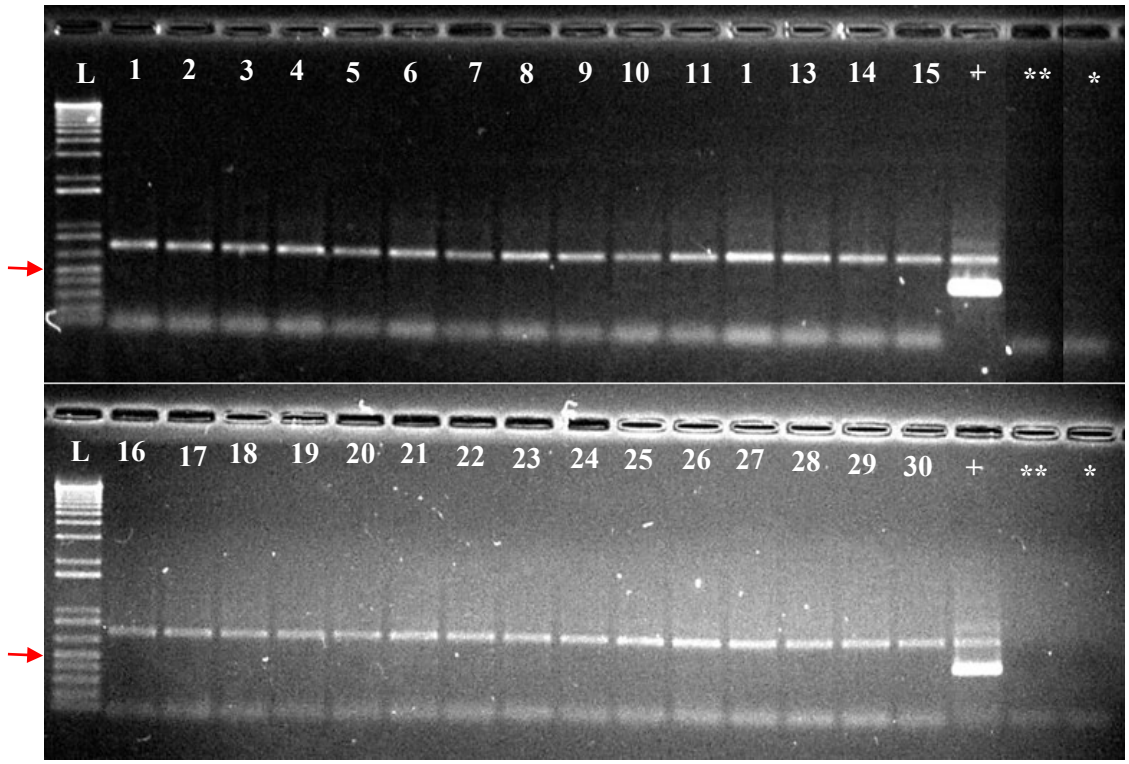

Figure 5.8 Representative 1% agarose gel showing the results of the secondary PCR of the nested PCR for the stem samples taken from the region above the leaf inoculated at 0 months and 2 months after application of different fungicides or control treatments of asymptomatic boysenberry plants. Lanes 1-6 from phosphorus acid, 7-12 from potassium phosphite, 13-18 acibenzolar-s-methyl, 19-24 from negative control and 25-30 from positive control treated plants; ** diluted control (with PCR water) from primary PCR step of nested PCR (**) and * negative control, L = 1+KB DNA size marker. Red arrows indicate the expected molecular weight of the band for *Peronospora sparsa*. Each pair from 1-30 represent one replicate plant sample with the odd numbered samples being the stem from the 2 month sampling and the even numbered sample being the stem from the 0 month sampling.

None of the tissues sampled from the stem of three asymptomatic plants per fungicide treatment, taken from above the point of attachment of the top most leaf inoculated at 0 months and 2 months after application of the fungicides gave a positive result for detection of *P. sparsa* by PCR (Figure 5.8).

5.3.4 Evaluation of fungicides to prevent dryberry production on systemically infected plants

There was no significant interaction (P=0.855) between fungicide and assessment time on incidence of dryberry caused by *P. sparsa*. There was a significant effect of fungicide application (P<0.001) on dryberry incidence, with phosphorus acid and acibenzolar-s-methyl significantly reducing dryberry incidence compared with the untreated control (Table 5.9). There was a significant effect (P=0.003) of assessment time on dryberry incidence with significantly higher dryberry incidence at the 31 December assessment compared with the 26 December assessment. There was no significant effect (P=0.854) of the protectant metalaxyl-M+mancozeb spray applied to the berries on dryberry incidence (data not shown).

There was no significant effect of assessment time (P=0.234) or interaction between fungicide and assessment time (P=0.638) on mean dryberry drupelet score (data not shown). There was a significant effect (P=0.002) of fungicide application on the mean dryberry drupelet score, with phosphorus acid significantly reducing mean dryberry drupelet score compared with the untreated control (Table 5.9). Application of the protectant metalaxyl-M+mancozeb spray applied to the berries had no significant effect (P=0.950) on mean dryberry drupelet score(data not shown). Full statistical analysis is presented in Appendix D.5.

Table 5.9 Mean effects of application of different fungicides on the incidence (proportion of dryberries) and dryberry drupelet score (scale based on the number of symptomatic dryberry drupelets) of dryberry on systemically infected boysenberry (cv. Mapua) plants assessed on the 26 and 31 December.

Fungicides	Disease incidence at each assessment time		Mean fungicide effect	Dryberry drupelet score
	26 Dec	31 Dec		
Control	0.18	0.25	0.22 a[1]	1.7 A[2]
Phosphorous acid	0.07	0.11	0.09 c	0.8 B
Acibenzolar-s-methyl	0.11	0.19	0.14 bc	1.1 AB
Potassium phosphite	0.17	0.28	0.23 ab	1.2 AB
Mean assessment time effect	0.13 e	0.21 d		

[1]Values within the rows or columns followed by the same letter are not significantly different according to Tukey's HSD test at P = 0.05. There was a significant effect of fungicide treatment (a-b; P < 0.001) and assessment times (e and d; P=0.003) on incidence of dryberries.
[2]There was a significant effect of fungicides (A-B; P=0.002) on dryberry drupelet score of dryberry disease.
There was no significant effect of protectant spray on both incidence (P=0.854) and dryberry drupelet score (P=0.950)-therefore data was not shown.

The maximum percentage of asymptomatic red berries (20%) which were positive for *P. sparsa* using nested PCR were from the phosphorus acid, potassium phosphite and the untreated control plants which were also sprayed with metalaxyl-M+mancozeb at the berry stage (Table 5.10). No berries were positive for *P. sparsa* from acibenzolar-s-methyl treated plants which were also sprayed with metalaxyl-M+mancozeb at the berry stage, whilst 10% were positive when not sprayed with metalaxyl-M+mancozeb. Similarly, 10% of berries from the phosphorus acid and the untreated control plants not treated with metalaxyl-M+mancozeb were positive for *P. sparsa* whilst no berries were infected from the potassium phosphite treated plants not sprayed with metalaxyl-M+mancozeb.

Table 5.10 The effect of application of different fungicides to *Peronospora sparsa* systemically infected boysenberry (cv. Mapua) plants and metalaxyl-M+mancozeb (met/man) spray at the berry stage on latent infection by *P. sparsa* of asymptomatic red ripe berries assessed using nested PCR.

Fungicides	Percentage of berries +ve for *P. sparsa* by PCR	
	Without met/man	With met/man spray
Control	10	20
Phosphorous acid	10	20
Potassium phosphite	0	20
Acibenzolar-s-methyl	10	0

5.4 DISCUSSION

A method for producing *Peronospora sparsa* pathogen free boysenberry propagation material was developed, with heat alone or in combination with fungicide (phosphorus acid and mancozeb) treatment of systemically infected boysenberry plants prior to the use of new shoot tips for tissue culture being effective. Similar low levels of expression of systemic leaf lesions characteristic to *P. sparsa* were seen in the tissue culture plants from both treatments (13% from the heat only treatment and 17% from the heat and fungicide treatment) indicating that heat only treatment would be sufficient to reduce *P. sparsa* infection of the subsequently produced tissue culture plants. However, both of the asymptomatic plants which gave a positive *P. sparsa* reaction in the PCR test were from the heat only treatment suggesting that the heat+fungicide treatment maybe more effective. Though, due to the potential of producing fungicide resistance strains of the pathogen, which could be disseminated widely with the planting stock, the heat only treatment is suggested to be a better long-term option. This method along with the PCR confirmation of the uninfected status provides a valuable tool for the production of boysenberry planting material free of *P. sparsa* infection. This is an important outcome considering that anecdotal evidence and the findings of Chapters 2 and 3 indicate that a large proportion of material currently available to growers for setting up new gardens or replanting areas in existing gardens is likely to be systemically infected.

The exact mechanism by which the heat treatment reduces the infection of the growing cane tip tissue which is subsequently used to propagate the tissue culture plants is unknown. Most published studies which have investigated the use of heat treatment to limit pathogens from the growing tips of systemically infected host plants have done so for the sole purpose of producing clean plants rather than understanding the underlying principles of heat therapy (Baumann, 1982; Pateña *et al*., 2004; Grammatikaki *et al*., 2006 cited in Panattoni and Triolo, 2010; Wang *et al*., 2008). However, some of the suggested mechanisms reported by Nyland and Goheen (1969) by which heat therapy eliminates virus infection are: 1. high temperatures cause the destruction of essential chemical activities in both the virus and the host, but the host is better

able to recover from the damage, 2. at higher temperatures the virus is inactivated with no synthesis of new virus particles, 3. infectivity of the virus is lost at high temperatures (35°C) without significant changes in other virus properties. Since *P. sparsa* is a biotrophic pathogen, similar mechanisms may account for the elimination of *P. sparsa* from the host tissue by the heat treatment. However, in the current study the temperature used (34°C) was selected as it was postulated that at this temperature the growth of *P. sparsa* into the shoot tips would be restricted whilst allowing plant growth that could subsequently be used for tissue culture.

This is the first report on the *in vitro* efficacy of selected fungicides belonging to different chemical classes and activities to inhibit *P. sparsa* spore germination and infection on boysenberry leaf discs. The aim of this *in vitro* screening was to select the most potent fungicides for subsequent whole potted plant experiments in which infection was either systemic or initiated by spores. Chlorothalonil, mandipropamid, and fluazinam (protectants) and azoxystrobin and dimethomorph (protectant and systemic activity) were the most effective at inhibiting *P. sparsa* spore germination with the EC_{50} values not exceeding 2 mg active ingredient /L. Mandipropamid, azoxystrobin and dimethomorph also completely inhibited lesion development in the infection assay even at the lowest concentration tested (1 µg/mL) indicating the potential of these fungicides at preventing spore infection. Correspondingly, studies conducted on the grapevine downy mildew pathogen, *Plasmopara viticola,* found that 0.05% azoxystrobin (Nithyameenakshi *et al*., 2006) or 0.36% azoxystrobin+mancozeb (Pannerselvam Ahila devi and Prakasam, 2013) were the most effective at inhibiting spore germination *in vitro*. However, they did not test mandipropamid and dimethomorph. Similarly, azoxystrobin was the most effective of the three fungicides tested (azoxystrobin, trifloxystrobin, and kresoxim-methyl) at inhibiting spore germination of *Sclerospora graminicola* in pearl millet (Sudisha *et al*., 2005). None of these authors however tested either mandipropamid or dimethomorph. Cohen *et al*. (2008) found mandipropamid was more effective than dimethomorph in suppressing spore germination of lettuce downy mildew pathogen, *Bremia lactucae,* although azoxystrobin was not included in their study. The EC_{50} values for these chemicals are all within the recommended field application rates. However, the results from *in vivo* germination assays may not relate to disease control efficacy in pot or field experiments.

The least effective fungicides at reducing spore germination were the systemic fungicides with the EC_{50} values ranging 10.1-736 mg a.i./ L. For acibenzolar-s-methyl, phosphorus acid and potassium phosphite this was not unexpected as these systemic fungicides have not been reported to act directly to inhibit spore germination, but are reported to stimulate the plant's natural defence response against pathogen attack (Hukkanen, 2008). However, phosphorous acid has been reported to inhibit mycelial growth of *Phytophthora* spp. *in vitro* (Horner and Hough, 2013). In addition, phosphorus acid reduced sporulation, disease incidence and severity by *Peronosclerospora sorghi* on maize, but had no effect on *in vitro* spore germination (Panicker and Gangadharan, 1999). Hukkanen (2008) demonstrated that no lesions developed

on *P. sparsa* inoculated leaves detached from artic bramble plants 4 days after spraying with phosphorous acid or BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester syn. acibenzolar-s-methyl; http://hazmap.nlm.nih.gov/category-details?id=3435&table=copytblagents). This result indicated that prior treatment with these chemicals was effective at priming the plant's immunity to avoid or limit spore infection. In contrast, in the current study spores were mixed with each fungicide solution and this used to inoculate detached leaf discs. Therefore, an inhibitory effect by these fungicides was unlikely to be seen as there was no time allowed for stimulation of the plant natural defences to occur before being challenged by the pathogen. Further, leaf discs detached from living plants were used and most likely limited the ability of the plant tissue to respond to the chemical. Cymoxanil has also been shown to inhibit zoospore production by *Phytophthora capsici* (Keinath, 2007) in addition to reducing infection. For fungicides where the reported activity is not direct inhibition of pathogen growth or germination, pre-screening using *in vitro* assays is not a useful way of selecting fungicides for further pot or field evaluations (Everett and Timudo-Torrevilla, 2007).

Although cymoxanil was more effective than acibenzolar-s-methyl, potassium phosphite or phosphorous acid in inhibiting spore germination and infection of leaf discs, it was not evaluated for ability to inhibit spore infection of potted boysenberry plants. The main reasons for its exclusion were for resource reasons, it is not registered for use on boysenberry in New Zealand and overseas research has shown that it has poor residual activity, so that mixing with a protectant was required (McGrath, 2006). However, cymoxanil mixed with mancozeb has been recommended in New Zealand to control downy mildew disease on onion and garlic (Novachem agrichemical manual, 2015) and mixing with a protectant may be worth evaluating (M. Walter, pers. comm., 2015). Similarly, fluazinam was also not included in the potted plant experiment as it has been shown to leave residues in fruit which would affect market access (Walter *et al*., 2004).

In vitro fungicide efficacy tests are extensively used in pre-screening to identify potentially effective fungicides prior to application in pot or field experiments. The most common *in vitro* test used involves the evaluation of fungicides at a range of concentrations on their ability to inhibit spore germination, generally conducted on glass slides (Neely, 1969). Such assays have been conducted using cavity slides for *P. viticola* by Nithyameenakshi *et al.* (2006) and PannerselvamAhiladevi and Prakasam (2013). However, in the current study, the germination assessments were conducted on boysenberry leaf discs incubated at high humidity since this was the best method to determine germination in Chapter 2. Further, the use of host tissue as the substrate may also have improved the accuracy of the results in this study with reference to activity *in planta*. In addition, the spores were directly mixed with the fungicide solution in both the germination and infection assays instead of making a spore suspension in water prior to adding to the relevant fungicide solution with the aim of minimising errors due to the initiation

of germination processes as the spores imbibed water prior to exposure to the fungicides. Therefore, both *in vitro* tests conducted in this study were designed to provide a more accurate estimation of the effectivity of the fungicides on reducing infection on whole plants.

The *in vitro* results in the current study accurately predicted the efficacy of some of the tested fungicides (azoxystrobin, dimethomorph) to limit spore infection of boysenberry plants in the pot experiment. Azoxystrobin and dimethomorph (Table 5.1) which were two of the most effective fungicides at both inhibiting spore germination and infection in the *in vitro* tests were seen to completely inhibit *P. sparsa* spore infection on young disease-free boysenberry plants in the shadehouse experiment.

Mandipropamid, which was also effective in reducing spore germination and infection *in vitro,* did not reduce spore infection on potted boysenberry plants in the current study. However, mandipropamid has been reported to be highly active against foliar oomycete pathogens, including the grape downy mildew pathogen *Plasmopara viticola* (Blum *et al*., 2010), and *Peronospora* spp. on potted greenhouse coleus (*Solenostemon scutellarioides*) plants (Harlan and Hausbeck, 2011). Similar results for other fungicides have been reported , for example, *in vitro* tests did not accurately predict the field performance of fluazinam in reducing avocado postharvest fruit rot caused by a group of fungi including *Colletotrichum acutatum*, *C. gloeosporioides*, *Botryosphaeria parva*, *B. dothidea* and *Phomopsis* spp. in New Zealand (Everett *et al*., 2005; Everett and Timudo-Torrevilla). However, these *in vitro* tests were conducted on 1.5% water agar or PDA to evaluate inhibition of spore germination or mycelial growth, respectively (Everett *et al*., 2005). The known mode of action of mandipropamid is as a protectant inhibiting spore germination, appresorium development, penetration and hyphal growth, and sporulation of oomycetes pathogens through inhibiting cell wall synthesis (Fernandez-Northcote, 2003; Blum *et al*., 2010). The reasons for the inconsistency between the results of the *in vitro* and potted plant experiments is not known but could be due to fluctuations in environmental conditions affecting fungicide activity, or application issues.

All three fungicides with both systemic and protectant activity (azoxystrobin, metalaxyl-M+mancozeb and dimethomorph) significantly reduced the incidence of *P. sparsa* leaf infections on young disease-free boysenberry plants. In addition, none of the leaves which developed after application of these fungicides developed *P. sparsa* leaf lesions. This indicated that the fungicides were effective at both preventing spore infection of the inoculated leaves through their protectant activity, and infection of newly developed leaves by spores in the shadehouse through their systemic activity. Similar results were reported for metalaxyl-M+mancozeb which provided good control of *P. sparsa* on blackberry leaves (O'Neil *et al.,* 2002) and boysenberry leaves and primocanes (Tate, 1981; Tate and Van Der Mespel, 1983; Walter *et al*., 2004). However, unlike the current study, the plants in those studies were systemically infected. In addition, azoxystrobin alone (Walter *et al*., 2004) or in combination

with phosphorous acid (M. Walter, pers. comm., 2014; A. Rebollar, pers. comm., 2015) provided control of dryberry disease on systemically infected boysenberry plants in New Zealand and blackberry in Mexico respectively, at low disease pressure. Azoxystrobin has also been shown to provide effective control of *Sclerospora graminicola* on pearl millet (Sudisha *et al*., 2005) and *Plasmopara viticola* downy mildew on grapevine (Nithyameenakshi *et al*., 2006) in both greenhouse and field studies. Cohen *et al*. (2008) reported that mandipropamid was more effective than dimethomorph in providing control of the lettuce downy mildew pathogen *Bremia lactucae* in growth chamber, greenhouse and field experiments. This is in contrast to the current study where dimethomorph provided better control than mandipropamid.

Dimethomorph (Sovrin® Flo) was the only fungicide which was seen to reduce the PCR detection of *P. sparsa* latent infection in asymptomatic tissues sampled 44 days after inoculation of the disease-free boysenberry plants. In addition, no upward colonisation of *P. sparsa* in the asymptomatic plants was detected which may indicate pathogen spread towards the newly developing foliage was prevented, although since only three replicate samples of any tissue from each treatment were sampled, further experiments are required to verify this result. These results, however, indicated that dimethomorph is a promising fungicide both to protect boysenberry plants from spore infections whilst also potentially limiting systemic colonisation. Dimethomorph, a morphine belonging to the carboxylic acid amide group, has been reported to be active against oomycetes, by inhibiting cell wall synthesis (Morton and Staub, 2008). It is also categorised as a fungicide with low-medium risk of inducing resistance (Morton and Staub, 2008). Currently dimethomorph is registered in New Zealand for use against downy mildew in grapes (*Plasmopara viticola*), onions (*Peronospoa destructor*), and lettuce (*Bremia lactucae*), and early and late blights of potatoes (*Alternaria solani* and *Phytophthora infestans*) (Novachem agrichemical manual, 2015); however at present it is not registered for use in boysenberry.

In the experiment to determine the effect of fungicides reported to act through induced resistance (Hukkanen, 2008) to limit spore infection, the incidence of *P. sparsa* infections in the inoculated positive control in the third inoculation conducted in March (0.63) was significantly higher than the previous two inoculations conducted in February (0.21) and January (0.42). This was probably due to environmental conditions in the shadehouse being more conducive to infection during the third inoculation (mean RH 90%, mean temperature 24ºC) compared to the drier and relatively warm conditions during the other two inoculation times (79% RH and 19ºC during the first, and 51% RH and 27ºC during the second inoculations). This corresponded with the results from the *in vitro* germination assays in Chapter 2 which showed maximum germination of *P. sparsa* spores at 20ºC under 100% RH. In addition, the other experiment evaluating the effect of fungicides to control spore infections which was run in parallel and inoculated in January also had relatively low levels of infection (50% infection in the *P. sparsa* inoculated control). This indicated that the absence of optimum conditions during inoculation

did not facilitate *P. sparsa* spore infections. In addition, there is the potential that the quality with respect to viability or infectivity of the spore inoculum between inoculations could have varied, however to minimise variability the inocula were produced on artificially inoculated leaf discs incubated at 20ºC under high RH.

None of the single applications of the systemic fungicides tested (phosphorous acid, potassium phosphite and acibenzolar-s-methyl) were effective at controlling leaf infections in young disease-free boysenberry plants. Since three fungicide applications have been reported to be required to induce systemic resistance (Walter *et al*., 2004), it may suggest that a single application was not sufficient to effectively induce the plant immunity against *P. sparsa* spore infection. Effective control of downy mildew in boysenberry in field experiments was only achieved with three applications of phosphorous acid (PA) with a single application not providing control (Walter *et al*., 2004; Walter *et al*., 2008a, Walter *et al*., 2009; Walter *et al*., 2012a; Walter *et al*., 2012b) indicating multiple applications of phosphorous acid is required. Similar results were reported by Rebollar-Alviter *et al*. (2012) where three-four applications of potassium phosphite on systemically infected blackberry field plants controlled the incidence and severity of downy mildew infection of both leaves and fruits. Single application of potassium phosphite was not studied by these authors. Even though a single spray of PA did not significantly reduce the incidence of downy mildew symptoms on leaves after repeated inoculations in the current study, there was an indication that the incidence of latent infection as detected by PCR was reduced, especially after the third *P. sparsa* inoculation, but again this needs to be verified further.

The results of the current study demonstrated that three applications of PA or acibenzolar-s-methyl to *P. sparsa* systemically infected boysenberry plants significantly reduced dryberry incidence. This result for PA is, as previously discussed, in agreement with the studies conducted by Rebollar-Alviter *et al*. (2012) and Walter *et al*. (2008b) for downy mildew of blackberry and boysenberry, respectively. Although the plant physiological stages at which PA was sprayed varied in the different studies, similar levels of control were achieved. Under low disease pressure (2000/01 season) Walter *et al*. (2004) applied PA as a single spray pre-flowering followed by two sprays at flowering (11 days apart) whereas under high disease pressure (2001/02), the three sprays were applied at pre-flowering stages (at one weekly interval). Further in the 2007/08 season the three sprays were applied at pre-flowering, flowering and pre-harvest, the latter two sprays being 2-3 and 4-6 weeks after the first application, respectively (Walter *et al*., 2008b) similar to the spray timing in the current study. It must however be noted that the field studies of Walter *et al*. (2004, 2008, 2012) were all conducted in Nelson where the timing of the different boysenberry development stages is likely to be different to that in Canterbury where the current study was conducted. Additional research is required to determine the most effective physiological stages to apply the fungicides for optimum control.

Whilst the three applications of acibenzolar-s-methyl (Actigard) reduced dryberry incidence it was not effective at reducing severity. This is in contrast to PA where three applications reduced both dryberry incidence and severity. Hukkanen (2008) reported that three applications of Bion (acibenzolar-s-methyl, syn. Actigard) were effective at reducing *P. sparsa* leaf symptoms on arctic bramble grown in the greenhouse; however the effect on berry infection was not studied. The same application rate of the active ingredient (acibenzolar-s methyl) was used in the current study (5 g/100 L water) as in the study of Hukkanen (2008). Acibenzolar-s methyl is known to induce the systemically acquired resistance (SAR) response in the host plant (Hukkanen, 2008; Morton and Staub, 2008) but it has been reported to be more effective when incorporated into a spray program with other fungicides rather than being applied alone (Morton and Staub, 2008). Therefore, whether replacing one or two sprays of the current three PA applications with acibenzolar-s methyl application(s) would result in more effective control warrants further investigation. In addition the substitution of one or two PA sprays with acibenzolar-s methyl may also help to minimise any potential risk of resistance developing in the pathogen that may occur with continuous application of PA (Rebollar-Alviter *et al*., 2012).

The three applications of potassium phosphite (PerkSupa) were not effective at controlling dryberry incidence although the active ingredient (a salt of PA; Thao and Yamakawa, 2009) was applied at a higher concentration (200 g/L) than PA (Table 5.1). A related product, Perk (potassium phosphonate) was reported by Walter *et al*. (2004) to increase the percentage downy mildew infection of emerging primocanes of boysenberry in the field in New Zealand. In contrast, Hukkanen (2008) observed that three applications of potassium phosphite products (Phosfik, Phostrol and Aliette) at 10 day intervals had similar efficacy against *P. sparsa* in greenhouse arctic bramble plants, but were less effective than Bion. Salts of PA are known as phosphites with phosphite esters referred to as phosphonates (Thao and Yamakawa, 2009), however, phosphonates are also converted into phosphites in the plants, but not to phosphate which is the direct phosphorous source utilised by plants (Walter *et al*., 2012a). Phosphite competes with phosphate in fungi and oomycetes thus blocking key enzymes required for growth (Walter *et al*., 2012a). Phosphite has been reported to act directly against *Phytophthora* by inducing the accumulation of pyrophosphates and polyphosphates resulting in the depletion of ATP and inhibition of pyrophosphatase activity which causes phosphate starvation and reduced growth of the pathogen (Niere *et al*., 1994 cited in Hukkanen, 2008). It has also been reported to stimulate the pathogen defence mechanisms in plants (Lovatt and Mikkeelson, 2006) with an increase in phytoalexin accumulation been reported to occur in *Phytophthora palmivora* infected plants in response to phosphonate application (Daniel and Guest, 2006). The exact mechanism(s) by which these compounds inhibit *P. sparsa* infection is not known. Although potassium phosphite products effectively reduced *P. sparsa* infection of artic bramble (Hukkanen, 2008), it was ineffective at controlling the pathogen on boysenberry in the current study, and may indicate that different host plants respond differently to phosphite applications.

Further, it is likely that different types of PA (PA, phosphite, phosphonate products) result in different disease control efficacy. However, although potassium phosphite did not provide control in the current study, control was achieved using PA.

Metalaxyl-M+mancozeb (Ridomil Gold MZ WG) which had been very effective at preventing spore infections of leaves in the pot experiment on disease-free boysenberry plants, was not effective at reducing the incidence of dryberry when sprayed to protect the unripe berries which developed on the systemically infected plants from *P. sparsa* spore infection. Similarly, Walter *et al*. (2004) observed the same fungicide applied at the same rate did not reduce dryberry incidence at the final harvest in the field in Nelson in either the 2000/01 and 2001/02 seasons, however leaf, emerging primocane and early fruit infections were reduced in the latter season. This indicated that either metalaxyl-M+ mancozeb were effective only at preventing infection of foliage and not fruit, or that the timing of application in the current study was not effective at controlling infection, or that dryberry disease resulted from systemic only infection (Chapter 4) or a combination of spore and systemic infection. In this study, metalaxyl-M+mancozeb was sprayed when the berries were at the green, unripe hard stage approximately half of the fully matured berry size. The berries may have already been infected by this stage, through spore infection of pollen at the flower stage followed by fusion of the infected pollen with the ovum and subsequent systemic growth of the pathogen into developing berry initials may also have occurred. In the current study, potential infection periods such as flowering and red ripe berry stage (Chapter 3) that may also result in dryberry disease were not targeted by the protectant fungicide application. The other possibility is that protectant application could have increased the humidity around the berries and contributed to the higher disease.

The sporulation on petals (Appendix D.6b) that was observed on a few plants which had been sprayed three times with phosphorous acid, potassium phosphite, acibenzolar-s-methyl and the untreated control suggests a need to apply a protectant at both pre-flowering and flowering. Therefore, it is recommended that three PA applications at pre-flowering, flowering and berry stages along with metalaxyl-M+mancozeb as a protectant at pre-flowering and/or flowering should be evaluated in the field to control dryberry disease in systemically infected plants. Depending on the disease risk in a particular season it may also be important to spray a protectant at the red, partially ripe, berry stage. However, mancozeb and metalaxyl-M based products have been reported to result in fruit residues in fruit which affect market access for export boysenberry (Walter *et al*., 2004). In addition, the risk of developing resistance to phenylamide fungicides such as metalaxyl, as has been reported for *P. cubensis*, the downy mildew pathogen in cucumber (Reuveni *et al*., 1980 cited in Lebeda and Cohen, 2011), indicates monitoring the frequency of application of metalaxyl-M+mancozeb is crucial in the field. Therefore, it is worth investigating whether azoxystrobin and dimethomorph, which were also shown to prevent leaf infection in the current study, could be used to replace at least some of the proposed metalaxyl-M+mancozeb sprays. However, it is also important to note that since

resistance to QoI (Quinoneoutside Inhibitors) group, of which azoxystrobin belongs to, has been reported for *Pseudoperonospora cubensis* the causal agent of cucurbit downy mildew (Colucci, 2008) and although the risk of developing resistance to dimethomorph is low-medium the reliance on one fungicide throughout a whole season is not recommended and their use in combinations is advised (Beresford and Vanneste, ND).

In conclusion, a protocol to produce *P. sparsa* -free boysenberry plants by tissue culture was developed in the current study and fungicides effective at reducing both disease expression in systemically infected boysenberry plants and spore infection were identified. Based on these results a downy mildew disease management strategy for implementation in the field can be recommended. Three applications of PA at pre-flowering, flowering (2 weeks after first application) and unripe young hard berry stage (4 weeks after first application) to control systemic pathogen development along with a protectant spray of metalaxyl-M and mancozeb at pre-flowering and flowering would be recommended. In order to avoid residues in the harvested fruit replacing metalaxyl-M+mancozeb with dimethomorph, which has both protectant and systemic activity, should be considered particularly to replace the recommended spray at flowering. Additionally, it is recommended that dimethomorph, mandipropamid and azoxystrobin are evaluated for their ability to protect *P. sparsa* disease-free plants from spore infection of foliage in the field. However, none of these fungicides are currently registered on boysenberry in New Zealand.

CHAPTER 6

Development of an improved molecular detection system for *Peronospora sparsa*

6.1 INTRODUCTION

The standard nested PCR methods (Lindqvist *et al.*, 1998; Williamson *et al.*, 1998; Aegerter *et al.*, 2002; Dodd *et al*., 2007) for detection of *P. sparsa* infection in *Rubus* and *Rosa* species are sensitive and can detect as little as two spores. However, as with all nested PCR they are time consuming due to the necessity of undertaking two PCR steps. Conventional nested PCR is also susceptible to contamination as it involves two PCR steps, requiring the transfer of amplified products from the first step to a second reaction tube for amplification with the nested primers (Mutasa *et al*., 1996; Liop *et al*., 2000). False negatives may also occur with the conventional nested PCR (Liop *et al*., 2000) unless they are multiplexed with a gene that acts as a positive control during PCR.

To overcome these issues single tube, nested PCR methods have been developed to detect latent infection of fungi (Mutasa *et al*., 1996) and bacteria (Liop *et al*., 2000) in host plants. In addition, these methods have been used for other organisms such as amoeba (Ahmad *et al*., 2011) and viruses (Lusi *et al*., 2005; Atkinson *et al*., 2014). These closed tube nested PCR methods rely on the annealing temperatures of each of the primer pairs being different and, therefore, allowing the separation of the two amplification cycles. Liop *et al*. (2000) developed a highly sensitive nested PCR procedure using a single closed tube for detection of *Erwinia amylovora* in asymptomatic apple, loquat, pear, quince, *Cotoneaster* spp., *Crataegus* spp. and *Pyracantha* spp. They achieved positive results with a range of plant materials such as flowers, buds, shoots, stems, fruits, and leaves. The method was sensitive enough to detect 0.7 CFU/ml, whereas, it was 70 CFU/mL with the standard PCR. Mutasa *et al*. (1996) produced a single tube nested method to diagnose *Polymyxa betae*, an obligate parasitic fungus in sugar beet roots, and this method was used successfully for the screening of plant breeding material. The method was sensitive enough to detect *P. betae* in as little as 1 pg of total DNA from infected roots (Mutasa *et al*., 1996).

Selection of the external and internal primer pairs by Liop *et al*. (2000) was based on several criteria such as (i) the external primer pair should amplify a fragment large enough to permit the design of an appropriate internal primer pair, (ii) the annealing temperatures of the primer pairs should allow for the separation of both PCRs by only this parameter, and (iii) the primers should be highly sensitive, such that the detection threshold of the nested PCR in one tube should be lower than for the two-tube method. The one-step method involves a single PCR cycle with two distinct stages; the first stage is a thermal cycle with an annealing temperature that is the same

as the temperature optimum for Taq DNA polymerase (72°C). This allows only the external primer pair to anneal and initiate extension by the polymerase. In contrast, the internal primer pairs bind to a site within the area amplified by the external primers but will only bind at a much lower temperature of (typically) 50-55°C. For example, the annealing temperatures for external primer pairs used by Mutasa *et al*., (1996) were 70.7/68.8°C and for internal primer pairs were 55/54.9°C. Thus, the binding of the different primer pairs to complementary sites on the target genomic DNA were separated by modifying the annealing temperature.

Improvement to the current nested PCR detection system to detect latent infection of *P. sparsa* in asymptomatic boysenberry plants was desired to improve throughput, reduce cost and decrease the potential for contamination. Therefore, the aims of this research were (i) to optimise an effective one step nested PCR and assess detection sensitivity and, (ii) to validate the one step nested PCR method for detection of latent infection of *P. sparsa* in systemically infected boysenberry plants during both dormant and active growth stages of the plant.

6.2 MATERIALS AND METHODS

6.2.1 Optimisation of a one step nested PCR for detection of *P. sparsa*

The one step nested PCR method was optimised for (i) primer concentrations (PS long F and PS long R2 external primer pair), (ii) primary PCR cycle number (Appendix E.1), (iii) secondary PCR cycle number, and (iv) sample/ DNA volume in the PCR. Primers were designed by Dr H. Ridgway (unpublished data).

6.2.2 Validation of optimised one step nested PCR for *P. sparsa*

Samples

Four replicates each of asymptomatic primocane tip, cane/stem, root and symptomatic (but non-sporulating) leaf and sporulating leaf samples were collected from four systemically infected boysenberry plants grown in the shadehouse (Lincoln University). DNA was extracted using the modified Plant & Food Research CTAB method (Section 3.2.1(i)).

PCR conditions

Each PCR contained 1 X PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3 and 0.01% (w/v) gelatin), 200 µM of each dNTPs, and 1.0 µL each of 10 nM of PS long F (5'-TTTGGCGGYGRCTGCTGGCATT-3') (forward) and PS long R2 (5'-GTCCAATAAGCGCCTGTTCAGCC-3') (reverse) *P. sparsa* specific primer pairs (Invitrogen Technologies, New Zealand), 200 nM PR3 short (5'-GCTGGCTGCTACTGGGCA-3') (forward) and PR4 short (5'-CCGACTGGCCACGCGGA-3') (reverse) (modified from the PR3 and PR4 primer pair developed by Lindqvist *et al*. (1998)), 1 U of FastStart Taq polymerase (Roche Molecular Biochemicals, Mannheim, Germany) and 1.0 or 2.0 µL of template DNA in a

total volume of 25 µL. The PS long F and PS long R2 external primer pair was used at an annealing temperature of 72°C, whereas the PR3 short and PR4 short internal primer pair at 55°C as to produce the amplification products at 55°C but not at 72°C. The non-template control included sterile water instead of the template DNA.

The amplification conditions (Figure 6.1) were in two stages as follows: ***Stage 1*** - 3 min initial denaturing at 94°C, followed by 32 cycles of 30 s at 94°C (1st stage denaturing), 45 s at 72°C (1st stage annealing and extension), followed directly by ***Stage 2*** - 30 s at 94°C (2nd stage denaturing) followed by 40 or 45 cycles of 30 s at 55°C (2nd stage annealing) and 30 s at 72°C (elongation), and a final elongation period of 7 min at 72°C using the Veriti 96-well Thermal cycler (AB Applied BioSystems). Gel electrophoresis and staining in the ethidium bromide were conducted as described in Section 3.2.1 (Chapter 3), however with 15 µL of each reaction mixture (instead of 10 µL) was separated by electrophoresis. Each sample loaded into the gel was prepared by 15 µL aliquot of the PCR product mixing with 3 µL of 6 x loading dye (0.025% bromophenol blue, 0.025% xylene cyanol FF, 40% (w/v) sucrose in water) to give a final volume of 18 µL. An amplimer of 660 bp was expected for *P. sparsa* specific amplification using these primers. An Invitrogen 1Kb plus DNA™ Ladder was used as the DNA size marker.

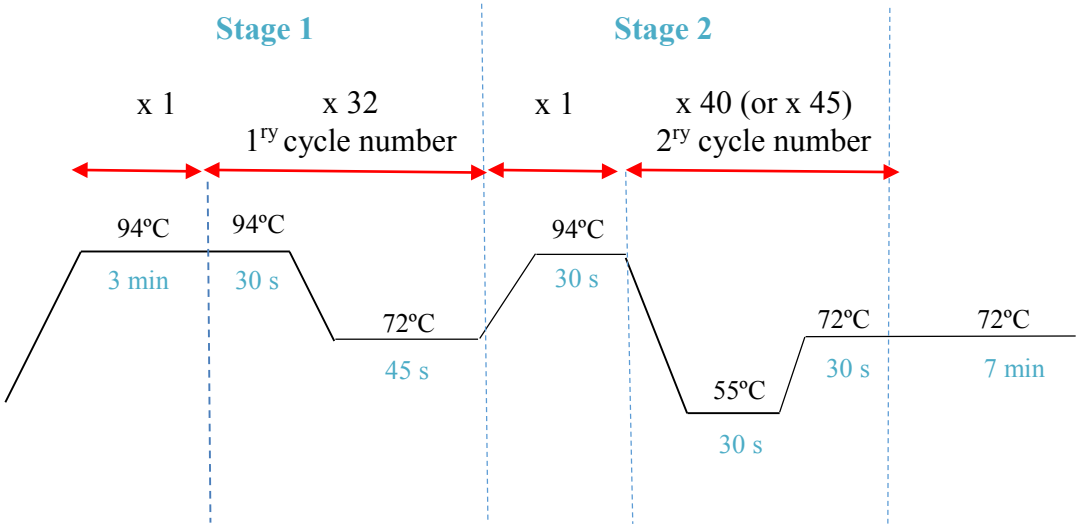

Figure 6.1 The amplification conditions of the one step PCR arranged in two stages; (i) Stage 1- first stage denaturing, annealing and extension followed by (ii) Stage 2- second stage denaturing, annealing and elongation. The PS long F and PS long R2 external primer pair was used at an annealing temperature of 72°C (Stage 1), whereas the PR3 short and PR4 short internal primer pair at 55°C (Stage 2).

6.2.3 Application of the optimised one step nested PCR to detect *P. sparsa* latent infection

Boysenberry plants (three months old) produced by tissue culture at the Plant & Food Research Station, Riwaka from systemically infected boysenberry (cv. Mapua) mother plants (Section 5.2.2.1) were dissected for the PCR assay. These plants were from the control group of an experiment which aimed to determine whether systemic infection could be reduced by heat and/or fungicide treatments. All of the young plants had expressed characteristic *P. sparsa* symptoms on leaves. Latent infection of *P. sparsa* in asymptomatic boysenberry plant tissues (primocane tips, leaves, leaf buds, petioles, stems, crowns and roots) was assessed using the optimised one step nested PCR, using 10 nM concentrations of PS long F and PS long R2 primers to amplify 1 µL samples of DNA in a primary PCR with 32 cycles and a secondary PCR of 40 cycles.

Plant samples

A) Tracking latent infection during dormancy

In August, 2013 (winter), a range of tissue samples were collected from the boysenberry plants (three months old), which had been grown in the shadehouse (Lincoln University). The asymptomatic tissue types sampled from each of ten plants included leaf (12 mm diameter), petiole of the same leaf (10 mm length), leaf bud, crown and root (10 mm length) samples (Figure 6.2). As the plants were not actively growing (referred to as 'dormant' in this study), cane/stem and primocane tip samples were not available. From the leaf nearest to ground level, the basal leaflet (Figure 6.2B) was sampled for each of the plants, which were 'rosetting' during dormancy.

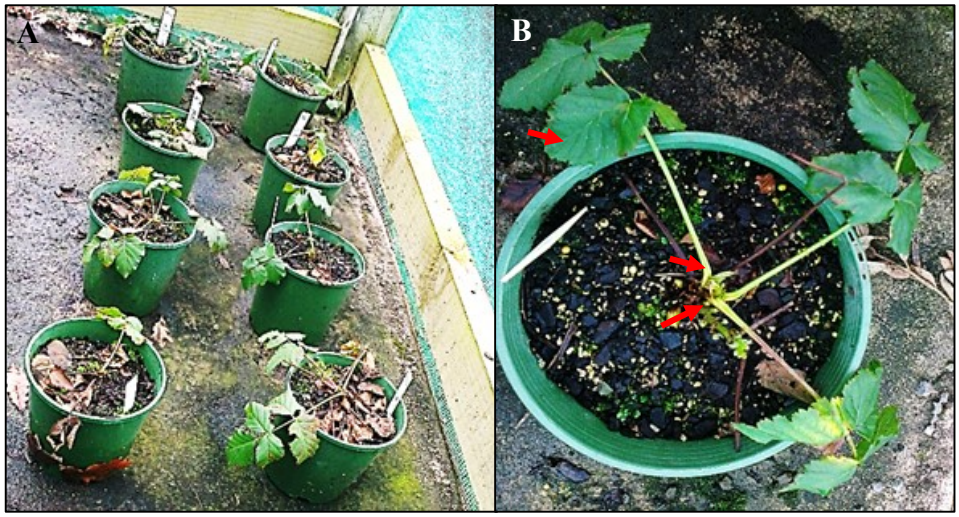

Figure 6.2 Three month old systemically infected boysenberry used for sampling tissues such as leaf, petiole, leaf bud, crown and root (A), tips and canes have not emerged, basal leaflet of the leaf nearest to ground level, petiole and leaf bud samples are shown by red arrows (B).

B) Detection of the pathogen *in planta* during active plant growth

Four boysenberry plants (three months old) from the same untreated control plants described in Section 6.2.2) were sprayed to run off with 100 ppm gibberellic acid (®ProGibb, Valent BioSciences Corporation, Illinois, USA) to promote cane growth. They were shifted to a clean (free of *P. sparsa* spores) greenhouse at Plant & Food Research, Lincoln the following day (mid-July, 2013) and were placed under continuous lights (24 h) to break dormancy at temperature ranged 12-20°C. After seven weeks (early September) the four plants had developed 12 replicate primocanes overall, which were each dissected into two sections: top (average length 15 cm) and bottom (average length 8.5 cm). For each section, leaf, petiole of the same leaf and stem samples were dissected. From each section, the following samples were also collected, a primocane tip from each top section, and a crown and root sample from the bottom section of each plant. Altogether there were 12 replicate samples for each tissue type except the crown and roots, for which there were four replicates.

Fluorescent microscopy and/or PCR

A) Tracking latent infection during dormancy

Each leaf petiole (10 mm) and root (10 mm) section was bisected into two 5 mm sections, one to be used for fluorescent microscopy (Section 3.2.3 A) and the other for PCR, to compare the effect of microscopy and PCR to detect *P. sparsa* latent infection. The ten crown sections, leaf discs (12 mm) and the leaf buds were also bisected with half for microscopy and half for PCR. Ten replicates of each tissue type were used for fluorescent microscopy to assess presence of mycelium or other structures characteristic of *P. sparsa* as described in (Section 3.3.3). DNA extraction was conducted for the other 10 replicates of each tissue type according to the Section 3.2.1 (i).The one step nested PCR was conducted as described in Section 6.2.1 followed by gel electrophoresis and staining, as described in Section 3.2.1.3 (Chapter 3), however with 15 µL of each reaction mixture (instead of 10 µL) was separated by electrophoresis As mentioned in the Section 6.2.2.

B) Detection of the pathogen *in planta* during active plant growth

DNA was extracted and the one step nested PCR conducted as described in Section 6.2.1 followed by gel electrophoresis and staining.

6.3 RESULTS

6.3.1 Optimisation of a one step nested PCR for detection of *P. sparsa*

The one step nested PCR using 40 cycles in Stage 2 was able to detect *P. sparsa* DNA in all symptomatic leaf tissues as well as one asymptomatic root and stem (Figure 6.3). At least one sample from each tissue type, except primocane tips, was *P. sparsa* positive using 40 cycles. In

contrast, only one sample (stem) was *P. sparsa* positive using 45 cycles and this was unaffected by increasing the sample DNA volume from 1 to 2 µL. Therefore, the optimised one step PCR method was found to be 32 cycles at the first stage with 40 cycles at the second stage.

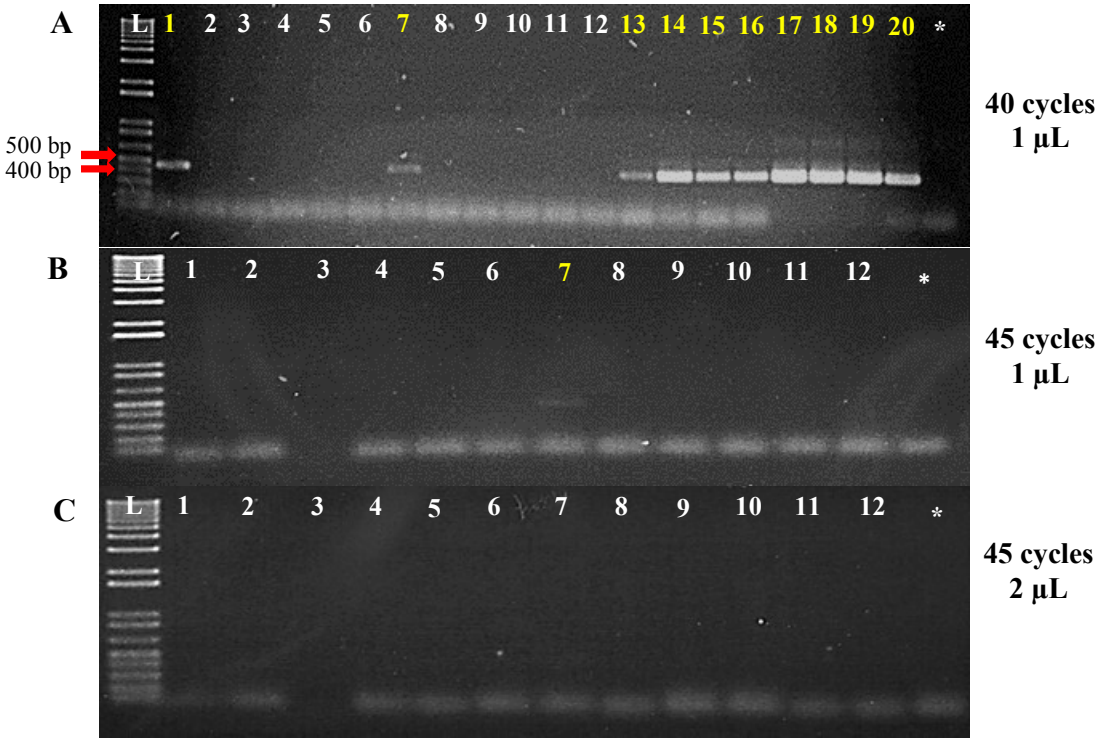

Figure 6.3 1% agarose gel of the one step nested PCR for *Peronospora sparsa*. (A) Using 1 µL DNA extract and 40 cycles in Stage 2 of the PCR, (B) Using 1 µL DNA extract and 45 cycles in the Stage 2 of the PCR, (C) Using 2 µL DNA extract and 45 cycles in Stage 2 of the PCR. Far left lane: L=1 Kb plus DNA ladder (Invitrogen, Carlsbad, California), root (1-4), stem (5-8), asymptomatic primocane tips (9-12), symptomatic leaves (13-16), sporulating leaves (17-20), negative control (*). Samples positive for *P. sparsa* DNA are in yellow.

The sensitivity of the optimised one step nested PCR method for *P. sparsa* spore DNA (Dr H. Ridgway, unpublished data) was 0.4 pg, the same as for the optimised two step nested PCR (Chapter 3) method which could also detect as little as 0.4 pg (400 fg).

6.3.2 Application of the optimised one step nested PCR to detect *P. sparsa* latent infection

A) Tracking latent infection during dormancy

Fluorescent microscopy showed wide aseptate hyphae characteristic of *P. sparsa* in crown and root samples of all plants, and leaf samples from 20% of plants (Figure 6.4). None were observed in other tissue types i.e. petioles or leaf buds.

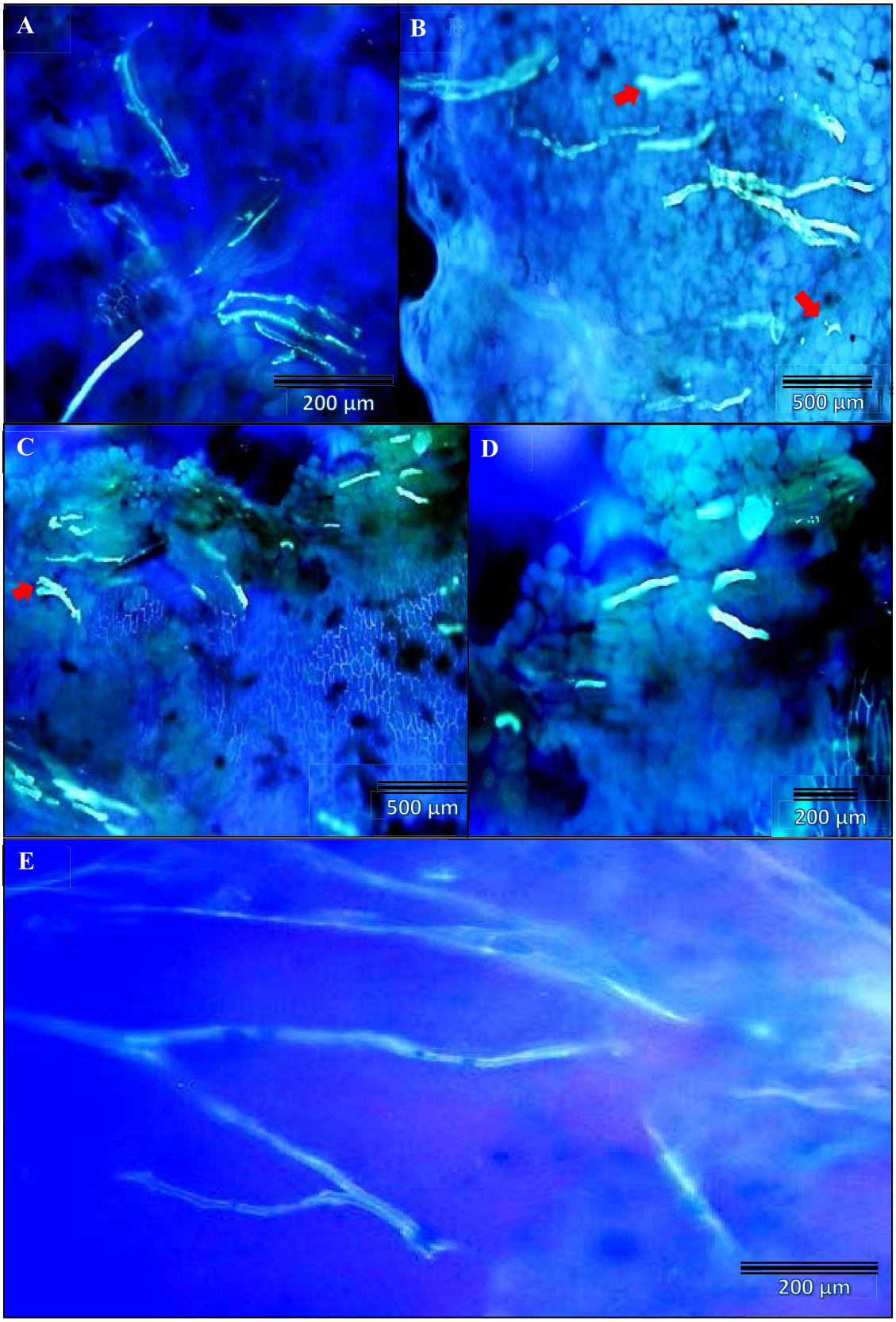

Figure 6.4 Hyphae characteristic of *Peronospora sparsa* in systemically infected boysenberry plant tissues visualised using fluorescence microscopy (fix specimen/clearing in NaOH (Chapter 3)). Root (A, B), crown (C, D) and leaf (E). Haustoria are shown by red arrows.

Peronospora sparsa was present in 60% and 40% of plants when petiole and crown DNA extracts were amplified, respectively (Figure 6.5). Positive PCR results were produced by 30% and 20% of plants for root and leaf bud DNA extracts, respectively. Only 10% of plants were PCR positive using DNA extracted from leaf samples. In plans 3 and 9, characteristic *P. sparsa* leaf lesions and hyphae had been observed in crown and root samples by fluorescent microscopy (Figure 6.3) (Table 6.1), although none of the tissues tested in these plants were PCR positive for *P. sparsa* (Figure 6.5, Table 6.1)

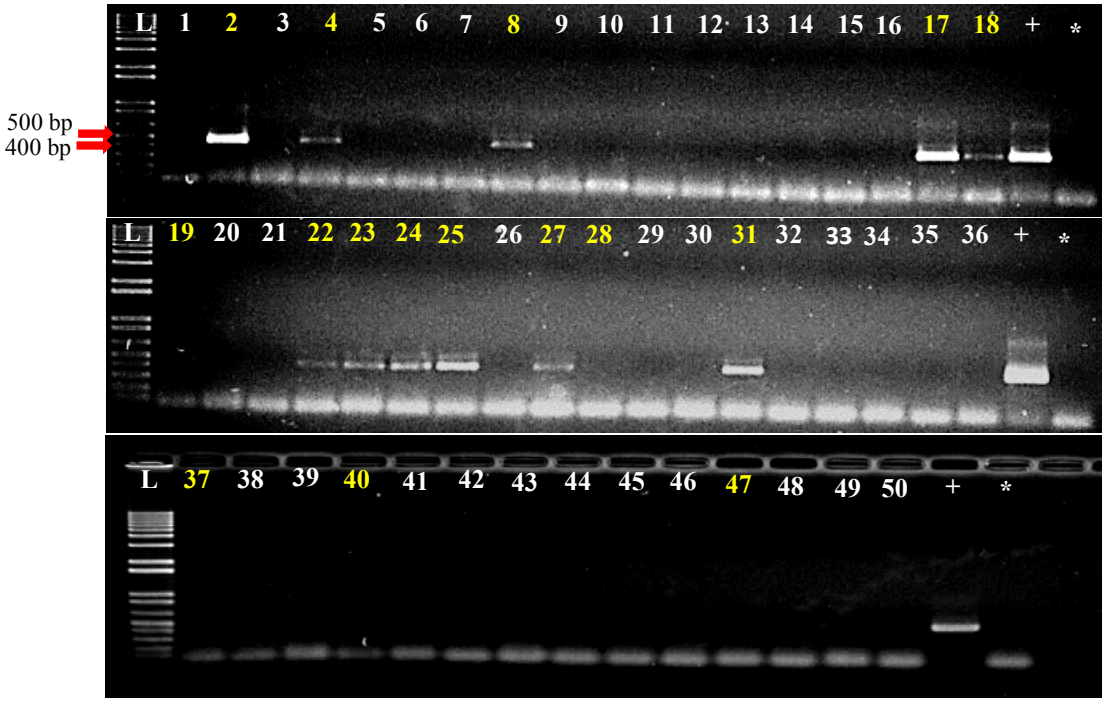

Figure 6.5 1% agarose gel of the one step nested PCR used to evaluate latent infection of *Peronospora sparsa* in young boysenberry plants during dormancy. L=1Kb plus DNA ladder (Invitrogen, Carlsbad, California), tissue samples in order of leaf, petiole, crown, root and leaf bud in plant.1 (1-5), plant.2 (6-10), plant.3 (11-15), plant.4 (16-20), plant.5 (21-25), plant.6 (26-30), plant.7 (31-36), plant.8 (37-40), plant.9 (41-45), plant.10 (46-50), positive control (+) and negative control (*).

Table 6.1 Comparison of fluorescent microscopy and one step nested PCR detection of *Peronospora sparsa* in different tissues of dormant, systemically infected young boysenberry plants (n=10).

Plant	Method of assessment	Plant tissue type[a]				
		Leaf	Petiole	Crown	Root	Leaf bud
1	PCR	-	+	-	+	-
	Microscopy	-	-	+	+	-
2	PCR	-	-	+	-	-
	Microscopy	-	-	+	-	-
3	PCR	-	-	-	-	-
	Microscopy	-	-	+	+	-
4	PCR	-	+	+	+	-
	Microscopy	+	-	+	+	-
5	PCR	-	+	+	+	+
	Microscopy	+	-	+	-	-
6	PCR	-	+	+	-	-
	Microscopy	-	-	+	+	-
7	PCR	+	-	-	-	-
	Microscopy	-	-	+	+	-
8	PCR	-	+	-	-	+
	Microscopy	-	-	+	+	-
9	PCR	-	-	-	-	-
	Microscopy	-	-	+	+	-
10	PCR	-	+	-	-	-
	Microscopy	-	-	+	+	-
Total	PCR	1	6	4	3	2
	Microscopy	2	0	10	8	0

[a] +, positive; -, negative

Of the crown and root samples in which wide aseptate hyphae characteristic of *P. sparsa* were observed in 100% and 80% respectively of the plants, only 40% and 25%, respectively, were positive for PCR.

B) Detection of the pathogen *in planta* during active plant growth

All plants were *P. sparsa* positive using the one step nested PCR with at least one tissue type per plant giving a positive amplimer. Of the tissues sampled, positive results were produced by 75% of root, 50% crown, 25% primocane tip samples. Only 17% of the leaves collected from the top cane section were PCR positive (Table 6.2). None of the leaves collected from the bottom section of the cane were PCR positive. In addition, 8% of the total petiole samples (from the same detached leaves) and total cane/ stem samples collected from both the top and bottom sections of the canes were PCR positive. None of the petiole samples detached from PCR positive leaves were PCR positive for *P. sparsa*, and *vice versa*. Each plant had different numbers of canes from two to four (Appendix E.2). At least one tissue type (in addition to the root or crown tissues) from approximately 25, 50 and 67% canes (4, 2 and 3 total canes per

plant, respectively), of each plant gave a positive PCR. Only one tissue sample (crown) was PCR positive for Plant-4 despite four canes being examined.

Table 6.2 Number of systemically infected boysenberry plant tissues testing positive for *Peronospora sparsa* using the one step nested PCR after breaking dormancy.

Tissue type	Total number of samples from 4 plants	Number of samples testing positive for *P. sparsa* by one step PCR
Primocane tip (top cane)	12	3
Leaf (top cane)	12	2
Petiole (top cane leaf)	12	1
Stem (top cane)	12	1
Leaf (bottom cane)	12	0
Petiole (bottom cane leaf)	12	1
Stem (bottom cane)	12	1
Crown	4	2
Root	4	3

6.4 DISCUSSION

This Chapter describes optimisation and validation of a one step nested PCR as a faster and more robust detection process for *P. sparsa in planta* than the two step nested PCR (Chapter 3). This method improved the integrity of detection by decreasing the opportunity for contamination to occur. The method could detect latent infection of *P. sparsa* in boysenberry at the same detection sensitivity (0.4 pg) as the two step nested PCR described in Chapter 3. The method was evaluated on the DNA extracted from asymptomatic root, crown, petiole, cane, leaf, leaf bud and primocane tips and symptomatic/ sporulating leaves of systemically infected boysenberry plants. The results showed that the asymptomatic crown and root tissues were more likely to return a positive result than petiole, cane, leaf, leaf bud and primocane tips which was similar to the results of the nested PCR described in Chapter 3.

This was the first report of a single tube nested PCR to detect latent infection of *P. sparsa* in boysenberry; however, it is not the first application of this method to oomycetes. Thongsri *et al*. (2013) recently developed a one step nested PCR method for the human and animal pathogen *Pythium insidiosum.* As for *P. sparsa* their primers also targeted the rRNA, however, they only achieved a sensitivity of 21 oospores or 2.7 pg. There are no other reports of this technique being applied to oomycetes. However, there are many successfully developed one step nested PCR methods for bacteria (Liop *et al*., 2000; Bertolini *et al*., 2003), fungi (Mutasa *et al*., 1996), viruses (Kiatpathomchai *et al*., 2001; Lusi *et al*., 2005; Atkinson *et al*., 2014) and protozoans (Ahmad *et al*., 2011; Foo *et al*., 2012).

The one step nested PCR method was sensitive enough to detect as little as 0.4 pg (400 fg) of *P. sparsa* genomic spore DNA. This was a similar (0.25 pg) detection limit to the PCR used to

detect *P. sparsa* in arctic brambles (Lindqvist *et al*.,1998) and more sensitive than the PCR conducted on rose (2 pg) (Aegerter *et al*., 2002). However, visual detection required a much higher loading volume in the gel (15 µL) than for the two step nested PCR (10 µL), which suggested that there was approximately 30% less amplification. A similar, comparison of two step nested PCR and one step nested PCR methods to detect human immunodeficiency virus (HIV-1) by Lusi *et al*. (2005) showed that the one step nested PCR required a higher gel loading volume (20 µL) than two step (10 µL). For *Pythium insidiosum* the 2.7 pg detection sensitivity was achieved by a 10 µL gel loading volume (Thongsri *et al*., 2013).

For fungi, detection limits of one step nested PCR are highly variable. In comparison with the current study, some, such as those for *Polymyxa betae* (in sugar beet roots) (Mutasa *et al*., 1996) were higher (1 pg) and some, such as for *Plasmodiophora brassicae* (in artificially infested soil) (Ito *et al*., 1999) and *Fusarium culmorum* (in cereals) (Klemsdal and Elen, 2006) were lower (1 and 5-50 fg, respectively). The sensitivity of detection does not seem to correlate with the gene region chosen or substrate. For example, the rRNA gene region was chosen for both *Polymyxa betae* and *Fusarium culmorum* (Mutasa *et al*., 1996; Klemsdal and Elen, 2006) with a 20 fold difference in detection limit. For viruses and amoeba similar variation was noted. Ito *et al*. (1999) achieved a detection limit with *P. brassicae* of 1 fg in soil which is a substrate known to contain PCR inhibitors, whereas, Mutasa *et al.* (1996) could only detect 1 pg (1000 fold higher) when extracting *P. betae* from root fragments. However, for the bacteria *Erwinia amylovora* and *Pyracantha* spp. in apple, loquat, pear, quince, *Cotoneaster* spp. and *Crataegus* spp., the detection limits were 0.7 CFU/ mL (Liop *et al*., 2000) and for *Pseudomonas savastanoi* pv. *savastanoi* in olive it was 1 CFU/ mL (Bertolini *et al*., 2003), which were similar detection sensitivities. The ultimate outcome therefore seems to be specific for particular organisms and detection limit is likely to be influenced by a number of factors including primer design, substrate for detection, pathogen type and target gene.

The success of one step nested PCR relies on primer design such that the annealing temperatures of two sets of primers are well separated. The separation between primers in the current study (72ºC and 55ºC) was 17ºC which is typical of primers for this methodology. For example, in the one step nested PCR for *Erwinia amylovora, Pythium insidiosum, amoeba, Pseudomonas savastanoi* pv. *Savastanoi,* cytomegalovirus and WSSV the differences between primer annealing temperatures were 11-16ºC (Liop *et al*., 2000; Kiatpathomchai *et al*., 2001; Bertolini *et al*., 2003; Ahmad *et al*., 2011; Thongsri *et al*., 2013; Atkinson *et al*., 2014). In contrast, smaller temperature differences were used for the one step nested PCR method for HIV virus (2ºC) (Lusi *et al*., 2005) and the thermostabilized, one-step, nested, tetraplex PCR for *Entamoeba* (3.5ºC) (Foo *et al*., 2012). However, in these cases the one step PCR systems were not as sensitive (39 or 78 pg respectively) when compared to the current study.

The differences (Δ) between cycle numbers of the two phases of the one step nested PCR methods developed to detect bacteria (Liop *et al*., 2000; Bertolini *et al*., 2003), viruses (Lusi *et al*., 2005; Atkinson *et al*., 2014) and protozoans (Ahmad *et al*., 2011) were typically Δ15. For example, 20 cycles in Phase 1 and 35 in Phase 2 for HIV (Lusi *et al*., 2005) and protozoan amoeba (Ahmad *et al*., 2011). In contrast, the Δ was zero for the oomycete *Pythium insidiosum* (Thongsri *et al*., 2013), and 8 for the non-zoospore forming *P. sparsa* in the current study. This suggests that cycle numbers can vary and should therefore be optimised for the specific host DNA as was done in this study. For *Pythium insidiosum* which is normally detected in blood samples 30 cycles were used in both phases (Thongsri *et al*., 2013) whereas, in the current study 32 cycles were used for Phase 1 and 40 for Phase 2 with *P. sparsa*. Forty cycles in Phase 2 were also reported for *Erwinia amylovora* (Liop *et al*., 2000) and *Pseudomonas savastanoi* pv. *savastanoi* (Bertolini *et al*., 2003). Different optimal cycle numbers may reflect variation in the co-purification of inhibitors and/or ratio of target to host DNA in the contrasting tissue types (e.g. plant versus blood).

Variability was observed between the fluorescence microscopy observations and the PCR for *P. sparsa* infection in the 3 month old boysenberry plants (systemically infected) incubated in the shadehouse to evaluate the effect of dormancy. Some plants (20%) appeared to be disease-free with none of the asymptomatic tissues testing positive in the PCR. However, for those same plants aseptate hyphae characteristic of *P. sparsa* were observed in crown and root samples with fluorescent microscopy. Characteristic *P. sparsa* leaf lesions were also observed on these plants. This apparent contradiction between PCR and microscopy could be due to one of three reasons; i) the pathogen was discontinuous throughout the plant or ii) infection was below the detection threshold of the PCR or iii) PCR was affected by the inhibitory compounds from the plant tissues. In the current study, the detection limit was assessed for spores and not the systemically infected tissues or a combination of these.

The sporadic detection of *P. sparsa* in canes and leaves was possibly due to discontinuous pathogen growth or uneven distribution of nuclei and is consistent with previous findings in this thesis using the two step nested PCR (Chapter 3). In agreement with this, Fraymouth (1956) reported that *Peronospora ficariae* hyphae in the leaves of *Anemone coronaria* are very short-lived, and run for only the distance between three stomata and then turn out and grow through the next one and develop a conidiophore. Also Nicolson (1959) reported that the nuclei in coenocytic hyphae were not always distributed evenly along hyphae but congregated in segments where branching took place and that some hyphal lengths lacked nuclei. In addition, absence of nuclei in young haustoria (Grenville-Briggs and van West, 2005) or more nuclei in young rather than older haustoria for the obligate oomycete *Albugo candida* in Brassicaceae (Baka, 2008) have been reported. In accordance with these reports, the samples selected in the current study, for PCR or fluorescence microscopy, were not always derived from the same

tissue and the inconsistency of PCR detection may be due to sporadic presence of *P. sparsa* nuclei.

The presence of other endophytes in boysenberry plants, such as other oomycetes and fungi, in addition to *P. sparsa* that may interfere with the accuracy of microscopy observations is a less likely explanation for sporadic detection by PCR. *Septoria rubi* (leaf spot), *Septocyta ruborum* (purple blotch), *Cercosporella rubi* (rosette) and *Botrytis cinerea* (Botrytis disease) are documented as other fungal pathogens in boysenberry (Langford *et al*., 2007). However, those pathogens have septate mycelium and are not biotrophs. Boysenberry are known to be mycorrhizal and arbuscular mycorrhizal fungi (AMF) in roots have slightly constricted, large, branched coenocytic hyphae in the cortical cells in a manner similar to the haustoria of Peronosporaceae (Hawker *et al*., 1957). In the current study, there could have been confusion between *P. sparsa* haustoria in intracellular growth and arbuscular fungi. These do differ (Kemen and Jones, 2012) but as they were unclear in the current study, more advanced microscopy may have been required rather than bright field or fluorescent microscopy. Thus, it seems more likely that the discontinuous infection by *P. sparsa* is the reason for inconsistent PCR detection rather than infection by another species of oomycete.

The experiments with both the nested PCR (Chapter 3) and one step nested PCR have demonstrated that leaves are not suitable for robust detection of the pathogen. This contrasts with the root or crown which was the most reliable for detection. This is probably because the pathogen overwinters in the crown and moves slowly into the canes at a rate that is variable and affected by host physiology and the environment. This hypothesis was supported by the PCR results from young boysenberry plants (3 months old) incubated in a greenhouse under lights, where extremely low levels of infection in above ground plant parts was observed [canes (8%) or leaves (17%)] compared to underground plant parts [roots (75%) or crowns (50%)]. It is also possible that amounts of DNA (both plant and microbial) present in different plant tissues have affected the PCR. More mature leaves have large amounts of phenolic compounds which can interfere with the extraction processes, thereby causing DNA yields to be low in amount and quality compared to young leaf buds (C. Winefield, pers. comm., 2015). Roots are also expected to yield low DNA amounts with low quality because of the vascular fluid, water and large vacuolated cells present behind the root cap, which leads high production of phenolics (C. Winefield, pers. comm., 2015). Despite the potential for phenolics in root extracts *P. sparsa* was detected frequently in roots suggesting that these tissues are more likely to contain larger amounts of the pathogen than the other tissues. Therefore, sampling of roots or crowns from adequate replicates of plants is the most appropriate tissue for detection of latent infection.

In conclusion, one step nested PCR can be used as a convenient, rapid and economical method for detection of latent infection of *P. sparsa* in boysenberry as it is less prone to contamination due to the reaction occurring in a single tube (Llop *et al*., 2000; Kiatpathomchai *et al*., 2001; Da

Silva *et al*., 2013) than the two step process. However, PCR inhibition can still be problematic in plant tissue extracts (Varma *et al.*, 2007). Root and crown tissues can be recommended as sampling sites to increase the probability of detecting latent infection. Future assessment of detection sensitivity of the one step PCR using systemically infected plant tissues is recommended.

CHAPTER 7

Concluding Discussion

The scope of this thesis was to examine factors affecting infection and disease expression by *P. sparsa* on boysenberry. As part of this work new methods were developed and validated for detection of *P. sparsa in planta*. The results of the work have several practical implications for boysenberry growers in New Zealand for disease management.

Spore trapping (Chapter 2) in a field in Upper Moutere showed that the disease was initiated by sporulation occurring in rainy early spring when the frequency of rainy days exceeds 38.7%. Typically this is in early September. The rain creates a high relative humidity within the bushes which coincides with warm temperatures (16-23ºC). Together these factors promote sporulation from the surfaces of the plant. Spore dispersal requires slightly dryer periods of approximately 70-85% RH which occur in mid-late spring. These results contrast to the recent study of Kim *et al*. (2014), which showed that the lowest disease incidences assessed in the Richmond boysenberry garden where when both high RH (frequency of days with rain per month >38.7%) and temperature thresholds (9.8 h) were met in December, 2011 and these differences may be related to local variation in microclimate. The disease forecasting model for *P. sparsa* in rose developed by Aegerter *et al*. (2003) included hours of leaf wetness (when temperature were less than 20ºC) in addition to the other two variables i.e. hours when temperatures were between 15 and 20ºC and when they exceeded 30ºC. These authors (Aegerter *et al*., 2003) have considered that leaf wetness duration data are of great importance in the microclimate [the environment within and around the crop canopy including sunlight exposure, air temperature, wind speed, and leaf wetness (Kurtural *et al*., 2008)] models, and failure of the mesoclimate [the climate of a given site affected by its local topography such as absolute elevation, slope, aspect, and soils (Kurtural *et al*., 2008)] model was due to the absence of the leaf wetness duration data compared to the microclimate model. Microclimate models applied on other biotrophic pathogens such as *Pseudoperonospora cubensis* and *Bremia lactucae* (Cohen, 1977, cited in Aegerter, 2001; Scherm and van Bruggen, 1993; Scherm and van Bruggen, 1995) also include leaf wetness duration data. Therefore, although infection is facilitated through spore release in late spring-early summer at temperatures higher than 20ºC as reported by Walter *et al.* (2012a), leaf wetness duration is also likely to affect infection and, thus it is important to validate Kim *et al*. (2014) Peronospora Sparsa model incorporating leaf wetness duration data.

In the first research chapter (Chapter 2) several methods were developed to facilitate experiments on this biotrophic pathogen. These methods and their standardization were essential to reduce variability inherent in working with a pathogen which is unable to be cultured in the absence of the host plants. These methods aimed to reduce the added variation that comes from isolate variation and variation in culture production. The novel *in vitro* leaf disc

method combined with knowledge of the optimum spore numbers required for infection (200 spores and the minimum being 20 spores) was an effective and consistent method for subsequent spore production for experiments. Previous studies (Breese *et al*., 1994; Aegerter *et al*., 2003; Hukkanen *et al*., 2006) have used spores from naturally infected leaves as an inoculum; however this process is dependent on year round access to infected plant material. The leaf disc method developed here avoids the need to maintain plants in growth rooms or the uncertainty of relying on optimum conditions to occur in the field for collection of inoculum. The leaf disc method incorporates surface sterilisation (70% ethanol) of the leaf discs and thereby minimises other contaminants. Genetic consistency of the inoculum was also improved by all inoculum originating from the *P. sparsa* isolate(s) in the systemically infected boysenberry plants (cv. Mapua) grown in the Lincoln University facilities. Some of the variability between the studies presented here and others may be due to genetic diversity of the pathogen itself. Ideally knowledge about the genetic diversity of this pathogen would be included, together with experiments on multiple isolates to gain a perspective of the species as a whole. However, this is very difficult with biotrophic pathogens. In future it would be interesting to see if sporulating leaf discs could be produced from single spores as a way to generate inocula from genetic individuals. This could then be used for molecular studies to examine the genetic diversity of this pathogen in New Zealand and elsewhere. Cooke *et al*. (2000) conducted the first sequence analysis for oomycetes that included *Phytophthora* spp., *P. sparsa, Pythium* spp., and *Albugo candida* based on the ITS sequences of genomic rDNA. The culturable oomycetes were grown on oatmeal agar, V8 or Frenchbean agar whereas the biotrophic *Albugo candida* from spores isolated from fresh plant material and the *P. sparsa* were from extracted spores. The authors did not seem to have produced single spore isolates from any of the biotrophic pathogens in this study. In addition, a high throughput DNA extraction method has been developed by Gobbin *et al*. (2003) for molecular analysis of the obligate pathogen *Plasmopara viticola* in grapevine directly in the host without any isolation procedure. However, in some cases more than one genotype was identified from a single oil spot lesion which the authors suggested was because what appeared to be a single lesion was effectively a mixture of two (or more) adjacent oil spots. Also, failure of some of the SSR primers may have been due to the contamination of the DNA used to construct the *P. viticola* genomic library by DNA from other microbes also occurring on the leaf lesions. These factors would need to be considered if this method was used to investigate the genetic diversity of *P. sparsa* isolates.

Given the biotrophic nature of the pathogen, molecular tools were used for detection and these were optimised in Chapter 3. A previous PCR study by Lindqvist *et al*. (1998) claimed a detection threshold of 0.25 pg. However, this is unusually sensitive for standard PCR compared to studies on various *Phytophthora* spp. with the highest detection being 1 pg (Zhang *et al*., 2006) with others being much less 2.5 pg (Grote *et al*., 2002 cited in Zhang *et al*., 2006), 5 pg

(Silvar *et al*., 2005 cited in Zhang *et al*., 2006) and 10 pg (Ippolito *et al*., 2002 cited in Zhang *et al*., 2006), and could not be replicated by Dodd *et al*. (2007) who developed a nested PCR based on the primers of Lindqvist *et al*. (1998). In this chapter the extraction of *P. sparsa* DNA from infected plant material was optimised. This was combined with an optimised nested PCR protocol for which the biological relevance in terms of detection sensitivity and its relationship to spore numbers was determined. The resulting system could detect as little as 0.4 pg (400 fg) of *P. sparsa* DNA equivalent to a single spore and in a DNA extract derived from a minimum of 40 diploid spores. This was more sensitive than the method of Aegerter *et al*. (2002) which could detect 2 pg *P. sparsa* DNA on rose and the method could detect *P. sparsa* in diverse asymptomatic boysenberry plant tissues.

The use of the nested PCR was combined with fluorescent microscopy and used to determine the presence or absence of *P. sparsa in planta*. Sporadic detection of *P. sparsa* in boysenberry tissues was repeatedly detected in Chapters 3, 4, 5 and 6 using nested PCR and microscopy. There were also several instances where the microscopy and PCR were not in agreement with each other. This may be due to discontinuous pathogen growth (Fraymouth, 1956) which is a new finding for *P. sparsa*. Another explanation is uneven distribution of nuclei (Nicolson, 1959; Grenville-Briggs and van West, 2005; Baka, 2008). The pathogen was present in different forms such as mycelium, haustoria, spores, sporangiophores and these structures may have different proportions of nuclear content (Schena *et al*., 2013). Intermittent detection by fluorescent microscopy may be because the pathogen weaves in and out of cells in a three dimensional manner, something that is not visible in a 2D microscope image with a thin section of tissue. Other reasons for the variability observed between the fluorescence microscopy observations and the PCR may be that infection fell below the detection threshold of the PCR or the PCR was inhibited by the compounds co-purified from the plant tissues. Live-cell imaging using confocal microscopy with a pathogen transformed with green fluorescent protein (GFP) would provide better options than the conventional fluorescent microscopy to study host cell colonisation by *P. sparsa*. This has been used for other oomycetes such as *Hyaloperonospora arabidopsidis* (obligate biotroph) and *Phytophthora infestans* (hemibiotroph) in *Arabidopsis thaliana* and *Nicotiana benthamiana*, respectively (Lu *et al*., 2012), and can be suggested as future work required, as little is known about the subcellular rearrangements in plant–oomycete infections (Lu *et al*., 2012).

A qPCR method was available in the literature for *P. sparsa* with the same detection limit as the nested PCR (Hukkanen, 2006). However, the inconsistent detection, apparent discontinuous nature of the pathogen and the presence of multiple fungal structures led to the conclusion that it would be of little advantage to the studies presented here. Presence/absence data had sufficient usefulness. There was an intention to use the qPCR on spores trapped on Vaseline coated slides but the degradation of the spores during storage and the subsequent poor recovery of DNA meant that this was abandoned.

The most reliable detection of the pathogen was in roots and crowns which can be considered as the reservoir for the pathogen. Although the canes were also observed to be colonised in cooler or warmer climates (Chapter 3), the pathogen was largely absent from the canes of plants grown at much higher temperatures under lights in a greenhouse (Chapter 6). Thus, *P. sparsa* may overwinter in the canes (Tate, 1981; Williamson *et al*., 1995), in addtion to the root and crown tissues. However, for continuous monitoring of the infection status of plants across all seasons in New Zealand the most reliable tissues are the roots and crowns.

The study of the biology of *P. sparsa* in the current research enabled production of a more detailed disease cycle of the pathogen. It is clear that this disease cycle relies on both systemic infection and new infections. Spring and autumn are the major seasons where the pathogen is active. In winter, the pathogen may remain quiescent in the root, crown and canes as mycelium. Presence of *P. sparsa* in the cane tips in winter transfers the infection into tip-rooted daughter plants in the field and allows pathogen transmission into new plants (Tate, 1981). Evidence showed that processes such as tissue culture did not remove that systemic infection and this remains a major pathway for pathogen transmission. In early spring (September), when there is high rain frequency, high RH and warm temperatures ≤ 15°C sporulation is favoured on young tissues such as stem/cane, foliage, calyx, and flower (petals and filaments). These spores are then available for dispersal onto neighbouring plants. In mid-late spring (October-November) a decrease in rain frequency promotes spore dispersal created where hygroscopic twisting of sporangiophores is facilitated, releasing spores (Erhardt, 2009; Lacey, 1996; McCartney and West, 2007). This may initiate the epidemiology in the field by spore infection of more susceptible young tissues. It is unclear how far these spores can travel but the close planting of boysenberry in most gardens favours the transmission of the pathogen by this route. In early summer (December) under warmer temperatures (20ºC) and high RH, spores landing on other tissues initiate germination and infection. Infection progresses by systemic colonisation at 20°C. Mid-late summer is unfavourable for the pathogen and the higher temperatures may kill the pathogen in/on the above ground tissues. However, this requires more research. The pathogen can remain dormant as mycelium or oospores in the much cooler roots or crowns during this period.

Thus, two infection pathways of *P. sparsa* in boysenberry to cause dryberry were identified:

1) *Systemic infection of berry initials*: crown infection moves to canes and then into flower buds, sepals and petals (Chapter 4). Under warmth and high humidity (early spring), sporulation on flowers (sepals, petals, filaments and pollen) infects pollen. Fertilisation of infected pollen with ova results in the systemic infection of the drupelets of the developing berry initials. However, the pathogen may be inactive in the unripe berry due to the presence of higher levels of polyphenols that inhibit *P. sparsa* enzyme activity (Verhoeff, 1974). Therefore, the pathogen is quiescent until the fruit start to ripen after which the pathogen becomes active, expressing

dryberry symptoms (non-glossy, shrivelled, hardened, early premature reddish drupelets) in early-late summer. The transition from latency is likely to be linked with physiological changes at fruit maturation especially with changes in the middle lamella of fruit cell walls (Verhoeff, 1974). This pathway of infection will be affected by the sporulation potential in the field at flowering, which depends on the weather. If environmental conditions are not conducive to sporulation, drupelets (berry) remain uninfected, even though the pathogen is still present in the sepals/ calyx. Uninfected unripe berries transition into healthy mature berries and only possibility of infection at ripening is by spores (summer).

2) *Spore infection at green unripe and red partially ripe stages*: *Peronospora sparsa* can infect drupelets at the green unripe and red partially ripe stages. Therefore, external infection from spores can infect berries and establish the infection in the field.

According to these two pathways of infection, dryberries appearing in the field where all of the drupelets are dried are most likely due to systemic infection of *P. sparsa* and dried drupelets observed randomly amongst healthy drupelets in red partially ripe berries due to secondary infection (spore infection). However, the disease cycle is still inconclusive and could be improved by (i) increasing the number of replicate plant tissues tested for each season and (ii) sampling tissues in early and late winter (June and August), mid and late autumn (April and May), and mid and late summer (January and February) – which was not done in the current study. It is also possible that the sporadic growth of *P. sparsa* may allow it to escape detection by PCR and that the variable ratio of plant: pathogen DNA in some tissue confounds detection of DNA (C. Winefield, pers. comm., 2015; Varma *et al.*, 2007).

One of the most difficult aspects of this work was the lack of clean plant material for experiments. This made experiments aimed at determining the relative importance of systemic versus spore initiated infection very difficult. It was evident from the purchase of nursery plants that systemic infection by this pathogen was widespread and a major route for transmission. One of the most significant outcomes for growers of this work was the identification of a novel method for producing *P. sparsa* free boysenberry propagation material without using fungicides along with the PCR confirmation. A heat only treatment of tissue cultured plants was sufficient to substantially reduce *P. sparsa* infection. This method was of similar efficacy to plants heat treated in combination with fungicides (phosphorus acid and mancozeb). Uptake of the heat treatment method will provide a commercially viable process for nurseries without the potential of producing fungicide resistance strains of the pathogen which could be disseminated widely with the planting stock. For an industry in which a large proportion of material currently available to growers for establishing or replanting gardens is systemically infected this is likely to have a major effect.

Based on the fungicide studies conducted in Chapter 5, a downy mildew disease management strategy for implementation in the field could be identified. Three applications of PA at pre-

flowering, flowering (2 weeks after first application) and unripe young hard berry stage (4 weeks after first application) to control systemic development along with a protectant spray of metalaxyl-M and mancozeb at pre-flowering and flowering would be recommended. Currently the recommendation to growers is for three applications of PA, similar to that recommended by this study. However, in addition to the current recommendation, it is suggested that growers also include two sprays of metalaxyl-M and mancozeb to protect developing flowers from spore infection. In order to limit residues in the harvested fruit replacing metalaxyl-M+mancozeb with dimethomorph, which has both protectant and systemic activity, should be considered particularly to replace the recommended spray at flowering. Additionally, it is recommended that dimethomorph, mandipropamid and azoxystrobin are evaluated for their ability to protect *P. sparsa* disease-free plants from spore infection of foliage in the field. However, none of these fungicides are currently registered on boysenberry in New Zealand and the effect of dimethomorph and mandipropamid to reduce dryberry incidence under New Zealand field conditions has not been tested. However, these extra fungicide application could add to the costs and would need to be evaluated based on a cost:benefit ratio. Additionally, dimethomorph is currently registered for oomycetes control in grapes, onions, potatoes and lettuce (Novachem agrichemical manual, 2015). Future field trials with these fungicides, along with fungicide residue analysis of the fruits, can be recommended to facilitate their registration for boysenberry in New Zealand.

In the final chapter, a novel one step nested PCR method was optimised to detect latent infection of *P. sparsa.* This was more convenient, reliable, economical and faster than the conventional two step nested PCR (Dodd *et al*., 2007). The detection sensitivity of this method was 0.4 pg (400 fg) of *P. sparsa* genomic spore DNA, sensitive enough to detect single spores. This is biologically adequate as a higher numbers of spores are likely to be required for optimum infection of *P. sparsa* (Chapter 2) in intact boysenberry plants; hence the detection limit in this nested PCR should be sufficient for accurate early diagnosis. Further work to create a multiplex PCR for this system should be done so that the issue of false negatives is removed.

In summary, a berry infection pathway was identified whereby dryberry was caused by both systemic and spore infection of *P. sparsa*. As the majority of boysenberry plants in New Zealand are systemically infected, disease management is required by thorough early diagnosis of the latent infection. For that, the current nested PCR method was optimised, in addition to the more convenient novel one step nested PCR method which was optimised to detect a minimum of a single spore of *P. sparsa* with detection sensitivity of 0.4 pg of *P. sparsa* genomic DNA. Although the sporadic growth of *P. sparsa* was observed *in planta* to cause inconsistency in the PCR results, the current study identified that root or crown samples were the most reliable asymptomatic tissues for PCR throughout the year in New Zealand for sampling. One of the most significant practical implications for boysenberry growers in New Zealand is development of a novel method for producing *P. sparsa* free boysenberry propagation material without using

fungicides that limits the development of fungicide resistance strains of the pathogen. In addition, for disease management in the field, three PA applications as is the current grower practice was slightly improved with recommendation to apply mancozeb+metalaxyl-M to limit spore infection of the flowers. Also, it is recommended that dimethomorph, mandipropamid and azoxystrobin be evaluated for their ability to protect *P. sparsa* disease-free plants from spore infection of foliage in the field followed by fungicide residue analysis of the fruits.

Presentations and publications from this thesis

Conference presentation

Herath Mudiyanselage, A.M., Jaspers, M.V., Ridgway, H.J., Walter, M., and Langford, G. (2012). *Evaluation of methods for sterilising boysenberry leaves for downy mildew infection studies*. Poster presented at New Zealand Plant Protection Society Conference 2012, Nelson, New Zealand.

Herath Mudiyanselage, A.M., Jaspers, M.V., Ridgway, H.J., Walter, M., and Langford, G. (2013). *Heat and chemical treatments to reduce systemic infection of tissue culture derived boysenberry plants (Rubus spp.) by the downy mildew pathogen* Peronospora sparsa. Paper presented at The Australasian Plant Pathology Society Conference, Auckland, New Zealand.

Herath Mudiyanselage, A.M., Jaspers, M.V., Ridgway, H.J., Walter, M., and Langford, G. (2013). *Evaluation of methods for long term storage of the boysenberry downy mildew pathogen* Peronospora sparsa. Paper presented at The New Zealand Plant Protection Society Conference 2013, Napier, New Zealand.

Herath Mudiyanselage, A.M., Jaspers, M.V., Ridgway, H.J., Walter, M., and Langford, G. (2013). *Evaluation of methods for long term storage of the boysenberry downy mildew pathogen* Peronospora sparsa. Oral presentation at Lincoln University Postgraduate Conference.

Journal publication

Herath Mudiyanselage, A.M., Jaspers, M.V., Ridgway, H.J., Walter, M., and Langford, G. (2013). Evaluation of methods for long term storage of the boysenberry downy mildew pathogen *Peronospora sparsa*. *New Zealand Plant Protection, 66*, 254-258.

Oral presentations at boysenberry growers meetings

Introduction to the planned PhD research project, annual boysenberry council meeting (2011), Nelson, New Zealand.

PhD research results and future experiments, annual boysenberry council meeting (2012), Nelson, New Zealand.

Progress of PhD research results after 18 months, annual boysenberry council meeting (2013), Nelson, New Zealand.

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Appendix A

Supplementary material for Chapter 2

A.1 Location of the three Boysenberry grower properties in the south island of New Zealand used for spore trapping and sampling.

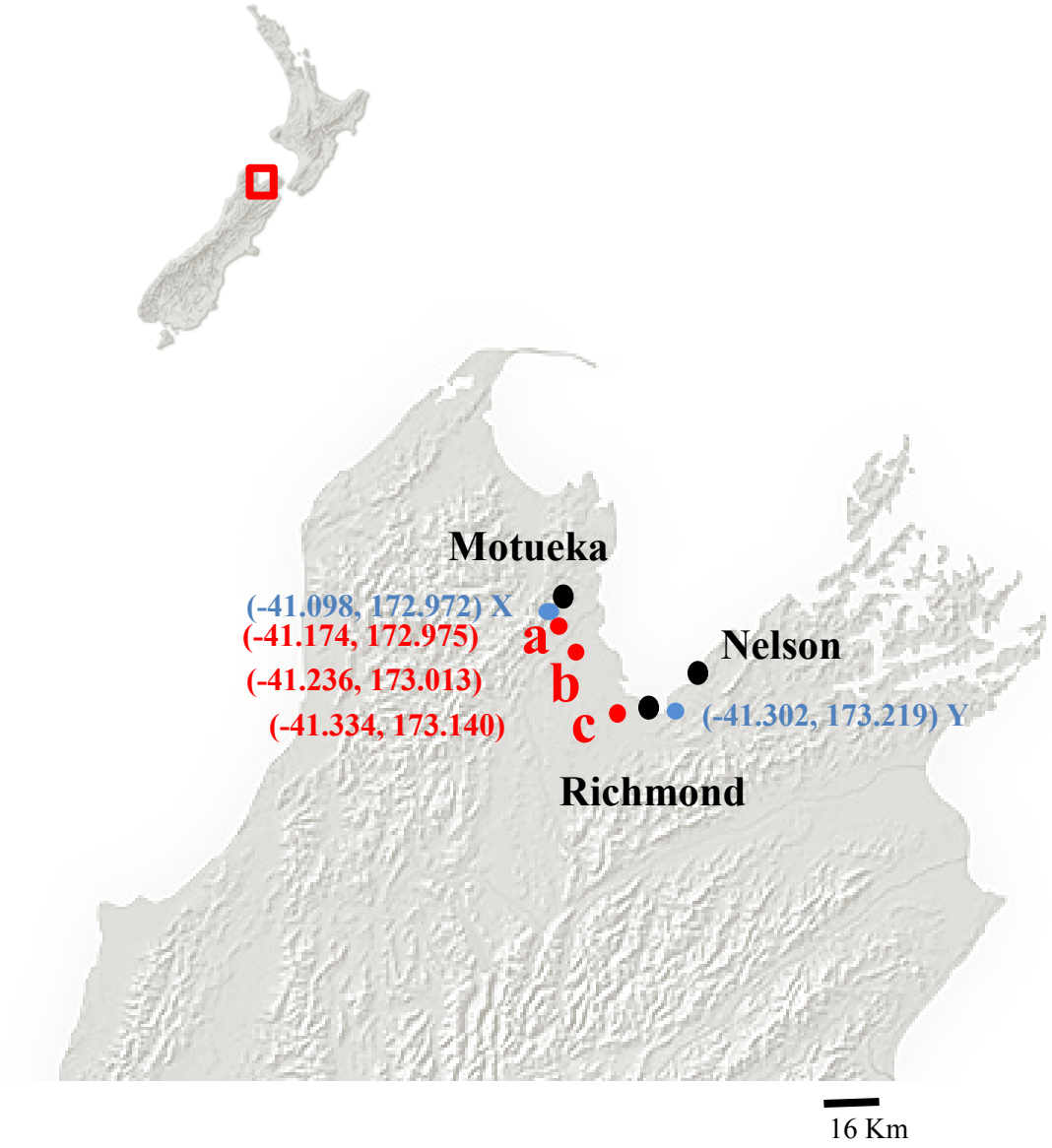

Figure A.1 Location of the three boysenberry grower properties in the Nelson region used to obtain boysenberry tissue materials and spore trapping for *P. sparsa.* (a) Holdaway Rd, Lower Moutere grower, (b) Gardners Valley, RD 1, George Harvey Rd, Upper Moutere grower, (c) Ranzau Rd West, RD 1, Richmond grower. (X) Riwaka Ews weather station and (Y) Nelson Aws weather station. Latitudes and longitudes for each grower property and weather stations are in parentheses.

A.2 12-14 month potting mix composition

For total volume of 200 L:

Bark 160 L
Pumice 40 L
Fertilisers- Osmocote exact 16-3.9-9.1 (12-14 months) 1000 g
Horticultural lime 200 g
Hydroflo 200 g

A.3 Recipes for media and stain types used

1. 1.5% Water agar (WA):

   15 g agar powder (Danisco® Bacteriological Agar) in 1000 mL water. Autoclave at 121ºC for 15 min.

2. Lacto-glycerol cotton blue for staining fungi in wet amounts (for 100 mL):

Lactic acid	20 mL
Glycerol	40 mL
Distilled water	20 mL
Aniline blue	0.075 g

*Acid always added to water to prevent dangerous chemical reactions.

A.4 Analysis of Variance (ANOVA) (General Linear Model) for spore trapping data in the field.

A.4.1 ANOVA (GLM) results on the effect of assessment time (weeks) on the number (log_{10}) of *P. sparsa* spores trapped at three different grower properties in 2010.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Week	10	107.5468	107.5468	10.7547	15.50	0.000
Grower property	2	3.1013	3.1013	1.5507	2.23	0.111
Week x Grower property	20	83.9208	83.9208	4.1960	6.05	0.000
Error	132	91.5841	91.5841	0.6938		
Total	164	286.1530				
S = 0.832958 R-Sq = 67.99% R-Sq(adj) = 60.24%						

A.4.2 ANOVA (GLM) results on the effect of grower property and assessment time (weeks) in 2011 on the number (log_{10}) of *P. sparsa* spores trapped at three different grower properties.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Week	2	2.6835	2.6835	1.3418	7.95	0.001
Grower property	12	46.3243	46.3243	3.8604	22.86	0.000
Week x Grower property	24	13.7014	13.7014	0.5709	3.38	0.000
Error	117	19.7578	19.7578	0.1689		
Total	155	82.4671				
S = 0.410938 R-Sq = 76.04% R-Sq(adj) = 68.26%						

A.4.3 ANOVA (GLM) results on the effect of grower property and assessment time (23 and 29 December) in 2011 on the number of dryberries per plant in the rows where *P. sparsa* spores were trapped at three different grower properties.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Grower	2	3429.3	4574.2	2287.1	21.46	0.000
Assessment time	1	3030.3	3532.2	3532.2	33.15	0.000
Grower x Assessment time	2	4020.1	4020.1	2010.0	18.86	0.000
Error	17	1811.6	1811.6	106.6		
Total	22	12291.4				
S = 10.3230 R-Sq = 85.26% R-Sq(adj) = 80.93%						

A.5 Lesion expansion and sporulation score (scale 0-6) data for *Peronospora sparsa* symptomatic leaves of two ages (mature and young) detached from boysenberry (cv Mapua) plants from the Lincoln University shadehouse in early December, 2011 and incubated at 80% RH for 14 days at three different temperatures (10°C, 15°C, 20°C).

	Leaf rep	LL_i (mm)	LL_{14dpi} (mm)	LW_i (mm)	LW_{14dpi} (mm)	LE (mm^2)	SS
Mature symptomatic							
10°C	1	25.16	25.16	27.25	27.25	0	0
	2	14.97	14.97	14.93	14.93	0	0
	3	27.68	*	27.62	*	-	0
	4	19.13	19.13	32.15	32.15	0	0
	5	16.16	*	11.32	*	-	0
	6	42.32	*B. cinerea*	35.56	*B. cinerea*	-	0
15°C	1	18.44	*	14.73	*	-	0
	2	31.74	*	10.54	*	-	0
	3	20.34	*	15.37	*	-	0
	4	8.28	*	13.10	*	-	0
	5	12.36	*	12.97	*	-	0
	6	10.17	*	25.52	*	-	0
20°C	1	12.68	12.97	20.26	22.00	0.50	2
	2	39.39	48.95	11.79	11.79	112.71	4
	3	42.10	*B. cinerea*	6.32	*B. cinerea*	-	0
	4	23.51	*	14.03	*	-	0
	5	42.03	*B. cinerea*	16.26	*B. cinerea*	-	4
	6	18.16	*	3.83	*	-	0
Young symptomatic							
10°C	1	53.58	*	12.42	*	-	0
	2	19.42	*	17.79	*	-	0
	3	11.17	*	13.56	*	-	0
	4	42.79	*	23.60	*	-	0
	5	53.99	*	28.20	*	-	0
	6	35.23	*	25.49	*	-	0
15°C	1	32.32	*	9.21	*	-	0
	2	48.75	*	19.50	*	-	0
	3	23.94	*	13.74	*	-	0
	4	35.37	*	32.60	*	-	0
	5	51.56	*	21.57	*	-	0
	6	21.17	*	6.00	*	-	0
20°C	1	27.46	*	10.05	*	-	0
	2	8.88	*	11.00	*	-	0
	3	20.40	*	14.01	*	-	0
	4	34.33	*	12.9	*	-	0
	5	24.54	24.66	19.84	19.97	5.59	0
	6	14.06	*	7.98	*	-	0

* Dried leaves; - No lesion expansion observed; *Botrytis cinerea* (*B. cinerea*) growth was observed(All leaves and leaf discs obtained from field plants and leaf discs cut from leaves obtained from shadehouse plants incubated at 80%RH irrespective of incubation temperature were dried. Therefore only data for leaves from shadehouse plants was recorded). LL-Lesion length, LW-Lesion width, LE-Lesion expansion, SS-Sporulation score, i-initial, 14dpi

A.6 Analysis of Variance (ANOVA) on the effect of leaf surface sterilisation treatments.

A.6.1 ANOVA results on the effect of leaf surface sterilisation treatments on the number of fungal colonies recovered on PDA from the two leaf surfaces (abaxial and adaxial).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	3	60908.9	20303.0	40.36	<.001
Leaf surface	1	5280.7	5280.7	10.50	0.002
Treatment x Leaf surface	3	5745.9	1915.3	3.81	0.013
Residual	88	44267.5	503.0		
Total	95	116203.0			

A.6.2 ANOVA results of the effect of leaf surface sterilisation treatments on the number of fungal colonies recovered on both PDA and NA from the two leaf surface (abaxial and adaxial).

a) PDA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	3	195751	65250	46.95	<.001
Leaf surface	1	22447	22447	16.15	<.001
Treatment x Leaf surface	3	30788	10263	7.38	<.001
Residual	40	55587	1390		
Total	47	304573			

b) NA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	3	305714	101905	55.00	<.001
Leaf surface	1	35698	35698	19.27	<.001
Treatment x Leaf surface	3	36546	12182	6.57	0.001
Residual	40	74116	1853		
Total	47	452073			

A.6.3 Fungal and bacterial colonies developing on PDA and NA media from the boysenberry leaves after surface sterilisation treatments.

Figure A.4 Fungal and bacterial colonies developing on (A-D) PDA and (E-H) NA from the leaves after (A, E) untreated control, (B, F) sterile water, (C, G) 10% bleach and (D, H) 70% ethanol treatments. The top and bottom rows for each treatment are from the abaxial and adaxial leaf surfaces, respectively.

A.7 Analysis of Variance (ANOVA) on the effect of different treatments on spore germination.

A.7.1 ANOVA (GLM) results on the effect of substrate, incubation time and temperature on germination of *P. sparsa* spores.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Substrate	2	38049.3	38049.3	19024.7	187.06	0.000
Time/h	1	1.7	1.7	1.7	0.02	0.897
Temperature (Temp.)/C	2	2991.7	2991.7	1495.9	14.71	0.000
Substrate x Time/h	2	99.9	99.9	50.0	0.49	0.613
Substrate x Temp./C	4	1404.1	1404.1	351.0	3.45	0.011
Time/h x Temp./C	2	87.3	87.3	43.7	0.43	0.652
Substrate x Time/h x Temp./C	4	477.8	477.8	119.4	1.17	0.327
Error	90	9153.2	9153.2	101.7		
Total	107	52265.1				
S = 10.0847 R-Sq = 82.49% R-Sq(adj) = 79.18%						

A.7.2 ANOVA results for the effect of the enumeration methods on the percentage germination of *P. sparsa* spores incubated on leaf discs.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Method	2	8752.9	4376.5	31.75	<.001
Leaf_surface	2	1221.6	610.8	4.43	0.018
Method x Leaf_surface	4	649.4	162.3	1.18	0.333
Residual	45	6203.1	137.8		
Total	53	16827.1			

A.7.3 Raw data for verification of the accuracy of the cellotape strip method for assessing percentage spore germination on leaf discs. Spore germination was assessed on the cellotape strip and on the same leaf disc after clearing.

Before clearing of leaf disc			After clearing of the same leaf disc			No. spores lost from inoculum*
Total no. of spores	No. germinated	% spore germination	Total no. of spores	No. germinated	% spore germination	
78	47	60.26	61	5	8.20	61
70	40	57.14	52	4	7.69	78
89	51	57.30	49	4	8.16	62
69	43	62.32	75	8	10.67	56
74	47	63.51	73	8	10.96	53
67	37	55.22	64	5	7.81	69
97	59	60.82	80	11	13.75	23
72	47	65.28	50	4	8.00	78
81	49	60.49	68	7	10.29	51
74	47	63.51	91	13	14.29	35
Means		60.62			9.98	57

* Each inoculum droplet on average contained 200 spores.

A.8 Analysis of Variance (ANOVA) on the effect of different treatment on infection of leaf discs by *P. sparsa.*

A.8.1 ANOVA (Generalized linear model-Bernoulli distribution) results of the effect of temperature and source of the tissue type on the incidence of *P. sparsa* infection of leaf discs.

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Temperature	1	6.128	6.128	4.9	0.03
Tissue type	1	3.261	3.261	2.61	0.111
Temperature x Tissue type	1	0.789	0.789	0.63	0.43
Residual	68	85.09	1.251		
Total	71	95.265	1.342		

A.8.2 ANOVA (GLM) results of the effect of temperature and tissue type on the lesion size caused by *P. sparsa* lesion size.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Infected tissue	1	3296.4	3296.4	3296.4	7.34	0.009
Temperature/ºC	2	11099.3	4507.8	2253.9	5.02	0.009
Infected tissue x Temperature/ºC	2	2505.1	2505.1	1252.5	2.79	0.069
Error	66	29646.2	29646.2	449.2		
Total	71	46547.0				
S = 21.1940 R-Sq = 36.31% R-Sq(adj) = 31.48%						

A.8.3 ANOVA (GLM) results of the effect of temperature and tissue type on the number of spores per lesion produced by *P. sparsa.*

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Infected tissue	1	8417452	8417452	8417452	8.13	0.006
Temperature/ºC	2	22277725	11547584	5773792	5.58	0.006
Infected tissue x Temperature/ºC	2	2136445	2136445	1068223	1.03	0.362
Error	66	68308178	68308178	1034972		
Total	71	101139800				
S = 1017.34 R-Sq = 32.46% R-Sq(adj) = 27.35%						

A.8.4 Kruskal-Wallis Test results for the sporulation score data.

A.8.4a Kruskal-Wallis Test results for the effect of tissue type on *P. sparsa* sporulation score (scale 0-6).

Treatment	N	Median	Ave Rank	Z
Leaf discs	54	5.000	39.1	1.86
Leaves	18	0.000	28.6	-1.86
Overall	72		36.5	
H = 3.46 DF = 1 P = 0.063				
H = 3.87 DF = 1 P = 0.049 (adjusted for ties)				

A.8.4b Kruskal-Wallis Test results for the effect of temperature on sporulation score (scale 0-6) by *P. sparsa.*

Temperature	N	Median	Ave Rank	Z
10ºC	24	0.000	25.3	-3.21
15ºC	24	5.000	44.0	2.14
20ºC	24	5.000	40.3	1.08
Overall	72		36.5	
H = 10.70 DF = 2 P = 0.005				
H = 11.97 DF = 2 P = 0.003 (adjusted for ties)				

A.8.4c Kruskal-Wallis Test results for the effect of temperature and tissue type on sporulation score (scale 0-6) by *P. sparsa*.

Treatment	N	Median	Ave Rank	Z
Leaf discs 10ºC	18	0.000	25.9	-2.47
Leaf discs 15ºC	18	6.000	48.2	2.74
Leaf discs 20ºC	18	5.000	43.3	1.59
Leaves 10ºC	6	0.000	23.3	-1.61
Leaves 15ºC	6	2.500	31.2	-0.65
Leaves 20ºC	6	2.500	31.2	-0.65
Overall	72		36.5	
H = 15.27 DF = 5 P = 0.009				
H = 17.07 DF = 5 P = 0.004 (adjusted for ties)				

A.9 Analysis of Variance (ANOVA) on the effect of spore concentration on *P. sparsa* infection.

A.9.1 ANOVA (Generalized linear model-Bernoulli distribution) results of the effect of spore concentration and assessment time (dpi) on the incidence of infection caused by *P. sparsa.*

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Spore concentration	4	71.3208	17.8302	36.56	<.001
dpi	1	5.9789	5.9789	12.26	<.001
Spore concentration x dpi	4	5.6472	1.4118	2.9	0.026
Residual	90	43.8888	0.4877		
Total	99	126.8357	1.2812		

A.9.2 Kruskal-Wallis Test results for the sporulation score data.

A.9.2a Kruskal-Wallis Test results for the effect of spore concentration on sporulation score (scale 0-6) by of *P. sparsa.*

Spore concentration (spores/20µL)	N	Median	Ave Rank	Z
0.2	20	0.000	34.0	-2.84
2	20	0.000	34.0	-2.84
20	20	0.000	37.5	-2.24
200	20	5.000	73.3	-3.93
2000	20	5.000	73.7	-4.00
Overall	100		50.5	
H = 42.05 DF = 4 P = 0.000				
H = 60.65 DF = 4 P = 0.000 (adjusted for ties)				

A.9.2b Kruskal-Wallis Test results for the effect of assessment time (dpi) on sporulation score (scale 0-6) by *P. sparsa.*

assessment time (dpi)	N	Median	Ave Rank	Z
9	50	0.000	46.5	-1.37
12	50	0.000	54.5	1.37
Overall	100		50.5	
H = 1.87 DF = 1 P = 0.171				
H = 2.70 DF = 1 P = 0.100 (adjusted for ties)				

A.9.2c Kruskal-Wallis Test results for the effect of spore concentration and assessment time (dpi) on sporulation score (scale 0-6) by *P. sparsa*.

Spore concentration (spores/20µL)	N	Median	Ave Rank	Z
0.2 spores-12dpi	10	0.000	34.0	-1.90
0.2 spores-9dpi	10	0.000	34.0	-1.90
2 spores-12dpi	10	0.000	34.0	-1.90
2 spores-9dpi	10	0.000	34.0	-1.90
20 spores-12 dpi	10	0.000	41.0	-1.09
20 spores-9dpi	10	0.000	34.0	-1.90
200 spores-12 dpi	10	5.500	86.5	4.14
200 spores-9dpi	10	2.500	60.0	1.10
2000 spores-12dpi	10	5.000	76.8	3.02
2000 spores-9dpi	10	5.000	70.6	2.31
Overall	100		50.5	
H = 46.73 DF = 9 P = 0.000				
H = 67.40 DF = 9 P = 0.000 (adjusted for ties)				

A.10 Analysis of Variance (ANOVA) on the effect of leaf maturity on susceptibility to *P. sparsa* infection.

A.10.1 ANOVA (Generalized linear model) results of the effect of leaf age, source of the leaves and assessment time (dpi) on the incidence of *P. sparsa* infection.

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Leaf age	1	17.877	17.877	26.46	<.001
Source (leaf discs)	1	20.2082	20.2082	29.91	<.001
dpi	1	5.2472	5.2472	7.77	0.006
Leaf age x Source (leaf discs)	1	1.1738	1.1738	1.74	0.19
Leaf age x dpi	1	0.0043	0.0043	0.01	0.936
Source (leaf discs) x dpi	1	2.595	2.595	3.84	0.052
Leaf age x Source (leaf discs)x dpi	1	0.0002	0.0002	0.00	0.986
Residual	136	91.8766	0.6756		
Total	143	138.9823	0.9719		

A.10.2 Kruskal-Wallis Test results for the sporulation score data.

A.10.2a Kruskal-Wallis Test results for the effect of the source of leaves on sporulation score (scale 0-6) by *P. sparsa.*

Source of leaves	N	Median	Ave Rank	Z
Greenhouse	72	0.000	62.4	-2.91
Shadehouse	72	0.000	82.6	2.91
Overall	144		72.5	
H = 8.47 DF = 1 P = 0.004				
H = 18.29 DF = 1 P = 0.000 (adjusted for ties)				

A.10.2b Kruskal-Wallis Test results for the effect of leaf age on sporulation score (scale 0-6) by *P. sparsa.*

Age of leaves	N	Median	Ave Rank	Z
Old	72	0.000	62.4	-2.86
Young	72	0.000	82.6	2.86
Overall	144		72.5	
H = 8.18 DF = 1 P = 0.004				
H = 17.67 DF = 1 P = 0.000 (adjusted for ties)				

A.10.2c Kruskal-Wallis Test results for the effect of assessment time (dpi) on *P. sparsa* disease based on sporulation score (scale 0-6).

dpi	N	Median	Ave Rank	Z
10	72	0.000	67.9	-1.33
14	72	0.000	77.1	1.33
Overall	144		72.5	
H = 1.76 DF = 1 P = 0.185				
H = 3.80 DF = 1 P = 0.051 (adjusted for ties)				

A.10.2d Kruskal-Wallis Test results for the effect of source of the leaves, age and assessment time (dpi) on sporulation score (scale 0-6) by *P. sparsa.*

Categorised factors	N	Median	Ave Rank	Z
Greenhouse-old-10dpi	18	0.000	59.0	-1.47
Greenhouse-old-14dpi	18	0.000	62.3	-1.11
Greenhouse-young-10dpi	18	0.000	59.0	-1.47
Greenhouse-young-14dpi	18	0.000	69.2	-0.36
Shadehouse-old-10dpi	18	0.000	62.5	-1.08
Shadehouse-old-14dpi	18	0.000	66.4	-0.67
Shadehouse-young-10dpi	18	0.000	91.0	2.01
Shadehouse-young-14dpi	18	0.000	110.6	4.14
Overall	144	4.500	72.5	
H = 24.90 DF = 7 P = 0.001				
H = 53.76 DF = 7 P = 0.000 (adjusted for ties)				

A.11 Analysis of Variance (ANOVA) on the susceptibility of different *Rubus* sp. cultivars to *P. sparsa* infection.

A.11.1 ANOVA (Generalized linear model) results for the incidence *P. sparsa* infection on different *Rubus* cultivars.

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Cultivar	3	31.4679	10.4893	14	<.001
Residual	44	32.9756	0.7494		
Total	47	64.4435	1.3711		

A.11.2 One way ANOVA results of P. sparsa lesion size and number of spores per lesion.

A.11.2a One way ANOVA results of *P. sparsa* lesion size on different *Rubus* cultivars.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	3	27728.3	9242.8	10.25	<.001
Residual	44	39691.2	902.1		
Total	47	67419.6			

A.11.2b One way ANOVA results of *P. sparsa* number of spores per lesion on different *Rubus* cultivars.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cultivar	3	1.255E+09	4.184E+08	11.68	<.001
Residual	44	1.577E+09	3.583E+07		
Total	47	2.832E+09			

A.12 Journal article published on evaluation of methods for long term storage of the boysenberry downy mildew pathogen *Peronospora sparsa.*

Evaluation of methods for long term storage of the boysenberry downy mildew pathogen *Peronospora sparsa*

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Abstract Downy mildew of boysenberry is caused by the biotrophic pathogen *Peronospora sparsa*. To ensure supplies of viable spore inoculum for infection experiments, six storage methods were assessed: the leaf discs cut from sporulating areas of the leaf stored dry or in 20% glycerol, and spores were suspended in 20% glycerol, all three of which were stored at either -20°C or -80°C. After 1, 2, 4 or 6 months storage, spore viability and the capability to infect leaf discs were evaluated. Storage methods had no significant effects on spore germination or infection. Storage time and temperature significantly ($P<0.001$) affected spore viability and infection, being greatest after 1 month and at -80°C. Overall, viability of spores stored as suspensions at -80°C (the most effective treatment) was greater than all other methods tested, and spore germination decreased from over 60% after 1 month of storage to less than 5% after 4 months.

Keywords storage temperature, spore germination, infection, sporulating, dryberry.

INTRODUCTION

Downy mildew of *Rubus* and *Rosa* species is caused by *Peronospora sparsa* Berk. (synonym *P. rubi* Rabenh.) (Breese et al. 1994), and is considered the most economically important disease of boysenberry (*Rubus* spp.) (Ellis et al. 1991). This disease is a serious threat to boysenberry growers in New Zealand. In 2001/02 in the Nelson region, downy mildew on fruit reduced boysenberry yields by approximately 50% for crops grown under conventional management (Richards 2002), with total losses in 2002 valued at NZ$1.8 M (Dodd et al. 2007). For organic growers, the situation was worse, with a total loss of fruit for that season. More recently in Whakatane, downy mildew was estimated to reduce yields by up to 25% in 2009-2010 (C.I. Julian, Berry Devine Boysenberries, Whakatane, personal communication).

Peronospora sparsa is an obligate biotroph, and as such is difficult to maintain in the laboratory as it cannot be cultured on artificial media. Maintenance of the pathogen requires culturing on plants, which is both time- and space–consuming, and is difficult during plant dormancy over winter periods. A long term storage method is therefore required to ensure viable spore inoculum is available for infection studies. Breese et al. (1994) reported that *P. sparsa* isolates from *Rosa* and *Rubus* spp. when stored on freshly sporulating leaf disks or leaflets in sealed plastic boxes at -70°C, remained viable for up to 4 months, but longer storage was not reported. In addition, they also reported that *P. sparsa* viability was retained when sporulating leaf discs were stored in liquid nitrogen, but not the maximum storage time. For the related pathogen *P. viciae*, spores on sporulating pea leaves were reported to remain viable for at least 1 year when stored dry at -80ºC (Gill & Davidson 2005). Glycerol has been widely used as a cryopreservation protectant for different microorganisms including oomycetes (Hubálek 2003). This compound maintained viability of *P. parasitica* spores to 21% after 1 year of storage (Klodt-Bussmann & Paul 1995). In contrast, for *P. viciae*, spores were not viable after 45 days when stored on different cryoprotectants including glycerol, skim milk plus glycerol or dimethylsulfoxide (Gill & Davidson 2005). The aim of the present study was to evaluate different methods for long term storage of *P. sparsa* spore inoculum with or without a cryoprotectant (20% glycerol), to provide information on effective storage protocols for this pathogen.

MATERIALS AND METHODS

Six storage methods were assessed. Leaves obtained from boysenberry (cv. 'Mapua') plants systemically infected by *P. sparsa* and grown in the Lincoln University shadehouse were used as a source of the pathogen inoculum. In order to produce sufficient young spores, 400-500 systemically infected leaves were incubated on 1.5% (w/v) water agar (WA) at 15ºC in 12:12 h light:dark, and observed for sporulation under the stereomicroscope. The resulting spores were stored in the following ways:

1. The sporulating regions of leaves were cut aseptically as leaf discs (7 mm diameter), and six leaf discs were placed in each sterile vial and stored at -80°C or -20ºC
2. The sporulating leaf discs were placed in sterile vials containing 20% glycerol, mixed for 5-10 s and stored at -80°C or -20°C.
3. Spores were harvested from sporulating lesions by gently shaking sporulating leaf discs in 20% glycerol. Any leaf debris was removed using sterilised forceps and the concentration (assessed with a hemacytometer) was adjusted to 10_5 spores/ml with 20% glycerol. The final glycerol suspensions were pipetted into 1.7 ml cryogenic tubes and stored at either -80°C or -20ºC.

Three vials were prepared for each of the six storage regimes for each storage time period (1, 2, 4 or 6 months).

The viability of spores was determined after each storage time period using three randomly selected vials from each treatment. The leaf discs were placed in sterile water, gently shaken and the spore concentrations in the resulting suspensions were adjusted to 10_4 spore/ml based on haemocytometer counts. The stored spore suspensions were diluted with sterile distilled water to 10_4 spores/ml. To assess germination and infection, fresh leaf discs that had been washed with sterile water (Herath Mudiyanselage et al. 2012) to minimise contamination were placed on 1.5% WA, abaxial side uppermost. For each replicate spore suspension for each treatment, a 20 µl drop (approximately 200 spores) was placed on the abaxial surface of each of two separate leaf discs and incubated in the dark at 20°C for 24 h. Germination was assessed using a cellotape strip to pick up these spores and mounting them on a drop of lacto-glycerol cotton blue placed on a microscopic slide (best method from preliminary experiments). A spore was assessed as germinated when the germ tube length was at least half the diameter of the spore. For each sample, the total intact spores and numbers of germinated spores were counted using a light microscope at ×10 magnification.

To assess the infective capacity of the spores, a 20 µl drop of each replicate diluted spore suspension, was placed on the surface of each of three sterile water washed leaf discs placed on 1.5% WA and incubated at 15°C in 12:12 h light:dark, the optimum conditions for infection determined in preliminary studies. The presence of lesions was assessed after 14 and 24 days incubation. The experiments were repeated three times for the germination assessments and twice for the infection assessments.

Table 1 Percent spore germination after one, two, four and six months storage at -80 and -20°C for the different storage methods: leaf discs (LD) cut from sporulating regions stored dry, and in 20% glycerol, and spores suspended (SS) in 20% glycerol.

	Temperature/ ºC						Temperature x time [1]	
	-80			-20				
Method	SS in 20% glycerol	LD	LD in 20% glycerol	SS in 20% glycerol	LD	LD in 20% glycerol	-80ºC	-20ºC
Time/ months								
1	61.5	47.6	50.8	44.3	40.8	39.6	53.3 a	41.6 b
2	22.0	13.2	13.3	4.3	12.6	14.6	16.2 c	10.5 c
4	5.0	1.2	0.7	0.9	1.1	0.7	2.3 d	0.9 d
6	2.6	1.2	1.2	0.4	0.5	1.5	1.7 d	0.8 d
Temperature x method #	22.8 A	16.5 B	15.8 B	12.5 B	13.7 B	14.1 B		

[1] Values followed by the same letter are not significantly different. For temperature x method (A-B) and temperature x time (a-d) means were highly significant (P=0.006, P=0.004, respectively).
Means within a column followed by the same letters are not significantly different according to Tukey's HSD at P<0.001

Statistical analyses

The percentage of healthy spores and percentage germination for the different storage treatments after different storage times were analysed using general linear model (Minitab 16). When factors were statistically significant, means were compared between treatments using Tukey's honestly significant differences (HSDs) at P<0.001. The proportions of leaf discs infected and with lesions were assessed by generalized linear model in GenStat 14. Confidence intervals (95%) for binomial proportion means are indicated in Figure 1. The experimental repeats were included in the analyses as random factors.

RESULTS

There was no statistically significant effect of storage method (P=0.111; Table 1) on spore germination. Storage time (P<0.001) affected spore viability, being greater after 1 month storage than after longer storage times. The storage temperature also affected spore germination (P<0.001), with spore viability being greater after storage at -80°C than at -20°C. The interaction between the storage methods and temperatures (P=0.006), and time and temperatures (P=0.004) also significantly affected the viability of spores. After 1 month, storage viability was significantly higher (P<0.001) at -80°C (53.3%) than -20°C (41.6%). Viability of spores stored as a suspension at -80°C (22.8%) was significantly higher (P<0.001) than all other methods (16.5-12.5%). However, even in the most effective treatment (spore suspension at -80°C) spore germination decreased from over 60% after 1 month storage to below 5% after 4 months storage.

There was no statistically significant effect of storage method (P=0.464; Figure 1) on ability of the spores to subsequently infect and cause leaf lesions. The ability of the spores to infect was affected both by storage time (P<0.001) and temperature (P<0.001). The interaction between the storage method and temperature (P=0.013) also significantly affected infection. After 1 month storage, spores stored as suspensions or on dried leaf discs at -80°C gave greater levels of infection compared with spores stored as dry leaf discs or in glycerol at -20°C (P<0.001). There were no significant differences in the amounts of infection between the three storage methods at -80°C and spores suspended in glycerol at -20°C. Decreased infection occurred between 1 and 2 months storage for all storage treatments, except for leaf discs stored in glycerol at -80°C and -20°C, and dried leaf discs at -20°C. There were very low infection rates observed from spores after 3 months storage for all storage methods and temperatures, and no infection resulted from spores stored for 4 or 6 months.

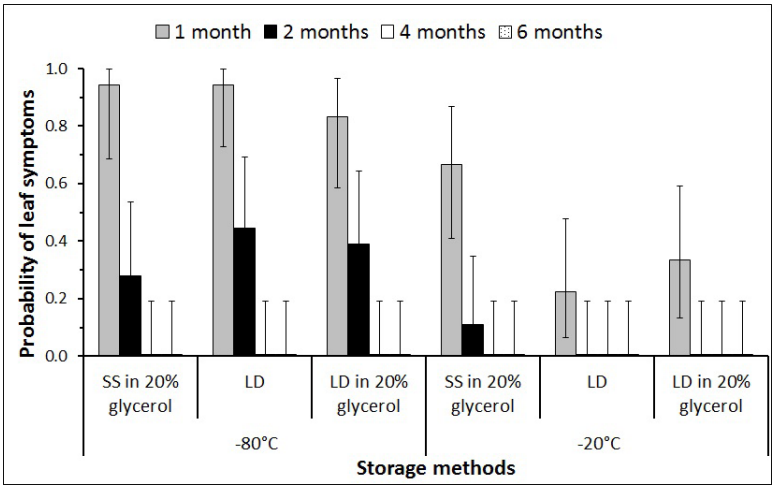

Figure 1 Probability of lesions (infection) on leaf discs (LD), resulting from *Peronospora sparsa* spores stored for different periods at either -80 or -20°C, using three different storage methods: leaf discs cut from sporulating regions stored dry (LD), leaf discs in 20% glycerol or spores suspended (SS) in 20% glycerol. Error bars represent the 95% confidence intervals of the probability of lesions (infection).

In addition, after 6 months spores stored on dried leaf discs at both temperatures were distorted and shrunken compared with the healthy spherical appearance of the spores stored as either suspensions or leaf discs in glycerol.

DISCUSSION

Spore viability of *P. sparsa* decreased with increasing storage time over 1 month, with none of the storage methods tested resulting in the maintenance of spore viability and ability of the spores to infect boysenberry leaves beyond 2 month storage. After 1 month storage, the viability of spores stored at -80°C was greater than those stored at -20°C, but this was not maintained after 2 months storage. In contrast, Breese et al. (1994) reported that *P. sparsa*, both from *Rubus* (blackberry and tummelberry) and *Rosa* species, retained viability for 1-4 months when stored as sporulating leaf discs or leaflets in a sealed plastic box at -70ºC. However, no information regarding the relative viability of the spores after the different storage periods was provided. Long-term storage of *P. sparsa* spores suspended in dimethylsulphoxide by cryopreservation using liquid nitrogen was also reported by these authors, but again no information on the viability after different storage periods was provided. In addition, this method required specialised equipment to enable the temperature to be lowered at a controlled rate. *Peronospora viciae* spores stored dry as sporulating infected pea leaves at -80°C retained viability for at least 1 year (Gill & Davidson 2005), infecting approximately 62% of pea seedlings compared with 93% seedling infection when fresh spores were used. However, these authors also suggested that sufficient spores should be stored to compensate for the decrease in infection rate. In contrast spores suspended in dimethylsulphoxide, glycerol, glycerol and skim milk or in sterilised water and stored at -80°C did not remain viable. However, in the present study with *P. sparsa*, the addition of glycerol did not significantly affect spore viability, indicating that different methods may be effective for different species of downy mildew pathogens.

It has been suggested that the sporangia (spores) of Peronosporales are relatively susceptible to damage and therefore lose viability due to their relatively thin and fragile walls (Laviola et al. 2006). Gulya et al. (1993) reported variable success of storage methods for different downy mildew species, with the spores of some species having extremely thin walls making them difficult to store. In this study, the shaking (mixing) and pipetting of the spores or osmotic shock due to the use of 20% glycerol (Laviola et al. 2006) may have contributed to the loss of viability due the physiological or mechanical stresses caused. However, the observation that the spores stored without the addition of glycerol were misshapen indicates that these spores were damaged during storage at low temperature. The addition of glycerol may also have protected the spores as it can penetrate cell walls and membranes as a cryoprotective additive even though the permeability is slow (Hubálek 2003). In the present study the leaf discs were not dried prior to storage, and this may have contributed to loss of viability, as Gill & Davidson (2005) recommended including filter paper to prevent condensation or ice formation, which could contribute to a reduction in spore viability. Since *P. sparsa* also produces thick walled oospores, the use of oospores for long term storage may be effective and warrants testing. However, a suitable method for consistent and rapid production of oospores of *P. sparsa* is required.

The relationship between spore viability and ability of the spores to infect and cause visible leaf lesions was expected. Previous work had indicated that at least 20 spores were required to initiate infection and produce a lesion (A.M. Herath Mudiyanselage, unpublished data). Therefore the numbers of lesions that developed during the assessment period were probably proportional to the numbers of viable spores applied to the leaf discs. However, there was an indication that some viable spores had reduced capacity for infection. The spores stored as sporulating leaf discs both dry and in glycerol at -20°C retained reasonable viability (approx. 40%) after 1 month storage. However, these spores were not as effective for infecting the boysenberry leaves, since spores with reduced viability (13-4.3%), such as those stored as leaf discs at -80°C after 2 months, resulted in comparable infection. Likewise, although viability of spores stored for 2 months on leaf discs at -20°C was similar to those stored at -80°C, resulting infection was much less from those spores stored at -20°C.
None of the methods used resulted in long-term storage of *P. sparsa*, with infection being low after only 1 month storage.

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A.13 Analysis of Variance (ANOVA) repeated measures analysis results on the effect of optimum incubation conditions on lesion expansion rate of *P. sparsa* systemic and spore initiated lesions on leaves detached from boysenberry plants grown in the shadehouse Lincoln University.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Subject stratum					
Lesion type	1	30.0297	30.0297	10.90	0.002
Residual	34	93.6644	2.7548	6.98	
Subject x Time stratum					
d.f. correction factor 0.5227					
Time	4	2.0664	0.5166	1.31	0.277
Time x Lesion type	4	1.7106	0.4277	1.08	0.346
Residual	136	53.6409	0.3944		
Total	179	181.1121			

A.14 Effect of the leaf surface on sporulation of *P. sparsa* on boysenberry leaf discs.

A.14.1 The percentage of boysenberry (cv. Mapua) leaf discs (n=18) sporulated by *Peronospora sparsa* when inoculated on upper or lower leaf disc surfaces with freshly prepared *P. sparsa* spore suspension (200 spores/ 20 µL) and incubated on 1.5% WA at 15°C (12 h/12 h light/ dark) for 14 days.

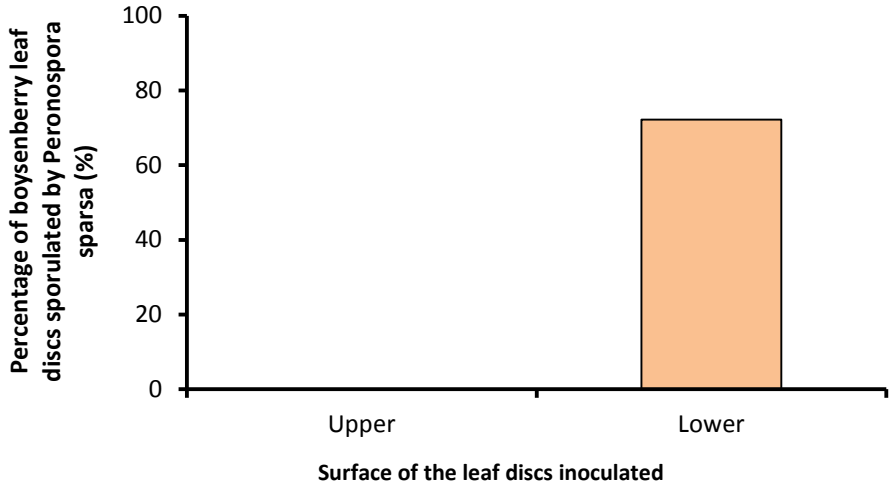


Figure A.5 The percentage of boysenberry (cv. Mapua) leaf discs (n=18) sporulated on lower surface of the leaf discs when inoculated on upper or lower leaf disc surfaces with freshly prepared *Peronospora sparsa* spore suspension.

A.14.2 Sporulation of *Peronospora sparsa* through stomata of the abaxial side of the boysenberry (cv. Mapua) leaf discs.

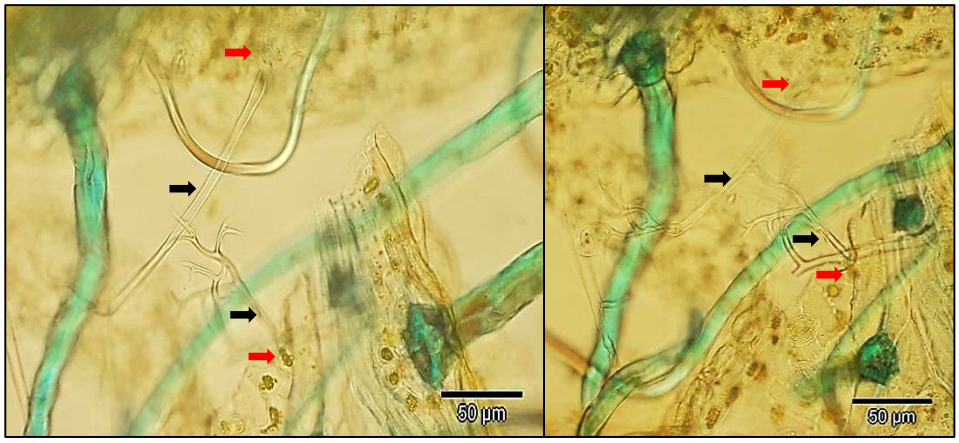


Figure A.6 Sporulation of *Peronospora sparsa* through stomata of the abaxial side of the boysenberry (cv. Mapua) leaf discs. Red and black arrows indicate the stomata and the sporangiophores immerging through the stomata respectively.

Appendix B

Supplementary material for Chapter 3

B.1 Buffer compositions used for DNA extraction

Plant & food CTAB buffer

Cetyltrimethyl ammonium bromide/Hexadecyltrimethylammonium bromide (CTAB)		2%
Sodium Chloride	0.8 M	
EDTA	20 mM	
Tris HCL	20 mM (pH 8)	

Aegerter CTAB buffer (Aegerter et al., 2002)

Cetyltrimethyl ammonium bromide/Hexadecyltrimethylammonium bromide (CTAB)		2.5%
Polyvinyl pyrrolidone (PVP 40)	1%	
Sodium Chloride	1.4 M	
EDTA	50 mM	
Tris HCL	100 mM (pH 8)	
2-mercaptoethanol[1]	0.5%	

[1](added just prior to use the buffer)

Aegerter modified CTAB buffer

Cetyltrimethyl ammonium bromide/Hexadecyltrimethylammonium bromide (CTAB)		2.5%
Polyvinyl pyrrolidone (PVP 40)	1%	
Sodium Chloride	1.4 M	
EDTA	50 mM	
Tris HCL	100 mM (pH 8)	

10 Mm TE Buffer (10 mM Tris, 1mM EDTA)

1 M Tris-Cl (Tris-HCl) pH 7.4	0.5 mL
0.5 M EDTA pH 8.0	0.1 mL
RO water	50 mL

5% Sarcosyl (Sodium Lauroylsarcosine)

Sodium Lauroylsarcosine salt	5.0 g
RO water	100 mL

*All the reagents were sterilised by autoclaving for 15 min. at 121°C

B.2 Manufacturer's protocol for PowerPlant® DNA isolation kit (MO BIO Laboratories, Inc, 2746 Loker Ave West, Carlsbad, CA).

Plant tissue sample (50 mg) was added to the PowerPlant® bead tubes (contained metal beads) followed by addition of 550 µL of PowerPlant® bead solution which contained a buffer that helped to wet the tissue surfaces and protect nucleic acids from degradation. It was vortexed gently to mix. Solution PB1 (containing sodium dodecyl sulphate (SDS) and other disruption agents required for complete cell lysis) was added (60 µL) and vortexed briefly. PB1 solution was checked carefully before addition if it had precipitated. If it had precipitated it was heated to 60ºC until the precipitate had dissolved before it was used. The PowerPlant® bead tubes were placed in a water bath at 65ºC for 10 min. Homogenization was done using the MiniBeadBeater cell disrupter (FRITSCH Pulverisette 23, Germany) at 26 oscillation per second for 10 min. This process was conducted twice, each time for 5 min with 1 min to cool the tubes in ice each time. Six tubes were able to process at once. The samples were centrifuged at 10,000 x g for 1 min. at room temperature. The supernatant was transferred to a clean 2 mL collection tube provided with the kit. The solution PB2 (containing a reagent to precipitate non-DNA organic and inorganic material including plant polysaccharides, cell debris and proteins) was added (250 µL) and tubes were inverted to mix the contents, followed by incubation at 4ºC for 5 min. Then the tubes were centrifuged at room temperature for 1 min. at 10,000 x g. The entire volume of the supernatant, except the pellet, was transferred to a clean 2.2 mL collection tube (provided with the kit). The solution PB3 (99% isopropanol) was added (1000 µL) and the tubes were inverted at least five times to mix the contents followed by incubation at room temperature for 10 min, then the tubes were centrifuged for 15 min at 12,000 x g. The supernatant was discarded and the pellet was re-suspended in 100 µL of solution PB6 (isopropanol). The solution PB4 (a concentrated salt solution) was shaken to mix well, and 500 µL of it was added to the tube which was briefly vortexed to mix. The entire volume (600 µL) was loaded onto a spin filter and centrifuged at 10,000 x g for 1 min. Next, the spin filter basket was removed, the flow through was discarded and the spin filter basket was replaced back in the tube. The solution PB5 (500 µL) which was an ethanol based wash solution was added and the tube centrifuged for 30 sec. at 10,000 x g. The flow through was discarded from the 2 mL collection tube and the tube was centrifuged again for 1 min. at 10,000 x g. The spin filter was placed carefully in a clean 2 mL collection tube (provided with the kit) avoiding any splashing of solution PB5 onto the spin filter. The solution PB6 (elution buffer, 50 µL) was added to the centre of the white filter membrane of the tube and it was centrifuged for 30 sec. at 10,000 x g.

B.3 Optimisation of the nested PCR cycle number.

Nested PCR amplification (Section 3.2.2) was conducted with changes in cycle number for the denaturation step (i) 30 cycles, (ii) 35 cycles, (iii) 40 cycles and, (iv) 45 cycles for 30 s at 95°C. Only one of the boysenberry tissue samples was positive for *P. sparsa* with the standard nested PCR cycle number (30 cycles), which was leaf tissue (Figure B.1). However, leaf and/or leaf bud samples of two plants (of the 3 replicate plants) were positive with 35 cycles but the positive result with the leaf sample for 30 cycles was not reproduced with 35 cycles.

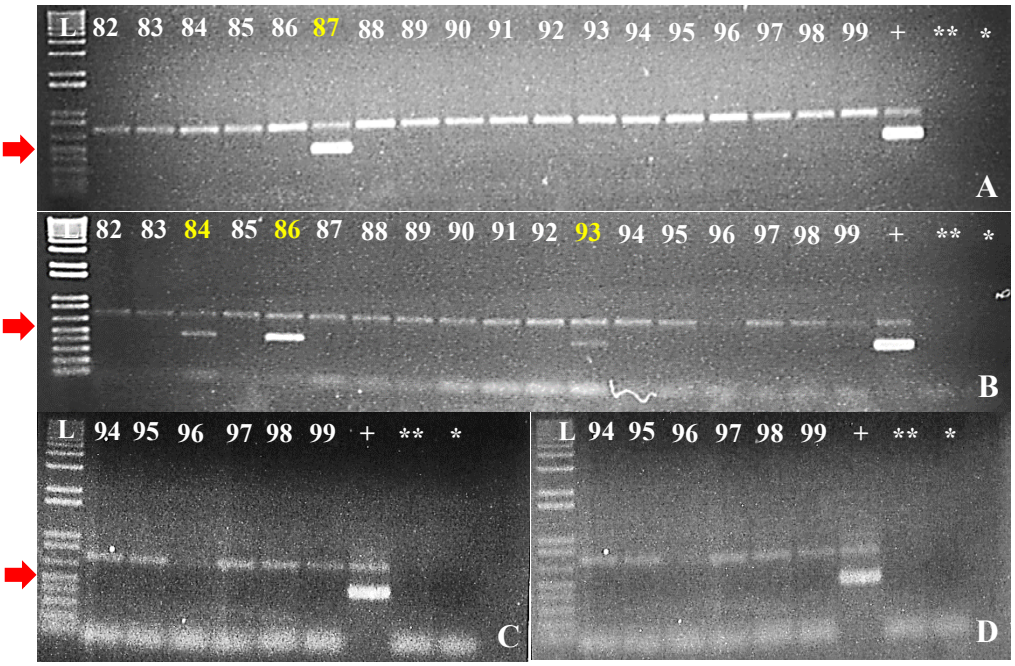


Figure B.1 1% agarose gel for the nested PCR method with increased cycle numbers, 30 (standard cycle number) (A), 35 (B), 40 (C) and 45 (D) for the asymptomatic plant tissues obtained from three boysenberry plants suspected to be infected (A and B), asymptomatic plant tissues obtained from the plant.3 suspected to be infected (C and D). Primocane tip, leaf bud and leaf samples of (i) plant-1, cane-1 (82-84), cane-2 (85-87), (ii) plant-2, cane-1 (88-90), cane-2 (91-93), and (iii) plant-3, cane-1 (94-96), cane-2 (97-99). Diluted control (with PCR water) from 1^{ry} PCR step of nested PCR (**), negative control (*). The samples with positive PCR are in yellow. L=1+KB plus DNA ladder. The expected band size (660 bp) is indicated by red arrows.

None of the tissue samples from the 3rd plant which was *P. sparsa* negative with previous cycle numbers became positive with the further increased cycle numbers 40 and 45 which indicated that the 3rd plant was disease free or that the level of infection was lower than the detection threshold. Therefore the optimised cycle number for nested PCR was 35 and the above results showed that the most sensitive detection of *P. sparsa* by this method was from asymptomatic leaves.

B.4 Estimation of C value for *Peronospora sparsa.*

Table 1 Estimate of DNA based on a putative C value of *Peronospora sparsa* for each spore suspension examined with the nested PCR with the 1, 2 or 3 µl DNA in the PCR reaction.

Sample	DNA concentration (pg/µl)	Number of *P. sparsa* spores in the PCR reaction with,			C value per *P. sparsa* spore (pg)			C value per single genome (pg)		
		1 µl DNA	2 µl DNA	3 µl DNA	1 µl DNA	2 µl DNA	3 µl DNA	1 µl DNA	2 µl DNA	3 µl DNA
1	3100	0	0	0	*	*	*	*	*	*
2	1800	12	24	36	150	75	50	75	38	25
3	1900	13	26	39	142	71	47	71	36	24
4	2000	32	64	96	61	30	20	30	15	10
5	1200	9	18	27	133	67	44	67	33	22
6	2200	1	2	3	2150	1075	717	1075	538	358
7	1700	1	2	3	1650	825	550	825	413	275
8	2600	0	0	0	*	*	*	*	*	*
9	2900	41	82	123	71	35	24	35	18	12
10	2600	4	8	12	638	319	213	319	159	106
11	1900	12	24	36	158	79	53	79	40	26
12	5500	9	18	27	611	306	204	306	153	102
13	2000	1	2	3	2000	1000	667	1000	500	333
14	3200	0	0	0	*	*	*	*	*	*
15	2300	0	0	0	*	*	*	*	*	*
16	1200	1	2	3	1150	575	383	575	288	192
17	1500	10	20	30	150	75	50	75	38	25
18	3100	0	0	0	*	*	*	*	*	*

*result is undefined. There are other fungi present which amplify well giving a band sizes between 300-850 bp.

B.5 Verification of the DNA extract from spores trapped on the Vaseline® coated slides.

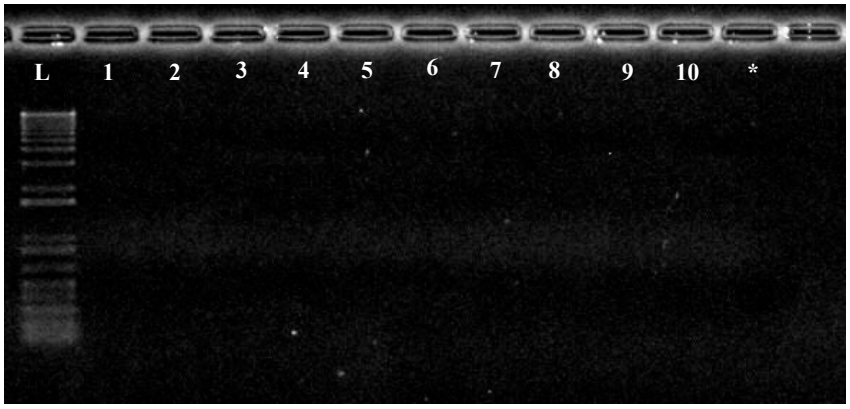


Figure B.2 1% agarose gel showing negative results for the ten representative samples of total DNA extracted from the spores trapped on the Vaseline® coated slides. Samples 1-5 are from the nested PCR positive (for *Peronospora sparsa*) and samples 6-10 are from the nested PCR negative samples. The first three sample of each category are from higher spore numbers and the last two samples are from zero or lower spore numbers counted for *P. sparsa* and the negative control (*). L=1+KB plus DNA ladder.

Table 2 DNA concentration, purity (A260/A280 ratio) and number of spores of *Peronospora sparsa* expected to be contained in 5 µL DNA used in the gel electrophoresis of the ten representative samples of DNA extracted from the spore trap Vaseline® coated slides.

Sample	DNA concentration (pg/µL)	DNA purity (A260/A280)	Number of *P. sparsa* spores in 5 µL DNA used in the gel	Nested PCR reaction for *P. sparsa* in the figure 3.9 (Chapter 3)
1	2200	1.50	5	Positive
2	2600	1.64	20	Positive
3	5500	1.41	45	Positive
4	2600	1.56	0	Positive
5	2300	1.49	0	Positive
6	1800	1.44	60	Negative
7	2000	1.47	160	Negative
8	2900	1.60	205	Negative
9	1200	1.52	45	Negative
10	3100	1.40	0	Negative

B.6 Establishing detection sensitivity (limit of detection) of the standard PCR.

The standard PCR method was sensitive enough to detect 0.4 ng of *P. sparsa* genomic DNA that had been extracted from spores (Figure B.3).

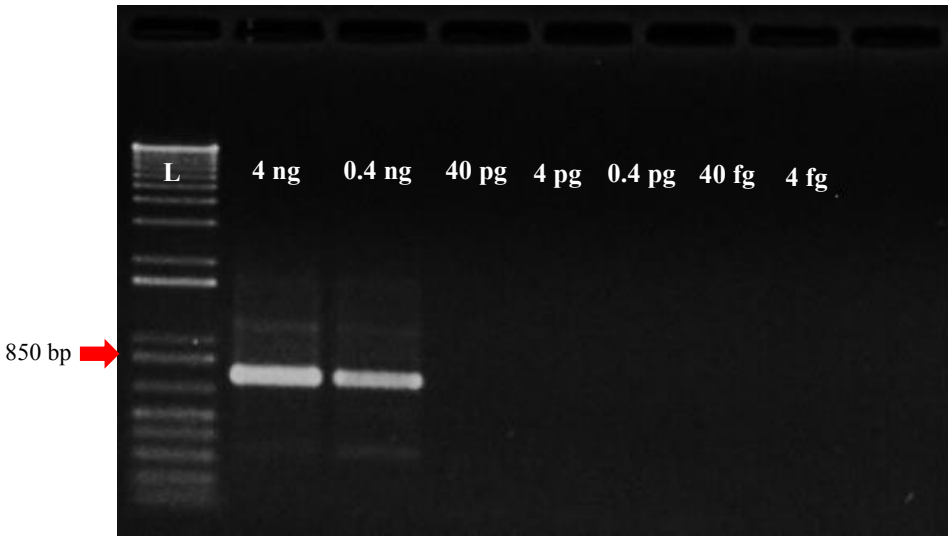


Figure B.3 1% agarose gel of *Peronospora sparsa* spore genomic DNA (4 ng to 4 fg) amplified using standard PCR and DNA extracted using the modified Plant & Food Research protocol. DNA quantity is designated at the top of the gel. L=1+KB plus DNA ladder.

Appendix C

Supplementary material for Chapter 4

C.1 Potting mix composition (3-4 months),

For Vol 1 L:*:* Composted Bark 80%

Pumice 1-4 mm 20%

Fertilisers- Osmocote exact 16-3.5-10 (NPK) (3-4 months fertiliser) 3 g

Horticultural lime 1 g

Hydroflo (wetting agent) 1 g

C.2 Hoagland's solution recipe

To make full strength solution 1 L:

Main solution;

1 M Calcium nitrate 7 mL

1 M Potassium nitrate 5 mL

1 M Potassium dihydrogen phosphate 2 mL

1 M Magnesium sulfate 2 mL

Solution A-trace element 1 mL;

Boric acid (H_3BO_3) 0.5 PPM

Manganese chloride ($MnCl_2.4H_2O$) 0.5 PPM

Zinc sulfate ($ZnSO_4.7H_2O$) 0.05 PPM

Copper Sulfate ($CuSO_4.5H_2O$) 0.02 PPM

Sodium molibdate ($Na_2MoO_4.2H_2O$) 0.01 PPM

Solution B-FeEDTA 1 mL;

Dissolve 26.1 g EDTA in 268 mL of 1N KOH. Then add 24.9 FeSO4.7H2O and dilute to 1 L. after aerating overnight to produce the stable ferric complex, the pH should be about 5.5. 1 mL provides 5 PPM to 1 L of solution.

Add 1 mL of solution A and 1 mL of solution B to main solution. Made to a total volume of 1 litre with distilled water.

C.3 Effect of mancozeb and metalaxyl-M on *Peronospora sparsa* spore germination.

C.3a Effect of concentration of mancozeb and metalaxyl-M on the mean percent germination of *Peronospora sparsa* spores on microscopic slide incubated at 20ºC, for 4 h in darkness.

Fungicide concentration	Mean % germination of *P. sparsa* spores after 4 h on microscopic slide (%)	
	Mancozeb	Metalaxyl
1	0.0	7.5
10	0.0	5.9
50	0.0	6.0
100	0.0	3.6
1000	0.0	0.0
Control	12.0	

C.3b Details reported for mancozeb and metalaxyl-M.

Active ingredient	Trade name	Conc. (a.i)	Manufacturer	Mode of action
Dithiocarbamate Mancozeb	Manco 75 WG[1]	750 g/Kg	Syngenta Crop Protection Ltd	Inhibits germination of the spores (protectant)
Phenylamide Metalaxyl-M (Mefenoxam)	APRON®XL[2]	350 g/L	Syngenta Crop Protection Ltd	Inhibits ribosomal DNA synthesis which affects mycelial growth and formation of spores (systemic)

[1]Novachem manual-2013
[2]http://www.syngenta.com/global/corporate/en/products-and-innovation/product-brands/seed-care/Pages/apron-xl.aspx and Novachem manual-2013

C.4 ANOVA results (General linear model) of the effect of fungicides and concentration on germ tube length and spore size (length/width ratio) for *P. sparsa* spores after 24 h on leaf discs.

C.4a germ tube length (µm).

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Fungicide	1	72418	72418	72418	7.81	0.006
Concentration	4	821426	821426	205357	22.14	0.000
Fungicide x Concentration	4	97940	97940	24485	2.64	0.034
Error	290	2689586	2689586	9274		
Total	299	3681370				
S = 96.3039 R-Sq = 26.94% R-Sq(adj) = 24.67%						

C.4b spore length/width ratio.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Fungicide	1	0.003982	0.003982	0.003982	0.99	0.320
Concentration	4	0.003225	0.003225	0.000806	0.20	0.938
Fungicide x Concentration	4	0.003375	0.003375	0.000844	0.21	0.932
Error	290	1.161399	1.161399	0.004005		
Total	299	1.171981				
S = 0.0632837 R-Sq = 0.90% R-Sq(adj) = 00.00%						

C.5 Regression analysis (Generalized linear model) results for the effect of fungicides and concentration on the incidence of *P. sparsa* infection on leaf discs after 14 days.

Experiment 1

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Fungicide	1	1.4982	1.4982	3.48	0.067
Concentration	1	16.6272	16.6272	38.59	<.001
Fungicide x Concentration	1	0.976	0.976	2.27	0.138
Residual	56	24.1263	0.4308		
Total	59	43.2277	0.7327		

Experiment 2

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Fungicide	1	1.2005	1.2005	2.72	0.111
Concentration	1	10.4368	10.4368	23.67	<.001
Fungicide x Concentration	1	0.4597	0.4597	1.04	0.317
Residual	26	11.4635	0.4409		
Total	29	23.5605	0.8124		

C.6 ANOVA results (General linear model) of the effect of mancozeb spray at either flower or berry stage on the proportion of dryberries produced.

C.6a Mancozeb spray at the flower stage.

C.6a.1 Production of dryberries from king flowers.

Source	DF	SS	MS	F	P
Incubation	1	1.2230	1.2230	4.52	0.049
Spray	1	0.6071	0.6071	2.24	0.154
Incubation x Spray	1	0.1719	0.1719	0.64	0.437
Error	16	4.3273	0.2705		
Total	19	6.3293			
S = 0.520055 R-Sq = 31.63% R-Sq(adj) = 18.81%					

C.6a.2 Production of dryberries from non-king flowers.

Source	DF	SS	MS	F	P
Incubation	1	1.0654	1.0654	6.67	0.020
Spray	1	0.5489	0.5489	3.44	0.082
Incubation x Spray	1	0.0728	0.0728	0.46	0.509
Error	16	2.5560	0.1597		
Total	19	4.2431			
S = 0.399684 R-Sq = 39.76% R-Sq(adj) = 28.47%					

C.6b Mancozeb spray at the berry stage.

C.6b.1 Production of dryberries from green berries.

Source	DF	SS	MS	F	P
Incubation	1	2.0233	2.0233	11.65	0.004
Spray	1	0.2360	0.2360	1.36	0.261
Incubation x Spray	1	0.7006	0.7006	4.03	0.062
Error	16	2.7791	0.1737		
Total	19	5.7389			
S = 0.416766 R-Sq = 51.57% R-Sq(adj) = 42.49%					

C.6b.2 Production of dryberries from red berries.

Source	DF	SS	MS	F	P
Incubation	1	1.1541	1.1541	7.48	0.015
Spray	1	0.2005	0.2005	1.30	0.271
Incubation x Spray	1	0.2005	0.2005	1.30	0.271
Error	16	2.4674	0.1542		
Total	19	4.0225			
S = 0.392702 R-Sq = 38.66% R-Sq(adj) = 27.16%					

C.7 Regression analysis (Generalized linear model) results on the effect of *P. sparsa* spore inoculation of bud/flower stage of excised flower laterals (shadehouse) on the incidence of *P. sparsa* sporulation and dryberry production after incubation at 100% RH at ambient temperature.

C.7a *P. sparsa* sporulation on flowers.

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Treatment	1	88.54	88.54	49.47	<.001
Residual	34	60.846	1.79		
Total	35	149.386	4.268		

C.7b Dryberry production

C.7b.1 Regression analysis (Generalized linear model) results for the effect of *P. sparsa* spore inoculation of king flowers on the incidence of king dryberry as a proportion of the total king berries produced.

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Treatment	1	1.51	1.51	1. 1	0.311
Residual	15	20.565	1.371		
Total	16	22.074	1.38		

C.7b.2 Regression analysis (Generalized linear model) results for the effect of *P. sparsa* spore inoculation of non-king buds on the incidence of non-king dryberry as a proportion of the total non-king berries produced.

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Treatment	1	2.093	2.093	1.16	0.330
Residual	5	8.997	1.799		
Total	6	11.09	1.848		

C.7b.3 Regression analysis (Generalized linear model) results for the effect of *P. sparsa* spore inoculation of buds and flowers on the total incidence of dryberries as a proportion of the total berries produced.

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Treatment	1	2.915	2.915	1.56	0.230
Residual	15	27.963	1.864		
Total	16	30.878	1.93		

C.8 Regression analysis (Generalized linear model) results for the effect of *P. sparsa* spore inoculation of bud/flowers of excised flower laterals (shadehouse) on the incidence of *P. sparsa* sporulation after incubation at 100% RH and 15ºC under growth lights.

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Treatment	1	15.268	15.268	15.27	0.023
Residual	34	91.848	2.701		
Total	35	107.117	3.06		

C.9 Regression analysis (Generalized linear model) results for the effect of *P. sparsa* spore inoculation of berries at the unripe stage on excised fruiting laterals (field) on the incidence of dryberry after incubation at 100% RH at ambient temperature.

C.9a. Dryberry incidence for king berries.

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Spore numbers	1	2.388	2.388	1.84	0.183
Region	1	0.022	0.022	0.02	0.897
Spore numbers x Region	1	0.126	0.126	0.1	0.757
Residual	41	53.263	1.299		
Total	44	55.799	1.268		

C.9b. Dryberry incidence for non-king berries.

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Spore numbers	1	5.128	5.128	2.68	0.110
Region	1	0.068	0.068	0.04	0.851
Spore numbers x Region	1	0.083	0.083	0.04	0.837
Residual	41	78.593	1.917		
Total	44	83.872	1.906		

C.9c. Total dryberry incidence.

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Spore numbers	1	6.446	6.446	3.34	0.075
Region	1	0.077	0.077	0.04	0.842
Spore numbers x Region	1	0.000	0.000	0.00	0.992
Residual	41	79.183	1.931		
Total	44	85.707	1.948		

C.10 Regression analysis (Generalized linear model) results of detached berries (shadehouse) on the incidence of *P. sparsa* sporulation and dryberry production after incubation at 100% RH and 15ºC under growth lights.

C.10a. Effect on incidence of *P. sparsa* sporulation.

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Block	3	0.194	0.0647	0.61	0.61
Inoculum	1	30.8285	30.8285	291.24	<.001
Maturity	1	0.2521	0.2521	2.38	0.126
Region	1	78.976	78.976	746.08	<.001
Inoculum x Maturity	1	0.0001	0.0001	0	0.981
Inoculum x Region	1	0.0002	0.0002	0	0.969
Maturity x Region	1	0	0	0	0.99
Inoculum x Maturity x Region	1	0	0	0	0.991
Residual	85	8.9976	0.1059		
Total	95	119.2486	1.2552		

C.10b. Effect on incidence of dryberry.

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Block	3	0.194	0.0647	0.61	0.61
Inoculum	1	30.8285	30.8285	291.24	<.001
Maturity	1	0.2521	0.2521	2.38	0.126
Region	1	78.976	78.976	746.08	<.001
Inoculum x Maturity	1	0.0001	0.0001	0	0.981
Inoculum x Region	1	0.0002	0.0002	0	0.969
Maturity x Region	1	0	0	0	0.99
Inoculum x Maturity x Region	1	0	0	0	0.991
Residual	85	8.9976	0.1059		
Total	95	119.2486	1.2552		

Appendix D

Supplementary material for Chapter 5

D.1 Recipe of the tissue culture medium

MT basal medium (Murashige and Tucker medium):

Recipe for stock solution (g/Litre)

MT Macronutrient stock (50x)	
NH_4NO_3	82.5
KNO_3	95.5
$MgSO_4.7H_2O$	18.5
KH_2PO_4 (monobasic)	7.5
MT Micronutrient stock (100x)	
H_3BO_3	0.62
$MnSO_4.H_2O$	1.68
$ZnSO_4.7H_2O$	0.86
KI	0.083
$Na_2MoO_4.2H_2O$	0.025
$CuSO_4.5H_2O$	0.0025
$CoCl_2.6H_2O$	0.0025
MT Iron stock (200x)	
Na_2EDTA	7.45
$FeSO_4.7H_2O$	5.57
MT Vitamin stock (100x)	
Myo-inositol	10.0
Thiamine-HCl	1.0
Pyridoxine-HCl	1.0
Nicotinic acid	0.5
Glycine	0.2
MT Calcium stock (66x)	
$CaCl_2.2H_2O$	29.33

Recipe for 1/3 strength initiation medium or multiplication medium (g or mL/Litre)

MT Macronutrient stock (50x)	6.67 mL
MT Micronutrient stock (100x)	3.33 mL
MT Iron stock (200x)	1.67 mL
MT Vitamin stock (100x)	3.33 mL
MT Calcium stock (66x)	5.00 mL
Supplements:	
Sucrose	30 g
PPM	2 mL
Active charcoal*	0.67 g
6BA (dissolve in 1M NaOH)	1 g
IBA (dissolve in 1M NaOH)	0.5 g
Agar	7.2

All of the ingredients were mixed together using a magnetic stirrer, and the pH adjusted to 5.8 using NaOH (before adding agar and autoclaving). This was then autoclaved at 121ºC for 20 min. After allowing to cool the agar was poured into sterile plastic tubs with around 20 tubs poured from 1 L.

*Active charcoal only added to the multiplication medium and not for the initiation medium.

D.2 ANOVA results of the effect of fungicides on the incidence of *P. sparsa* infection of leaf discs *in vitro.*

D.2.1 ANOVA (Generalized linear model-Bernoulli distribution) results of the effect of fungicides and concentrations on the incidence of *P. sparsa* infection.

Change	d.f.	deviance	mean deviance	deviance ratio	approx. F pr.
Fungicide	9	1.11E+02	1.23E+01	236470.5	<.001
Concentration	1	9.52E+01	9.52E+01	1833647	<.001
Fungicide x Concentration	9	2.71E-02	3.01E-03	57.92	<.001
Residual	30	1.56E-03	5.19E-05		
Total	49	2.06E+02	4.20E+00		

D.2.2 Kruskal-Wallis Test results for the sporulation score of *P. sparsa* infection assessed using sporulation scale data.

D.2.2a Kruskal-Wallis Test results for the effect of fungicides on the sporulation score of *P. sparsa* infection assessed using sporulation scale data.

Fungicides	N	Median	Ave Rank	Z
Actigard	15	6.0	129.6	5.09
Amistar WG	15	0.0	42.5	-3.10
Bravo 720SC	15	0.0	54.8	-1.95
Curfew	15	3.0	77.3	0.17
Dichlofluanid	15	4.0	88.7	1.24
Foli-R-Fos400	15	6.0	112.0	3.43
Nando	15	0.0	51.6	-2.25
PerkSupa	15	6.0	113.5	3.57
Revus	15	0.0	42.5	-3.10
Sovrin Flo	15	0.0	42.5	-3.10
Overall	150		75.5	
H = 80.68 DF = 9 P = 0.000				
H = 99.09 DF = 9 P = 0.000 (adjusted for ties)				

D.2.2b Kruskal-Wallis Test results for the effect of fungicide concentrations on the sporulation score of *P. sparsa* infection assessed using sporulation scale data.

Concentrations	N	Median	Ave Rank	Z
1	30	4.0	94.1	2.62
10	30	2.0	82.5	0.99
50	30	1.5	80.8	0.75
100	30	0.0	71.0	-0.63
1000	30	0.0	49.1	-3.73
Overall	150		75.5	
H = 18.13 DF = 4 P = 0.001				
H = 22.26 DF = 4 P = 0.000 (adjusted for ties)				

D.2.2c Kruskal-Wallis Test results for the effect of interaction of fungicide and concentrations on sporulation score assessed as sporulation scale data of *P. sparsa* infection.

Fungicides	N	Median	Ave Rank	Z
Actigard 1	3	6.0	135.0	2.40
Actigard 10	3	6.0	135.0	2.40
Actigard 100	3	6.0	135.0	2.40
Actigard 1000	3	5.0	108.2	1.32
Actigard 50	3	6.0	135.0	2.40
Amistar WG 1	3	0.0	42.5	-1.33
Amistar WG 10	3	0.0	42.5	-1.33
Amistar WG 100	3	0.0	42.5	-1.33
Amistar WG 1000	3	0.0	42.5	-1.33
Amistar WG 50	3	0.0	42.5	-1.33
Bravo 720SC 1	3	4.0	103.8	1.14
Bravo 720SC 10	3	0.0	42.5	-1.33
Bravo 720SC 100	3	0.0	42.5	-1.33
Bravo 720SC 1000	3	0.0	42.5	-1.33
Bravo 720SC 50	3	0.0	42.5	-1.33
Curfew 1	3	5.0	108.2	1.32
Curfew 10	3	4.0	99.5	0.97
Curfew 100	3	0.0	42.5	-1.33
Curfew 1000	3	0.0	42.5	-1.33
Curfew 50	3	3.0	93.8	0.74
Dichlofluanid 1	33	5.0	108.2	1.32
Dichlofluanid 10	3	5.0	108.2	1.32
Dichlofluanid 100	3	2.0	88.0	0.50
Dichlofluanid 1000	3	0.0	42.5	-1.33
Dichlofluanid 50	3	4.0	96.7	0.85
Foli-R-Fos400 1	3	6.0	135.0	2.40
Foli-R-Fos400 10	3	6.0	135.0	2.40
Foli-R-Fos400 100	3	5.0	112.5	1.49
Foli-R-Fos400 1000	3	0.0	42.5	-1.33
Foli-R-Fos400 50	3	6.0	135.0	2.40
Nando 1	3	2.0	88.0	0.50
Nando 10	3	0.0	42.5	-1.33
Nando 100	3	0.0	42.5	-1.33
Nando 50	3	0.0	42.5	-1.33
Nando 1000	3	0.0	42.5	-1.33
PerkSupa 1	3	6.0	135.0	2.40
PerkSupa 10	3	6.0	135.0	2.40
PerkSupa 100	3	5.0	120.0	1.79
PerkSupa 1000	3	0.0	42.5	-1.33
PerkSupa 50	3	6.0	135.0	2.40
Revus 1	3	0.0	42.5	-1.33
Revus 10	3	0.0	42.5	-1.33
Revus 100	3	0.0	42.5	-1.33
Revus 1000	3	0.0	42.5	-1.33
Revus 50	3	0.0	42.5	-1.33
Sovrin Flo 1	3	0.0	42.5	-1.33
Sovrin Flo 10	3	0.0	42.5	-1.33
Sovrin Flo 100	3	0.0	42.5	-1.33
Sovrin Flo 1000	3	0.0	42.5	-1.33
Sovrin Flo 50	3	0.0	42.5	-1.33
Overall	150		75.5	
H = 120.78 DF = 49 P = 0.000				
H = 148.33 DF = 49 P = 0.000 (adjusted for ties)				

D.3 ANOVA results of the effect of fungicides to control *P. sparsa* spore infection on young disease free boysenberry plants.

D.3.1 ANOVA (General linear model) results for the effect of fungicides on the incidence of *P. sparsa* infection at two assessment times.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	8	2.9127	2.9127	0.3641	2.98	0.005
Assessment time	1	0.0338	0.0338	0.0338	0.28	0.600
Error	98	11.9542	11.9542	0.1220		
Total	107	14.9007				
S = 0.349259 R-Sq = 19.77% R-Sq(adj) = 12.41%						

D.3.2 ANOVA results for the effect of fungicides on the lesion size of *P. sparsa* infection.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Fungicides	8	3018.7	377.3	0.85	0.566
Residual	45	20004.1	444.5		
Total	53	23022.9			

D.3.3 ANOVA (General linear model) results for the effect of fungicides on the incidence of *P. sparsa* latent infection detected by PCR.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Fungicides	8	2.50294	2.50294	0.31287	3.85	0.008
Error	18	1.46216	1.46216	0.08123		
Total	26	3.96510				
S = 0.285011 R-Sq = 63.12% R-Sq(adj) = 46.73%						

D.4 ANOVA results of the effect of systemic fungicides to control *P. sparsa* spore infection (secondary infection) of young disease free boysenberry plants.

D.4.1 ANOVA (General linear model) results of the effect of fungicides on the incidence of *P. sparsa* infection.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	1.2070	1.2070	0.4023	1.47	0.230
Month inoculated	2	3.5337	3.5337	1.7668	6.44	0.003
Treatment X Month inoculated	6	0.5338	0.5338	0.0890	0.32	0.923
Error	84	23.0604	23.0604	0.2745		
Total	95	28.3349				
S = 0.523955 R-Sq = 18.61% R-Sq(adj) = 7.96%						

D.4.2 ANOVA results for the effect of fungicides on the lesion size of *P. sparsa* infection.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Fungicides	3	715.3	238.4	1.56	0.205
Inoculation	2	427.2	213.6	1.40	0.253
Fungicides x Inoculation	6	489.1	81.5	0.53	0.782
Residual	84	12843.8	152.9		
Total	95	14475.3			

D.5 ANOVA results of the effect of fungicides to prevent dryberry production on systemically infected plants.

D.5.1 ANOVA (General linear model) results of the effect of fungicides on dryberry incidence.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Fungicide	3	1.19875	0.96298	0.32099	8.45	0.000
Protectant	1	0.00129	0.00129	0.00129	0.03	0.854
Assessment time	1	0.38224	0.35378	0.35378	9.31	0.003
Fungicide X Protectant	3	0.10468	0.10468	0.03489	0.92	0.435
Fungicide X Assessment time	3	0.07967	0.04679	0.01560	0.41	0.746
Protectant X Assessment time	1	0.00127	0.00127	0.00127	0.03	0.855
Fungicide X Protectant X Assessment time	3	0.02778	0.02778	0.00926	0.24	0.866
Error	104	3.95166	3.95166	0.03800		
Total	119	5.74735				
S = 0.194928 R-Sq = 31.24% R-Sq(adj) = 21.33%						

D.5.2 ANOVA (General linear model) results on the effect of fungicides on mean dryberry drupelet score.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Fungicide	3	13.4472	11.1771	3.7257	5.15	0.002
Protectant	1	0.0029	0.0029	0.0029	0.00	0.950
Assessment time	1	1.4471	1.0369	1.0369	1.43	0.234
Fungicide X Protectant	3	0.1511	0.1511	0.0504	0.07	0.976
Fungicide X Assessment time	3	0.9467	1.2304	0.4101	0.57	0.638
Protectant X Assessment time	1	0.1209	0.1209	0.1209	0.17	0.683
Fungicide X Protectant X Assessment time	3	1.5815	1.5815	0.5272	0.73	0.537
Error	104	75.1662	75.1662	0.7228		
Total	119	92.8636				
S = 0.850148 R-Sq = 19.06% R-Sq(adj) = 7.38%						

D.6 Effect of heat and fungicide treatment on plant appearance and systemic growth of *P. sparsa.*

D.6a Figure showing the damage on boysenberry mother plants after heat treatment.

Figure D1. Growth of new shoots on boysenberry plants (with fungicides spray) after 4 weeks in the growth cabinet at 34ºC for the heat treated prior to use for tissue culture. The leaves appear burnt. Similar appearance was observed for the plants without fungicide spray.

D.6b Figure showing *Peronospora sparsa* on boysenberry petals

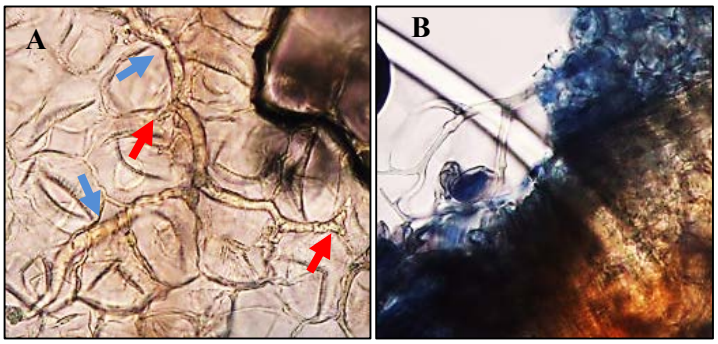
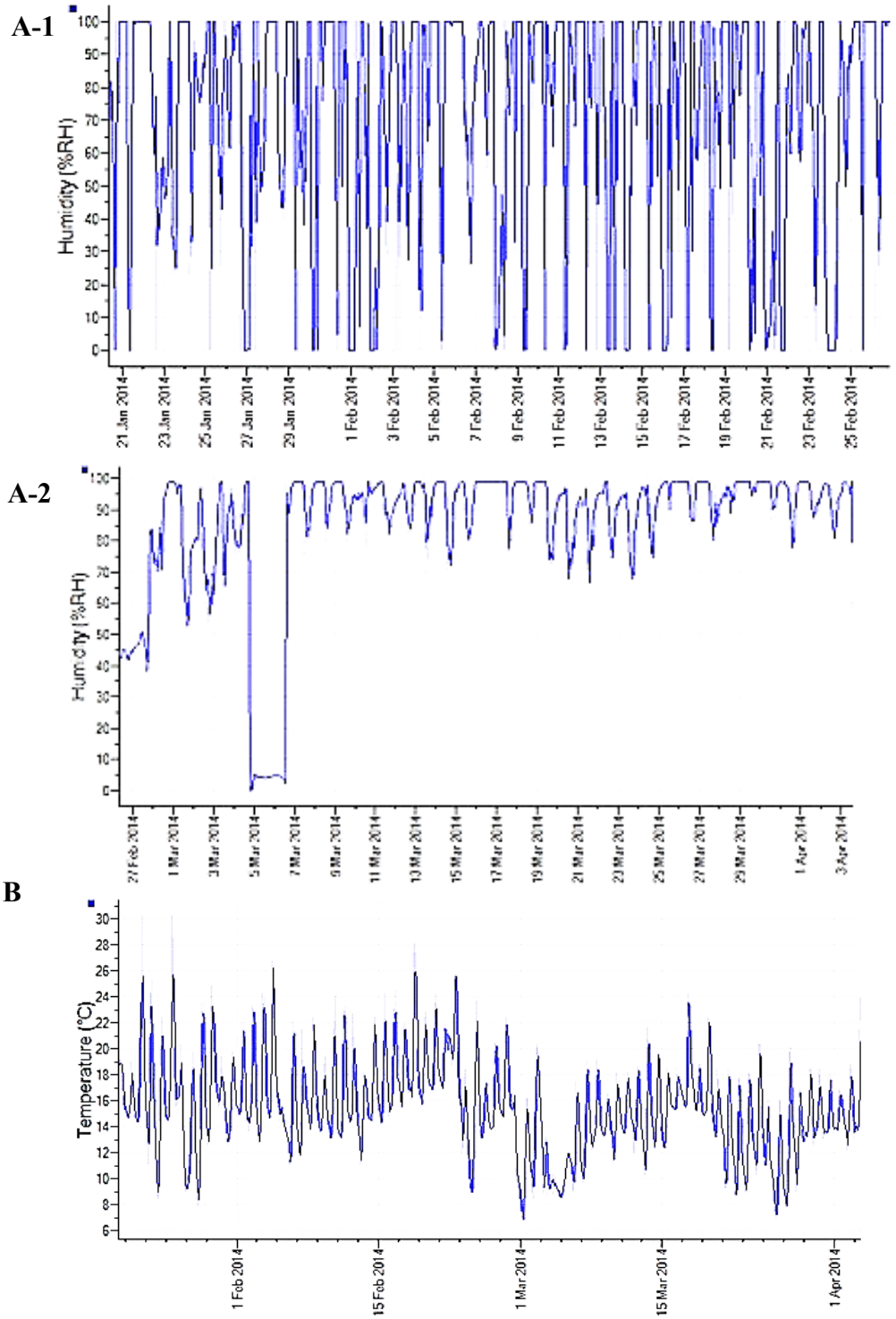


Figure D2 *Peronospora sparsa* (A) mycelium (blue arrow) and haustoria (red arrow) in cleared petals (glacial acetic acid 1: 99% ethanol 1 followed by staining with LGCB), and (B) a sporangiophore on a sporulated petal (stained with LGCB) collected from systemically infected boysenberry plants which had been sprayed three times phosphorous acid, potassium phosphite and acibenzolar-s-methyl to evaluate systemic spray effect to control dryberry.

D.7 Data recorded by the Tinytag® data loggers for humidity recorded from (A-1) 21 January to 25 February, (A-2) 27 February to 1 April and (B) temperature in the shadehouse (Lincoln University) over the duration of the experiment (21 January to 1 April) to evaluate the effect of fungicides to limit *Peronospora sparsa* infection of disease free boysenberry plants (Section 5.3.3).



D.8 Table outlining the mode of action of some of the fungicides tested.

Chemical group	Example of fungicide	Mode of action
Chloronitrile	Bravo® 750SC[1]	Multi-site inhibitor including depletion of thiols from germinating fungal cells, leading to disruption of glycolysis and energy production, fungistasis and fungicidal action
Mandelamide	Revus[2]	Inhibition of phospholipids biosynthesis and interference with cell wall deposition, however not fully understood.
Inorganics	Foli-R-Fos400[3]	Inhibits oxidative phosphorylation in oomycetes. In addition, indirect effect by stimulating the plant's natural defense response against pathogen attack.
Inorganics	PerkSupa[3]	Interrupting the metabolic processes of downy mildew
Benzothiadiazole	Actigard/Bion[4]	Plant activator which stimulates the natural systemic acquired resistance (SAR) response, no direct activity against target pathogens.
Strobilurin	Amistar WG[5]	Inhibits mitochondrial respiration in fungi
Morpholine	Sovrin® Flo[6]	Inhibits sterol biosynthesis.
Phenylamide & dithiocarbamate	Ridomil Gold MZ WG[7]	Metalaxyl-M: inhibiting fungal growth and reproduction, Mancozeb: inhibits germination of the spores

[1]http://www.syngenta.com/global/corporate/en/products-and-innovation/product-brands/crop protection/fungicides/Pages/bravo-daconil.aspx, http://www.oxon.it/AGROCHEMICALS/Products/Chlorothalonil.aspx, and http://www.agf.gov.bc.ca/pesticides/infosheets/chlorothalonil.pdf
[2]http://www.syngenta.com/global/corporate/en/products-and-innovation/product-brands/crop-protection/fungicides/Pages/revus.aspx
[3]http://pnwhandbooks.org/plantdisease/pesticide-articles/fungicidal-activity-and-nutritional-value-phosphorous-acid, http://www.vineyardadvising.com/wp-content/uploads/2013/04/Phosphorous-Acid-Factsheet_AgriLife_F.Westover.pdf
[4]Novachem manual-2013
[5]http://www.fatcow.com.au/c/syngenta/amistar-fungicide-available-from-syngenta-n811069
[6] http://resistance.nzpps.org/index.php?p=fungicides/morpholine
[7]http://www.triachem.com/Products/Fungicides/Ridomil%20Gold%20MZ%2068%20WP.pdf

Appendix E

Supplementary material for Chapter 6

E.1 Optimisation of Stage 1 conditions in a one-step nested PCR for detection of *P. sparsa* in boysenberry.

Optimisation for primer concentrations (PS long F and PS long R2 external primer pair) and primary PCR cycle number:

Method

A one-step nested PCR amplification was conducted with a reduced concentrations (8 nM *cf* the 10 nM use in Section 6.2.2) of PS long F and PS long R2 primers with 1.0 µL of template DNA in a total volume of 25 µL. Other reagents contained in the PCR were 1 X PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3 and 0.01% (w/v) gelatin), 200 µM of each dNTPs, and 1.0 µL each of 8 nM of PS long F (5'-TTTGGCGGYGRCTGCTGGCATT-3') (forward) and PS long R2 (5'-GTCCAATAAGCGCCTGTTCAGCC-3') (reverse) *P. sparsa* specific primer pairs (Invitrogen Technologies, New Zealand), 200 nM PR3 short (5'-GCTGGCTGCTACTGGGCA-3') (forward) and PR4 short (5'-CCGACTGGCCACGCGGA-3') (reverse) (modified from the PR3 and PR4 primer pair developed by Lindqvist et al. (1998)), 1 U of FastStart Taq polymerase (Roche Molecular Biochemicals, Mannheim, Germany) in addition to the 1.0 µL of template DNA. There were 25 or 30 cycles in the primary PCR (Stage 1) and 40 cycles in the secondary PCR (Stage 2) (Figure E.1).

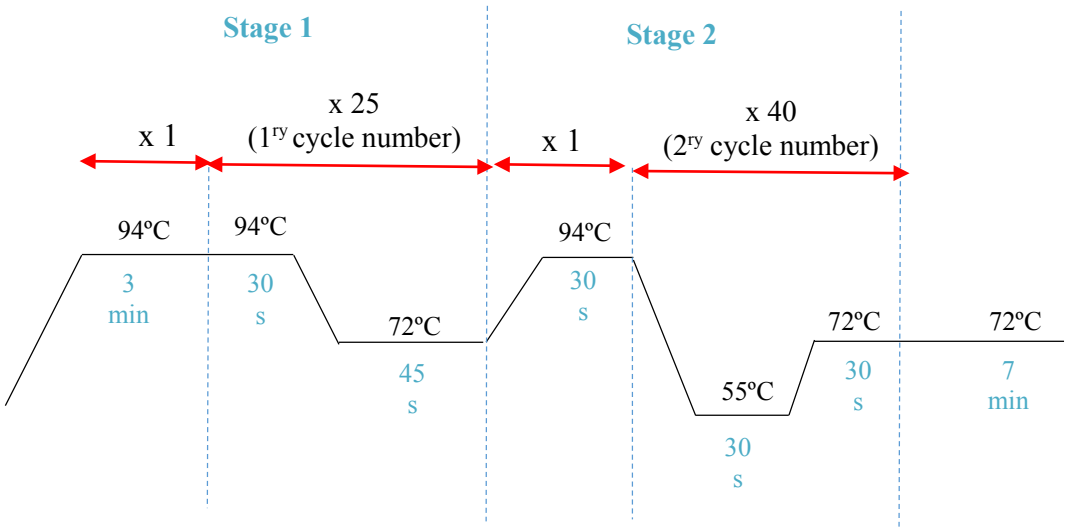


Figure E.1 The amplification conditions of the one step PCR arranged in two stages; (i) stage 1- first stage denaturing, annealing and extension followed by (ii) stage 2- second stage denaturing, annealing and elongation. PS long F and PS long R2 external primer pair was used at an annealing temperature of 72°C (stage-1), whereas the PR3 short and PR4 short internal primer pair at 55°C (stage 2).

One step PCR with 25 cycles in the primary PCR.

Samples

Two replicates each of symptomatic (non sporulating) leaf, asymptomatic leaf, asymptomatic primocane tip, old cane/stem, young cane/stem and leaf bud samples were collected from two systemically infected boysenberry plants (1 year old) grown in the shadehouse (Lincoln University). DNA was extracted using the modified Plant & Food Research CTAB method (Section 3.2.1.2) followed by gel electrophoresis and staining, as described in Section 3.2.1.3 (Chapter 3).

Results

Only one from each of symptomatic (non sporulating) leaves, asymptomatic primocane tip and old cane/stem tissue samples were positive for *P. sparsa* (Figure E.2). None of the tissues from asymptomatic leaves, buds and young canes/stems were positive for *P. sparsa.*

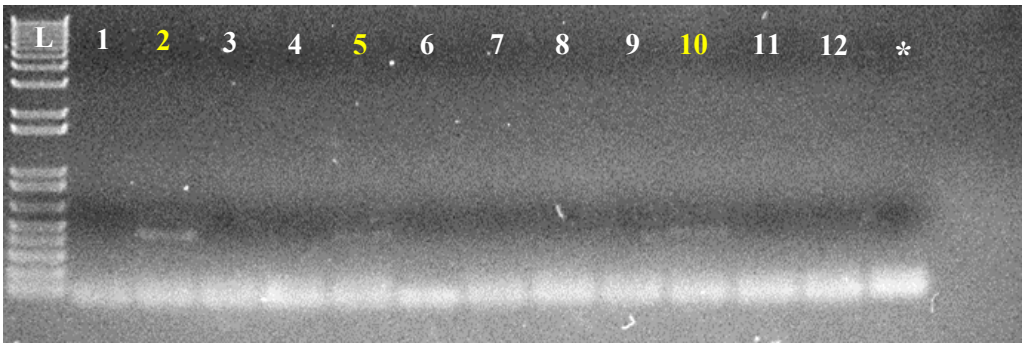


Figure E.2 1% agarose gel showing results of one-step nested PCR method with low primer concentrations (8 nM) of PS long F and PS long R2 external primer pair with plant tissues obtained from two boysenberry plants suspected to be infected. Symptomatic (non sporulating) leaf (1, 2), asymptomatic leaf (3, 4), asymptomatic primocane tip (5, 6), young cane/stem (7, 8), old cane (9, 10) and leaf bud (11, 12) samples and negative control (*). The samples with positive PCR are in yellow. Far left lane: L=1+KB DNA size marker (Invitrogen, Carlsbad, California).

Reduced primer concentration (8 nM) with 30 cycles in the primary PCR stage.

Samples

Four replicates each of symptomatic non sporulating leaves, symptomatic sporulating leaves, asymptomatic primocane tip, cane/stem and root samples were collected from four systemically infected boysenberry plants (1 year old) grown in the shadehouse (Lincoln University). DNA was extracted using the modified Plant & Food Research CTAB method (Section 3.2.1.2).

Results

Only symptomatic (3 of 4) and sporulating (all 4) leaf samples were positive for *P. sparsa* (Figure E.3). None of the other asymptomatic tissues were positive for *P. sparsa.*

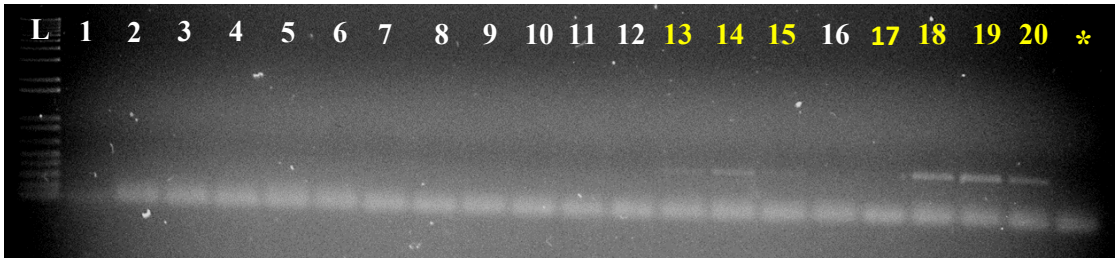


Figure E.3 1% agarose gel showing results of one-step nested PCR method with low primer concentrations (8 nM) of PS long F and PS long R2 external primer pair with plant tissues obtained from four boysenberry plants suspected to be infected. Asymptomatic root (1-4), cane/stem (5-8), primocane tip (9-12), symptomatic (none sporulating) leaf (13-16), symptomatic leaf (sporulating) (17-20) samples and negative control (*). The samples with positive PCR are in yellow. Far left lane: L=1+KB DNA size marker (Invitrogen, Carlsbad, California

E.2 Detection of the pathogen *in planta* during active plant growth.

Table E.1 Variability of detection of *P. sparsa* by one step nested PCR method from cane to cane in the same systemically infected boysenberry plants upon breaking-off the dormancy by warm conditions (greenhouse conditions).

Boysenberry plant	Cane	Tissue sample	One step nested PCR
1	Cane-1	Primocane tip (top cane)	-
		Leaf (top cane)	+
		Petiole (top cane leaf)	-
		Stem (top cane)	-
		Leaf (bottom cane)	-
		Petiole (bottom cane leaf)	-
		Stem (bottom cane)	-
	Cane-2	Primocane tip (top cane)	-
		Leaf (top cane)	-
		Petiole (top cane leaf)	-
		Stem (top cane)	-
		Leaf (bottom cane)	-
		Petiole (bottom cane leaf)	-
		Stem (bottom cane)	-
		Crown	+
		Root	+
2	Cane-1	Primocane tip (top cane)	+
		Leaf (top cane)	-
		Petiole (top cane leaf)	-
		Stem (top cane)	-
		Leaf (bottom cane)	-
		Petiole (bottom cane leaf)	-
		Stem (bottom cane)	-
	Cane-2	Primocane tip (top cane)	+
		Leaf (top cane)	+
		Petiole (top cane leaf)	-
		Stem (top cane)	-
		Leaf (bottom cane)	-

		Petiole (bottom cane leaf)	-
		Stem (bottom cane)	-
	Cane-3	Primocane tip (top cane)	-
		Leaf (top cane)	-
		Petiole (top cane leaf)	-
		Stem (top cane)	-
		Leaf (bottom cane)	-
		Petiole (bottom cane leaf)	-
		Stem (bottom cane)	-
		Crown	-
		Root	+
3	Cane-1	Primocane tip (top cane)	-
		Leaf (top cane)	-
		Petiole (top cane leaf)	-
		Stem (top cane)	-
		Leaf (bottom cane)	-
		Petiole (bottom cane leaf)	+
		Stem (bottom cane)	+
	Cane-2	Primocane tip (top cane)	+
		Leaf (top cane)	-
		Petiole (top cane leaf)	+
		Stem (top cane)	+
		Leaf (bottom cane)	-
		Petiole (bottom cane leaf)	-
		Stem (bottom cane)	-
	Cane-3	Primocane tip (top cane)	-
		Leaf (top cane)	-
		Petiole (top cane leaf)	-
		Stem (top cane)	-
		Leaf (bottom cane)	-
		Petiole (bottom cane leaf)	-
		Stem (bottom cane)	-
		Crown	-
		Root	+
4	Cane-1	Primocane tip (top cane)	-
		Leaf (top cane)	-
		Petiole (top cane leaf)	-
		Stem (top cane)	-
		Leaf (bottom cane)	-
		Petiole (bottom cane leaf)	-
		Stem (bottom cane)	-
	Cane-2	Primocane tip (top cane)	-
		Leaf (top cane)	-
		Petiole (top cane leaf)	-
		Stem (top cane)	-
		Leaf (bottom cane)	-
		Petiole (bottom cane leaf)	-
		Stem (bottom cane)	-
	Cane-3	Primocane tip (top cane)	-
		Leaf (top cane)	-
		Petiole (top cane leaf)	-
		Stem (top cane)	-
		Leaf (bottom cane)	-
		Petiole (bottom cane leaf)	-

	Stem (bottom cane)	-
Cane-4	Primocane tip (top cane)	-
	Leaf (top cane)	-
	Petiole (top cane leaf)	-
	Stem (top cane)	-
	Leaf (bottom cane)	-
	Petiole (bottom cane leaf)	-
	Stem (bottom cane)	-
	Crown	+
	Root	-