Genetic Diversity and Pathogenicity of Pea Downy Mildew (*Peronospora viciae* f. sp. *pisi*) in New Zealand

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Downy mildew of peas is caused by the biotrophic pathogen *Peronospora viciae* f. sp *pisi* (*Pvp*) which occurs sporadically throughout temperate pea growing regions across the world. Severe infections can completely disrupt commercial production by reducing crop quality and yield; early season systemic infections can ultimately prevent seed from being produced. Control strategies are largely preventative, with the use of tolerant cultivars being the most simple and cost effective, however, the genetic diversity within *Pvp* often correlates with varying tolerances towards cultivars. To date, there are 14 known pathotypes of *Pvp* throughout global pea growing regions but the New Zealand *Pvp* population has not been examined. This is the first study in New Zealand to assess the genetic diversity of *Pvp*. The results has indicated that the causal organism of pea downy mildew is *Peronospora viciae* f. sp. *pisi* with genetically different isolates being present in pea populations in New Zealand. Preliminary bioassays have been developed that has enabled the potential for future rigorous screenings of pea cultivars.

To characterise the genetic diversity of *Pvp* in New Zealand's pea growing regions pods were collected from infected plants from 7 sites across the North and South Islands of New Zealand in the 2018-2019 growing season. An examination of the partial ITS1, complete 5.8S and partial ITS2 region via sequencing and RFLP analysis did not indicate any genetic variation between the representative samples. In contrast, a RAPD analysis of selected representative samples examined a larger portion of the genome and indicated genetic dissimilarities within and between sites. Despite the limited number of analysed samples the results indicated that the surveyed pea growing regions in the North Island have a more variable *Pvp* population that the surveyed South Island sites. Overall, genetic variation within *Pvp* in New Zealand is minimal, however, it is expected that further studies with a more representative sample size would identify greater variation.

A range of bioassays tested various methods of inoculation and growth chamber conditions, whilst using different sources of inoculum to develop a method to screen pea plants against *Pvp* in a

controlled environment. Infection was achieved using fresh sporangia and soil collected from sites with a known history of hosting *Pvp* infected plants. No infection was achieved with dehydrated, infected field pods. Only two plants expressed signs of *Pvp* infection, thus no discernable laboratory conditions were identified to facilitate disease expression. The successful inoculation methods identified in this study could be used in future studies to investigate the most conducive conditions for disease expression in a controlled environment.

Keywords: *Peronospora viciae pisi*, *Pisum sativum*, peas, genetic, diversity, bioassay, infection, RFLP, RAPD, asymptomatic, disease expression

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May the peas be with you x

Contents

List of Tables

List of Figures

Chapter 1

Introduction

1.1 The Pea Plant

Pisum sativum, commonly known as the pea plant, is considered one of the world's most important and versatile non-cereal crops (Biddle 2017). Belonging to the Fabaceae family, the genus *Pisum* has two recognised species, *P. sativum*, which consists of all the cultivated forms, and *P. fulvum* (Kraft & Pfleger 2001). The first known domestication of pea plants dates from 7000-6000 B.C, in the Fertile Crescent of Southwest Asia (Cousin 1997; Kraft & Pfleger 2001). Cultivation subsequently expanded into the Indian subcontinent, eastern China, and upon discovery of the New World, into the Western Hemisphere (Kraft & Pfleger 2001). Modern cultivation saw peas introduced to North America, Europe, and to other temperate regions such as New Zealand (Biddle 2017). The various locations where peas have been domesticated and developed in agricultural systems are reflective of the great diversity within *Pisum* (Kraft & Pfleger 2001; Biddle 2017). This diversity has allowed for advances in production for both feed for domestic animals and food for human consumption (Kraft & Pfleger 2001). Peas for human consumption can be fundamentally categorised into dehydrated, fresh, or processed (canned or frozen) (Biddle 2017). Fresh and processed are the most commercialised and are commonly produced from vining pea varieties (as opposed to bush varieties), which often require additional support during development due to their naturally tenuous biology (Kraft & Pfleger 2001; Biddle 2017).

Although pea plants all undergo similar developmental stages, it is important to acknowledge that variation between cultivars and climatic or cultural conditions may influence observed timing and growth patterns (Knott 1987). Vining peas are typically sown in spring with seeds germinating soon after imbibition. Pea seeds contain meagre amounts of endosperm thus the cotyledons have embryonic respiring tissue. During the early stages of germination, the cotyledons remain below ground to supply the embryo with energy until the plumule emerges to form the primary leaves (Biddle 2017). Selective breeding for specific attributes to improve product quality and yield means that a description for the plant's morphology is difficult. Modern varieties have been bred to improve agricultural adaptability, with breeders selecting for differences in stem, stipule, leaf and tendril characteristics. Usually, the plant will produce one main stem, however variation in varieties means that some may produce one or more axillary stems. Naturally the plant is poor at supporting itself, thus the ability for breeders to produce a plant with a robust stem to aid in standing more erect is desirable. During development, leaves are produced alternately from an axillary meristem,

commonly referred to as a node. Leaves develop at each node from approximately the sixth node up. Each compound leaf consists of a petiole, which has 4-6 pairs of pinnate leaflets and ends in 3 tendrils. Leaves have a cuticle of wax on the upper surface and although colour is determined by the cultivar, leaves can range from a yellowy-green to a deep blue-green. Certain developments have led to many varieties having reduced stipules and tendrils instead of leaflets. These types are referred to in the industry as 'afila' types, or leafless peas. Further breeding has produced varieties with normal stipules whilst retaining afila attributes. These 'semi-leafless' types are predominantly used in vining pea operations (Knott 1987; Biddle 2017). Pea plants have a fine taproot which can penetrate approximately 80 cm into the soil, as well as lateral roots that grow along soil fissures. Roots develop nodules which contain nitrogen-fixing bacteria, providing the plant with a sufficient supply of nitrogen throughout its life. Flower initiation is triggered by temperature and photoperiod; however, the number of nodes and intrinsic earliness of the plant will also determine at which node the first flowers are produced. Modern varieties have a predetermined node at which flowering will begin, thus providing the grower with a known time of maturity and aiding harvest. The number of flowering nodes is determined by genotype, but most vining peas have 6-8 nodes which produce pods (Knott 1987). The flowers are reflective of the *Papilionaceae* family; five petals and self-pollinating (Cousin 1997). Insects visiting pea flowers can cause natural hybridizations, although Cousin (1997) reported that natural pea populations tend to be genetically stable. They may vary in colour, although usually only white flowers are produced in vining peas (Knott 1987; Biddle 2017). Modern varieties tend to have at least two pods per node, but some are known to produce multiple pods on each fertile node. The number of seeds per pod is generally 5-6 and they often differ in size and shape. Depending on the variety, seeds may appear as dimpled, round, or wrinkled, and range from 90 mg/seed to 400 mg/seed (Knott 1987; Biddle 2017).

1.2 New Zealand Pea Production

Peas are the main pulse crop grown in New Zealand (Millner & Roskruge 2013). Farmers are known to grow peas as a break crop for the control of *Gaeumannomyces graminis* var. *tritici* (take-all disease) and to increase soil fertility within cereal rotations (White 1987; Millner & Roskruge 2013). Peas have been estimated to increase soil nitrogen levels within a range of 17 and 83 kg/ha, thus they are often utilized in crop rotations (White 1987). A small quantity of peas produced in New Zealand are used in the compound feed industry, however, the pea crop is most valuable as an export crop and is sold for human consumption in a wide variety of forms; dehydrated, canned, or frozen (White 1987; Millner & Roskruge 2013). New Zealand grown peas have a high reputation for quality due to the climate at harvest (White 1987). Size, colour, purity, flavour and chemical

composition are all important factors to maintain and uphold for both green and dried peas (White 1987). Advances in both harvesting and production techniques have allowed for larger scale productions, generating a greater quantity of high-quality peas. For the year ending in June 2017, approximately 10 000 hectares were employed to produce field and seed peas, resulting in approximately 38 000 tonnes (t) of product (Statistics New Zealand 2018). Aitken and Warrington (2017) reported that processed peas had a value of $84.6 million in exports in the year ending June 2017. The investment into the productive area of New Zealand horticultural and post-harvest facilities for peas and beans, both on- and off-farm, was estimated at $1.1 billion for the year ending in June 2017 (Aitken & Warrington 2017). Off-shore, the United States was the greatest producer of vining peas in 2013, producing approximately 260 000 t of seed. In Europe, France and the United Kingdom produced a large amount of seed; a combined 340 000 tonnes (t). Other large producers in 2013 were Belgium (69 000 t), Spain (62 000 t), and Canada (50 000 t) (Biddle 2017).

Canterbury and Hawke's Bay are New Zealand's major producers of pea crops in New Zealand (White 1987). The Canterbury and Hawke's Bay regions have climatic similarities as they are both influenced by nearby mountain ranges. Compared to the rest of New Zealand, these regions experience relatively low annual mean rainfalls (Canterbury – <900mm; Hawke's Bay – 1000mm). Hawke's Bay is characterised by its mild to warm surface air temperatures, high sunshine hours, and a relatively mild wind regime (Fowler et al. 2013). Canterbury's climate is heavily influenced by the Southern Alps. The high country near the main divide experiences prevailing north-west winds which carry abundant precipitation and warm temperatures that can lead to rapid evaporation. Comparatively, the Canterbury plains experience prevailing north- and south-east winds which carries little precipitation and high annual temperatures (Macara n.d). The variation due to orographic patterns means that pea production practises will vary from west to east along the plains. Most pea crops are spring sown; however, some cultivars may be sown later (White 1987). Peas grow best in deep, well-drained soils, which can be found in over 220 000 hectares in Canterbury (White 1987). They are sensitive to poor soil aeration and waterlogging; as little as 12 hours of waterlogging can reduce yields, whilst two days of waterlogging will cause root necrosis and yellow foliage, with a slim chance of recovery (White 1987).

1.3 Common Pathogens of Peas in New Zealand

Pea production is constantly under threat from pests and diseases which can have a huge impact on both yield and quality. In New Zealand, there has been little research to investigate the definite level of infection required to impact the pea crop in such a way that leads to economical yield or quality loss, however, a report commissioned by the Ministry for Primary Industries (MPI) reported that

costs relating to plant disease in horticultural production are estimated to be approximately $35-70 million per year (Beresford & McKay 2012). Well recognised threats to peas include bacterial blights caused by the *Pseudomonas syringae* complex and other pathogens such as *Erysiphe pisi*, *Aphanomyces euteiches*, *Fusarium solani*, *Botrytis cinerea,* Ascochyta disease complex, and downy mildew caused by *Peronospora viciae* f. sp. *pisi* (Harvey 2003; Pea Industry Development Group 2010).

Powdery mildew is a disease caused by the pathogen *Erysiphe pisi*. It is an obligate biotroph, which is most devastating in areas experiencing warm, dry days, and cool nights (Barilli et al. 2014). It overwinters on infected plant debris and then spreads asexual conidia via wind currents to cause white or dull grey lesions on the upper side of the leaves (Fondevilla & Rubiales 2012; Barilli et al. 2014). These lesions may coalesce to cover the leaf surface whilst pod infections cause seed degradation and discolouration (Fondevilla & Rubiales 2012). Current management methods for *E. pisi* include the use of fungicides and planting early in the season, although the use of genetically tolerant cultivars is considered the most effective and most economical (Fondevilla & Rubiales 2012).

Aphanomyces root-rot disease is caused by *Aphanomyces euteiches*; an Oomycete which causes severe levels of disease in peas, as well as many of New Zealand's pasture species (PIDG 2008). Plants may appear to be stunted and yellow, with severe disease incidence causing the plant to wilt and die. The roots and stem bases will develop water-soaked, honey-coloured lesions (PIDG 2008). The pathogen can survive periods of adverse conditions and host absence by developing thick-walled, sexual oospores that can remain viable for up to 15 years within the soil (Hossain et al. 2012). Infection can occur at all soil temperatures that support pea development, however 16˚C is optimal for infection and between 20-28˚C will aid disease development. High rainfall often leads to high levels of disease, as increased soil moisture encourages the formation of asexual sporangia and dispersal of zoospores (Wu et al. 2018). Due to the survival capabilities of the pathogen, rotations out of susceptible hosts for 6 years or longer are recommended to prevent inoculum build-up (Hossain et al. 2012). It has been suggested that residues from both oat and brassica can help to reduce disease incidence (PIDG 2008; Wu et al. 2018).

Fusarium solani and *Fusarium oxysporum* f. sp. *pisi* are destructive, soil-borne pathogens which are difficult to eliminate as they can grow saprophytically in the absence of a compatible host (Bani et al. 2017). *Fusarium* spp. have broad genetic variability and can survive in the soil as thick-walled chlamydospores for many years, making disease management challenging (Cousin 1997; Bani et al. 2017). Chlamydospores infect the plant via the roots to cause disease symptoms that vary between *Fusarium* spp. and races; although symptoms include overall stunted growth, colour change which affects the entire plant, and discoloured or shrunken roots (Scott 1987; Biddle 2017). Problems with

Fusarium rots are more frequent during hot, dry seasons, when increased soil temperatures favour infection and disease development (Scott 1987). Management can be very difficult or near impossible to control with fungicides (Pea Industry Development Group 2010), thus the most economically viable solution is to use highly tolerant cultivars (Bani et al. 2017).

Outbreaks of *Botrytis cinerea* occur throughout the world on a multitude of crops (Biddle 2017). Semi-circular lesions first appear to be water-soaked but turn grey due to the production of spores when dry (Hagedorn 1991). After surviving adverse conditions on infected debris, the pathogen produces asexual conidia which are readily dispersed by the wind to cause secondary infections on nearby plants (Hagedorn 1991). The pathogen is most severe in moist, humid conditions (Biddle 2017), at temperatures between 16-21˚C, and often in fields with potassium deficiencies (Hagedorn 1991). Fungicides are often used to control *B. cinerea*, however it is recognised that there is pathogen resistance to some active ingredients (Biddle 2017).

The Ascochyta disease complex is comprised of three pathogens; *Mycosphaerella pinodes, Didymella pinodella,* and *Ascochyta pisi. Ascochyta pisi* is the most common of the three, and its symptoms are the most distinctive (Biddle 2017). Lesions appear to be slightly sunken and brown, with a prominent margin. On the leaves and pods spots are circular, whilst on the stems they are elongated (Ashby et al. 1987). Primary infection generally originates from seed-borne infection or from overwintering chlamydospores in crop debris or soil. Periods of leaf wetness over 3-5 days and moderate temperatures (15-23˚C) initiate wind-borne spore production, causing secondary infections. Effective control can be achieved by using clean seeds, crop rotation, growing in drier seasons and fungicide application to the seeds (Falloon & Armstrong 2002; Biddle 2017).

1.4 Peronospora viciae f. sp. *pisi*

Peronospora viciae f. sp. *pisi* is an obligate parasite which is often referred to as a fungus in the wider literature, despite being taxonomically an Oomycete. It is taxonomically described as follows; kingdom Straminipila, division Oomycota, class Peronosporomycetes, order Peronosporales, and family Peronosporaceae. This pathogen only infects plants within the *Pisum* genus, thus its *forma specialis* as *Peronospora viciae* f. sp. *pisi* (Chang et al. 2012). Throughout this research, *P. viciae* f. sp. *pisi* will be referred to as *Pvp*. Downy mildews are devastating pathogens, causing major crop losses throughout a multitude of cropping systems and control of these can be linked to approximately 17% of the global fungicide market (Clark & Spencer-Phillips 2011).

Stegmark (1994) described three types of *Pvp* infection; systemic, local and pod. Each infection type can produce differing symptoms of disease that can be identified within a single crop cycle. Systemic

infections produce the most severe symptoms, usually occurring before flowering and are associated with total stunting and distortion (Kraft & Pfleger 2001). A dull, mealy growth may develop on the plant causing it to wither and die (Fig. 1.1A). Early season systemic infections can limit growth so severely that plants may die 2-3 weeks after infection. Systemic infections during plant maturity may restrict the plant to its current growing point (Ashby et al. 1987) and produce copious amounts of inoculum (Kraft& Pfleger 2001). Local infections occur most commonly from wind-dispersed sporangia. Lesions on the upper surface of the leaves will form as yellow-brown blotches, approximately 0.2-2 µm in diameter, with white/grey, cotton-like mycelial growth on the underside (Ashby et al. 1987; Stegmark 1994). Further development produces chlorotic patches on the leaves and stems (Ashby et al. 1987). Though younger leaves are more susceptible, symptoms begin on the lower leaves, seemingly as the moist conditions produced by the canopy provide a high humidity required for disease development. Disease then progresses up the plant (Ashby et al. 1987). Pod infection is stimulated by high humidity and arises when a viable spore is deposited on the pod, rather than disease progression through the plant (Stegmark 1994). Yellow lesions will form on the outside of the pod (Ashby et al. 1987). Felt-like mycelial growth develops within the pod preventing the seed from maturing (Fig. 1.1B). Occasionally the mycelial growth protrudes from the pod. Systemically infected plants peas will rarely seed, and the pods appear flattened and yellow (Ashby et al. 1987). Early publications suggested that infected seeds do not seem to be a source of primary inoculum in the field, however subsequent to mycelia and oospores being discovered in a small quantity of tested seed, *Pvp* is now recognised as a seed-borne pathogen (Hagedorn 1974 (cited in Stegmark 1990); Stegmark 1990; Falloon et al. 2000; Chang et al. 2012; Feng et al. 2012).

Figure 1.1: A. Sporulating *Peronospora viciae* f. sp. *pisi* on an 'Utrillo' plant. **B.** Mycelial growth of *Peronospora viciae* f. sp. *pisi* on the inside of a pea pod.

Falloon and Sutherland (1996) described the asexual and sexual reproductive structures of *Pvp*. Sporangiophores are produced from the stomata in clusters and have elongated hyphae. Hyphae branch monoplodially to produce multiple terminal sporangia that are wider at the base than the apex. During development, the sporangia have smooth surfaces and become finely echinulate in maturity; 15-30 µm in diameter (Clark & Spencer-Phillips 2011). Gametangia are produced extensively on the inner surface of the pea pods from rounded hyphae adhering to the host epidermis. Each oogonium is surrounded by several antheridia. Fertilised oogonia develop into oospores enclosed in an oogonial membrane. Oospores are round, light brown to deep yellowish pink, and can range from 25-42 µm in diameter (Kraft & Pfleger 2001). They have thick epispores with large, raised reticulations and are heavily fissured between reticulations (Falloon & Sutherland 1996).

1.5 *Peronospora viciae* f. sp. *pisi* Life Cycle

Being a polycyclic pathogen, *Pvp* may undergo multiple infection cycles throughout a single growing season (Liu et al. 2013) (Fig. 1.2). It can survive unfavourable environmental conditions and host absence by laying dormant on plant debris or as oospores within the soil, remaining viable for as long as 10-15 years (Stegmark 1994; Kraft & Pfleger 2001; Liu et al. 2013). As a homothallic pathogen, oospores are formed within the internal pod walls, leaves, stems, and seed coats of *Pvp* after oogonia and antheridia are formed on the same mycelium, and fuse together (Gaag & Frinking 1996; Clark & Spencer-Phillips 2011). Gaag and Frinking (1996) examined the effect temperature had on oospore production and found that oospore formation was most noteable between 15-20˚C, with densities decreasing steadily as the temperature dropped. No oospores were observed at 5˚C. Root exudates and favourable environmental conditions initiate sporadic oospore germination, triggering the onset of primary systemic and local infections (Kraft & Pfleger 2001; Clark & Spencer-Phillips 2011). Oospores germinate to form a germ tube that directly infects the plant through epidermal cells. Secondary infections are caused by asexual sporangia and are initiated by cool to moderate temperatures and high humidities, which are frequent in New Zealand's spring and autumn seasons. Sporangia production is induced by 12-hour periods of at least 90% humidity, at temperatures between 1-20˚C (Stegmark 1994; Kraft & Pfleger 2001). Much like the oospores, sporangia germinate to produce a germ tube. Germ tubes develop appressoria on the host surface where a specailised hypha will penetrate the tissue either via a stoma or directly through the epidermal cell walls. Once *Pvp* has entered the plant, it grows intercellularly and sporadically produces penetration pegs to pierce the cell wall and invaginate the plant plasma membrane. Inside the plant cell the peg begins to enlarge, forming a specialised intracellular structure called a haustorium. The formation of the haustorium allows for molecular and nutrient exchange between *P. sativum* and *Pvp* that may not otherwise occur with intercellular hyphae. The pathogen continues to grow through the plant to the

underside of the leaf where it emerges through stomata to form another generation of sporangiophores. Changes in humidity cause the sporangiophores to twist and release their sporangia, where they are dispersed by wind currents or water splashes to nearby plants. Sporangia viability is temperature and humidity dependent; surviving for 24-hours at 20˚C, 7 days at 15˚C, and a month at 4˚C (Kraft & Pfleger 2001). These time periods are relative as sporangia are generally short-lived and may only survive a number of hours or days in periods of unfavourable humidities (Clark & Spencer-Phillips 2011). Disease due to *Pvp* can arise sporadically and requires a minimum of two asexual generations to succesfully establish itself and spread throughout the crop, thus the importance of implementing an appropriate mangement strategy (Kraft & Pfleger 2001).

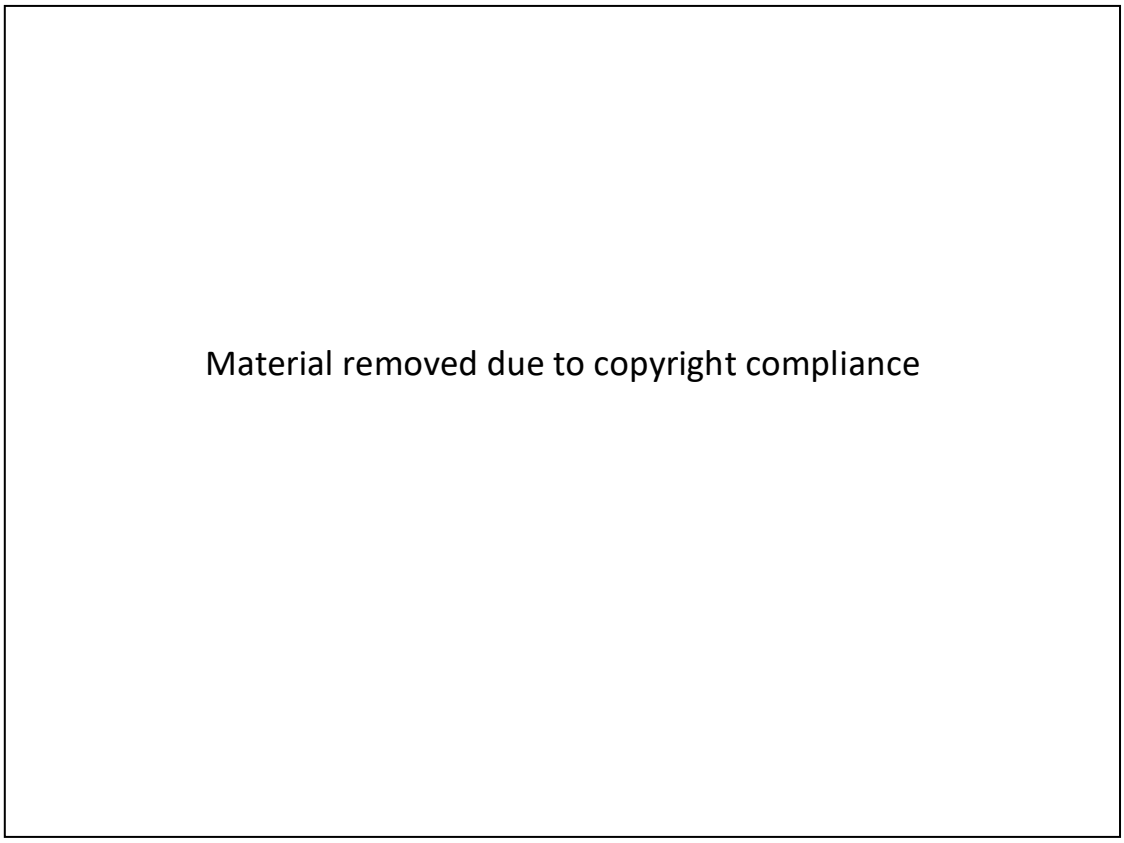

Figure 1.2: Basic disease cycle of *Peronospora viciae* f. sp. *pisi* on pea (CropPro n.d).

1.6 *Peronospora viciae* f. sp *pisi* Control Strategies

Controlling *Pvp* in vining pea cropping systems is difficult, thus an increased reliance on methods of disease prevention rather than attempting to eliminate the problem when it appears. Synthetic pesticides are disfavoured for control, although when chemicals are required the use of prediction forecasting and ongoing field monitoring is encouraged. Integrated approaches that may include aspects of husbandry, choice of field, healthy seeds, cropping rotations, and frequent monitoring are becoming more common amongst growers to best understand the relationship between crop and

disease and to provide the best outcome available (Biddle 2017). A wide literature search did not yield specific guidelines towards economic or action thresholds for pea crops in New Zealand, however a report commissioned by Plant and Food Research, aimed at vegetable growers, consultants, and crop scouts, outlines the basic aspects of integrated pest management programmes, and guides the reader towards sustainable practices that would be best suited for their crop and production system (Walker et al. 2019).

1.6.1 Cultural Control

Cultural strategies involve careful planning and preparation of the area to be cultivated to reduce the likelihood of primary invasions. As *Pvp* can lay dormant within the soil for many years, growers who wish to minimise the risk of serious disease levels often incorporate crop rotations into their management strategies. Research in the 1970's suggested a minimum of a five-year rotation out of peas to reduce the build-up of soil-borne pests. Five-year rotations are now considered standard practise for disease management, with Europe and the UK often choosing to extend the rotation to 6 or 7 years (PIDG 2008; Biddle 2017). In New Zealand, peas would be included in a livestock or a mixed livestock/cropping farm prior to winter feed species such as brassicas or grasses. Comparatively, in an arable farming rotation, peas would typically follow a cereal crop such as wheat or barley (White 1987).

1.6.2 Chemical Control

Fungicides are invaluable for the ongoing success of crop production but are often not so effective on Oomycetes as they are not 'true' fungi. Oomycetes tend to only be susceptible to a narrow range of fungicides such as phenylamide-type chemicals, whilst the use of soil/foliar fungicides are known to be problematic due to their toxicity to non-target organisms and disruption of soil ecosystems (Stewart et al. 2001). However, there are some chemical products which may be used as either seed or foliar treatments to achieve effective control of *Pvp*. The New Zealand NovaChem Agrichemical Manual currently lists ten products registered for controlling *Pvp*. Of those ten, five are inorganic coppers and two are Quinone outside Inhibitors (QoI) fungicides. Treatments may be applied preventatively if disease forecasting predicts major disease incidence or to gain rapid control in the case of a random outbreak. Coppers are contact action only fungicides, requiring complete spray coverage of the plant to ensure total protection. In comparison, QoI-fungicides are systemic, travelling throughout the plants vascular system to provide protection from the inside out, thus complete spray coverage is less important. Although the best results are observed when applied preventatively, some fungicides can reduce the likelihood of sporulation, thus impacting the pathogens ability to spread further. The Foundation for Arable Research (FAR) (2002) assessed the effect of foliar fungicides on the incidence and severity of *Pvp* infection. Although none of the

assessed products are currently registered for controlling *Pvp*, some of the products belong to the same chemical groups. Plants treated with two applications of Ridomil Gold (active ingredient: metalaxyl-m) and plants treated with one application of Foschek 5.0 (active ingredient: phosphorous acid) had significantly lower disease incidences (3% and 2%) compared to other treatments which ranged between 40-60%. The study also noted no significant difference in yield when comparing any of the treated plants with the non-treated plants, thus it was concluded that all foliar treatments reduced the gross margin of the crop. This conclusion is also reflected in a three-year study conducted by Chang et al. (2012) from 2009-2011, which assessed fungicides from different chemical groups, including a QoI-fungicide (azoxystrobin) and a metalaxyl based fungicide. Azoxystrobin significantly reduced plant mortality in 2009 compared to metalaxyl, however the following year there was no significant difference. Overall foliar applied fungicides reduced the severity of *Pvp*, however an increased yield was not consistently observed, thus making their use uneconomical.

The other three products registered for use in New Zealand are phenylamide seed treatments; each containing a metalaxyl based active ingredient. Seed treatments protect the plant from disease in the emergence stages of crop growth. Interrupting the disease cycle at the seed and seedling stages of the pea crop cycle is highly important when managing *Pvp*, hence the desirability for the added protection that seed treatments provide (Falloon et al. 2000). In 2002, FAR evaluated three seed treatment products for their efficacy of reducing *Pvp* disease incidence (Apron® XL, Aliette Super®, and Wakil® XL); two of which are currently registered (Apron® XL and Wakil® XL). All seed treatments significantly increased seed establishment, however at 9 weeks old only the Wakil-treated plants were disease free. Despite Wakil-treated plants remaining infection free and Aliette-treated plants experiencing 23% infection rate, both groups produced 5.8 t/ha. Apron-treated plants scored low compared to the others with a 59% infection rate and yield of 3.9 t/ha. Metalaxyl-based seed treatments have been registered for use on pea seeds in New Zealand since 1984, and from 1995 there have been reports of *Pvp* infected plants grown from metalaxyl-treated seed (Falloon et al. 2000). Falloon et al. (2000) investigated the efficacy of metalaxyl-based seed treatments against field populations of *Pvp*, possible alternative treatments, and compared seed treatments for effects on crop establishment, disease incidence, and yield. In summary, metalaxyl-based seed treatments were ineffective against 56% of the *Pvp* collections thus confirming resistance within New Zealand populations of *Pvp* to this active ingredient. To manage metalaxyl resistant populations Falloon et al. (2000) suggested using cymoxanil, fosetyl-Al, or mancozeb as a replacement, or as additives to phenylamide mixtures.

As observed with phenylamides, fungicides can rapidly be rendered ineffective as pathogens can develop a tolerance to active ingredients when a product is overused or used incorrectly. To reduce the chances of resistance developing, it is important to practise using fungicides with different modes

of action and applying appropriate dose rates. Fungicides with different modes of action may be applied together in a mixture or alternated between within a chemical programme. This ensures that the pathogen can be controlled by one fungicide even if it has a reduced sensitivity to the other. When implementing this practise growers should affirm that both fungicides are active against *Pvp* and their duration of activity is similar. To delay the onset of reduced sensitivity and to ensure that *Pvp* populations are effectively controlled growers must apply an appropriate dose rate of each fungicide. Reflective of any individual control method, fungicides are most valuable when used in conjunction with other management strategies rather than individually relied on (Stewart et al. 2001).

1.6.3 Varietal Selection

Globally accepted as the most effective management strategy in preventing *Pvp* infection, the selection of highly tolerant varieties is often heavily relied on. Breeding for resistance to *Pvp* began in the 1980's and has been rapidly advancing ever since, thus varieties with varying tolerances to *Pvp* are everchanging (Jermyn 1987). A reflection of the growing research can be reviewed in the annual Vining Pea Variety Guide, published by the Processors and Growers Research Organisation (PGRO), which summarises vining pea variety characteristics and their level of tolerance towards *Pvp* and other pathogens. The most current Vining Pea Variety Guide (2019) lists forty different varieties that are rated as having good field resistence towards *Pvp*. The use of tolerant cultivars accompanied with tillage and crop rotation is recommended by the PIDG (2008) to reduce *Pvp* incidence in a given field.

1.7 Pathotype Evolution

Breeding for a high tolerance towards pests and pathogens is important to consider when observing selection pressures on host specific pathogens, such as *Pvp*. Simple genetically based variation can arise from many sources; whether it be mutations, genetic drift or migration, recombination through sexual reproduction or somatic hybridisation (Burdon & Silk 1997). The effect of host related selection pressure is unpredictable and can lead to considerable variation within a population (Burdon & Silk 1997). Understanding the pathogen in an ecological context is crucial when attempting to understand its life history. A combined effect of *Pvp* biology and how it interacts with an ever-changing host due to selective breeding programmes may lead to a patchwork of individual pathogen virulence in a given area, or on a given host. These patchworks can become strengthened and genetically diverse from each other through an intricate and dynamic relationship often observed between a pathogen, its host, and environment (Groβkinsky et al. 2015).

1.8 *Peronospora viciae* f. sp. *pisi* Pathotypes

Many studies have investigated the virulence of *Pvp* and how informally identified pathovars interact with pea cultivars with varying tolerances towards the pathogen. The first recorded variation in the virulence of *Pvp* isolates was in the Netherlands by Hubbeling (1975; cited in Stegmark 1990) where five pathotypes were distinguished on seven differential pea cultivars ('Cobri', 'Cicero', 'Heralda', 'Koroza', 'Perfect Freezer', 'Recette, and 'Starnain'). Cultivars 'Starnain', 'Starcovert', and 'Gastro' were considered resistant to all pathotypes in the 1975 study (Stegmark 1990). Building on the results of Hubbeling (1975), Ester and Gerlagh (1979; cited in Stegmark 1990; Liu et al. 2013) identified three more pathotypes using 10 differential cultivars ('Clause-50', 'Katinka', 'Puget', in addition to the seven which Hubbeling (1975) used). 'Race 8' was a pathotype identified as being virulent on all pea genotypes tested (Ester & Gerlagh 1979; cited in Stegmark 1990). In Germany, Heyendorff and Hoffman (1978; cited in Stegmark 1990; Liu et al. 2013) reported four pathotypes of *Pvp* using cultivars 'Cobri' and 'Puget' as differentials. Later, Taylor et al. (1989) reported 11 pathotypes in the United Kingdom, on cultivars 'Clause-50', 'Cobri', 'Katinka', and 'Starnain'. Whilst researching and identifying parental material and lines within the Australian pea breeding germplasm which are resistant to a particular pathotype of downy mildew, Davidson et al. (2011) indirectly refers to two pathotypes present in South Australia. Davidson et al. (2011) describes how the most commonly grown pea cultivars in Australia are 'Kaspa' and 'Parafield'. Kaspa was released from the Australian Field Pea Improvement Program in 2001, known to have a high tolerance to a *Pvp* pathovar capable of infecting the Parafield cultivar (designated the 'Parafield' strain). In 2007, a new strain of *Pvp* was found to be virulent on Kaspa field peas in South Australia and was designated the 'Kaspa' strain. Together, these studies provide conclusive evidence of physiological specialization of *Pvp* and that individual pathotypes have varying capabilities of infecting specific pea genotypes.

An extensive literature search suggested that there is no evidence that prior to 2013 there had been any molecular research undertaken to examine the genetic diversity of field populations of *Pvp*. Liu et al. (2013) examined *Pvp* infected pea shoots in commercial pea production systems in Central Alberta, Canada, with the aims to identify the predominant pathotypes in Alberta and to assess the genetic diversity in regional pathogen populations. As a result, three pathotypes were found (UKP1, UKP2, UKP11), although these had previously been identified as being present in the United Kingdom by Taylor et al. (1989). Comparatively, UKP10 was identified as the predominant pathotype in the United Kingdom and UKP1 was the predominant pathotype in Alberta. There has been no published research to suggest that any work has been undertaken in New Zealand to examine the genetic diversity of field populations of *Pvp.*

1.9 Molecular Techniques

Molecular techniques are now common practise for answering questions based on inter- or intra-species genetic variation or evolution. Techniques vary in their applicability to taxonomic levels and the type of data produced, therefore, for the most suitable technique to be utilized, it is important to clarify what data is required to answer the research question. To determine the species identity, house-keeping genes or other regions of DNA that are often used in taxonomic studies may be amplified via polymerase chain reaction (PCR). This is a basic method with a wide range of applications and is often employed by researchers as a diagnostic tool. During PCR a section of genomic DNA is amplified, resulting in millions of copies of that section, allowing for the detection and analysis of one or more genes in a small DNA sample (Sinclair 2002). A PCR reaction requires a combination of heat tolerant enzymes (DNA polymerase), nucleotides, and oligonucleotides (primers) to be heated and cooled repeatedly. This allows the primers to anneal to and copy the targeted section of DNA. The amplified DNA is then separated during gel electrophoresis and visualised under UV, subsequent to an ethidium bromide stain (Ward et al. 2016). The targeted region of DNA is determined with the selection of specific primers. The Internal Transcribed Spacer (ITS) region is the most universally used region of DNA for identification to the species level due to its high sequence variation and easy amplification using universal primers (Sapkota & Nicolaisen 2015). Since the first database of ITS sequences was published, ITS became the most frequented DNA barcode for Oomycete species (Robideau et al. 2011). There are some cases where formally described species of Oomycota are extremely similar; sharing 99.9% ITS sequence (Robideau et al. 2011), thus the use of another region when examining the species diversity is desired. Cytochrome *c* oxidase subunit I (*cox*1) is a mitochondrially encoded locus universally accepted as a valuable DNA barcode for species identification across a variety of eukaryotes, primarily for *Pythium* and *Phytophthora* (Robideau et al. 2011; Choi et al. 2015). The *cox*2 locus has been broadly used in studies of downy mildew phylogenies as it more frequently allows for successful amplification when compares to *cox*1 (Choi et al. 2015).

Species specific primers have been developed for many *Peronospora* species, including *P. viciae*, which enables detection of the targeted pathogen within an impure sample (Kitz 2008; Liu et al. 2013; Herath Mudiyanselage et al. 2019). However, primers for *P. viciae* have only been used for pure sporangial samples (Liu et al. 2013). Sometimes the amount of DNA for the targeted pathogen can be minimal in comparison to the host tissue or secondary pathogens, such as in asymptomatic infections, making it difficult to obtain a pure DNA sample. Field populations of *Pvp* have been known to remain asymptomatic until conducive conditions for sporulation arise (Clark & Spencer Phillips 2011), therefore a reliable method to detect *Pvp in planta* is required. Herath Mudiyanselage et al. (2019) successfully used nested PCR to detect asymptomatic *Peronospora sparsa in planta*. Two-step

nested PCR uses two sets of primers; the first set amplifies a slightly larger region of the target DNA whilst the second set of primers amplifies a smaller section of the target DNA within the product of the first reaction, which increases the sensitivity and specificity of a PCR reaction, suitable for detecting *Pvp* within its host tissue.

Methods which could be applicable to examine the possible genetic variability between isolates of *Pvp* include but are not limited to; Random Amplification of Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Multi Locus Sequencing Types (MLST) and Simple Sequence Repeats (SSR). Random Amplification of Polymorphic DNA (RAPD) is a PCR-based technique that amplifies the DNA at random points along the genome, generating band patterns which vary between genetically dissimilar individuals. In comparison to a standard PCR reaction, RAPD uses arbitrary 10-bp primers that can detect polymorphisms in the absence of specific nucleotide sequences. The polymorphisms act as genetic markers which may be used to create genetic maps or evolutionary trees (Arif et al. 2010). Major polymorphisms may indicate a measure of genetic distinctness which can identify between unrelated species, whereas minor polymorphisms could signify genetic distinctness within species or populations (Liu et al. 2013). When Liu et al. (2013) assessed the genetic diversity of *Pvp* in Alberta, they performed a RAPD analysis as well as an analysis of the partial ITS1, 5.8S rRNA and partial ITS2 region. It was concluded that the sequence analysis was perhaps not as effective as the RAPD analysis, likely because a larger representation of the genome is examined in RAPD whilst only the targeted portion of DNA is amplified for analysis during sequence analysis. Restriction Fragment Length Polymorphism identifies variation in the patterns of fragments produced when the DNA is digested by the same restriction enzyme. This method may use one or more restriction enzymes to digest, or cut, the genomic DNA from the sample (Henry 2012). The digested DNA is separated by length during gel electrophoresis. Analysis of the fragments involves the use of specific probes to blot the gel which may result in the detection of genetic dissimilarities between samples or individuals (Henry 2012).

1.10 Aims and Objectives

The difficulties working with an obligate parasite and the fact that the pea genome is complex means that there is very little information on the physiology and genetics of tolerance to *Pvp* or the molecular basis for pathogenicity (Liu et al. 2013). Molecular approaches to investigate the genetic diversity amongst field populations of *Pvp* has not been reported in New Zealand. Despite estimates from pea breeders suggesting the existence of multiple *Pvp* pathotypes in New Zealand (Alexis Plouy, Crites Seeds, Personal communication, 2019), there has been no research conducted to support these suggestions.

Thus, the objectives of the research are as follows:

1. Characterise the genetic diversity of *Peronospora viciae* f. sp. *pisi* in New Zealand pea growing regions.
2. Develop a method to detect asymptomatic and symptomatic *Peronospora viciae* f. sp. *pisi* infection.
3. Develop a method to screen pea cultivars against *Peronospora viciae* f. sp. *pisi* which can be applicable to global pea growing regions.

1.11 Terminology

For the basis of this report, the following definitions will be associated with these terms; isolate, population, pathotype, tolerance, virulence. This is to give a precise meaning to these terms as in the wider literature these terms are used interchangeably.

Isolate – a sample of sporangia or mycelia that has been collected from a single pea plant or leaf and used for DNA extraction purposes (Liu et al. 2013).

Population – a collection of *Pvp* sporangia or mycelia resulting from a mixture of 2 or more isolates from a given field (Liu et al. 2013).

Pathotype – an isolate of *Pvp* which displays a substantially different pathogenicity profile on different pea cultivars compared to other isolates and are genetically dissimilar.

Resistance – the ability of a pea genotype to suppress the growth of *Pvp* (Stegmark 1990).

Tolerance – the ability of the genotype to express minimal disease from *Pvp* and still obtain a good yield.

Virulence – the capability of a *Pvp* isolate to cause disease on a certain pea genotype (Stegmark 1990).

Chapter 2

Peronospora viciae f. sp. *pisi* Isolate Characterisation

2.1 Introduction

Peronospora viciae f. sp. *pisi* (*Pvp*) is commonly found throughout temperate pea growing regions across the world (Falloon et al. 2000). Disease severity in New Zealand varies between growing seasons and is influenced by the local climate, how the crop came to be infected and individual cropping practises. Heavy early season infection can cause plant stunting and death in a matter of weeks (Stegmark 1994). Most commonly, *Pvp* will cause chlorotic patches and grey cotton-like growths on the underside of the foliage, however, it can also prevent the plant from producing seed (Ashby et al. 1987). Current control methods are preventative and cultural-based. Choice of cultivar, length of crop rotation and removal of infected debris are common practise and the most effective (Biddle 2017). Due to the genetic variability of the pathogen, cultivars with a higher tolerance to *Pvp* may not provide the desired level of protection when grown in an area when a certain pathovar is present (Stegmark 1990).

Globally, 14 pathovars of *Pvp* have been identified to date, however most were informally identified whilst screening pea lines for their susceptibility towards the pathogen and they were not molecularly inspected. However, in 2013, the first molecular analysis of *Pvp* isolates was performed to investigate the genetic diversity of *Pvp* in Alberta, Canada. Liu et al. (2013) used random amplification of polymorphic DNA (RAPD) analysis and an examination of the internal transcribed spacer (ITS) region; ITS1, 5.8S rRNA and partial ITS2 region. Random amplification of polymorphic DNA uses small, random primers to amplify arbitrary fragments of the DNA template. A primer anneals at places along the genome where a near complimentary sequence is located, then PCR amplifies the fragment between the two sites. Banding patterns are formed when fragments of various sizes are amplified and are specific to the DNA template in question (Tamang 2014). Liu et al. (2013) indicated that RAPD was the preferred method for analysing genetic diversity due to its ability to examine a larger representation of the genome rather than that targeted ITS region that may or may not present any dissimilarities. However, the reproducibility of RAPD DNA fingerprints is questionable as differences in DNA and PCR preparations can influence primer annealing, thus an alternative method should be used to confirm any differences between samples (Tamang 2014).

To identify and differentiate between species, Robideau et al. (2011) suggested using the ITS region and cytochrome oxidase 1 (*cox*1) as standard DNA barcode markers for oomycetes, yet, a study by Choi et al. (2015) compared the PCR performance of *cox*1 and *cox*2 and found that *cox*2 had a higher

species identification success. The *cox*2 also had a higher efficiency in amplifying DNA from dried herbarium specimens than *cox*1. PCR products from these loci can be sequenced to examine the base pairs and identify any nucleotide substitutions or be subject to restriction fragment length polymorphism (RFLP). The RFLP method digests a previously amplified DNA template with a restriction enzyme and the resulting DNA fragments are compared following gel electrophoresis (Gomi 2014).

No study to investigate whether different pathovars of *Pvp* are present in New Zealand has been completed. Understanding the genetic diversity of New Zealand's *Pvp* populations will have benefits across the industry. Seed producers and plant breeders will have the ability to screen pea lines against different pathovars of *Pvp* and confidently provide growers with seed which is highly tolerant to genetically dissimilar pathovars of the pathogen. Downy mildew infections are not always symptomatic (Clark & Spencer Phillips 2011; Herath Mudiyanselage 2015), thus it is important that a method to detect asymptomatic *Pvp* is developed to ensure that cultivars are able to be screened against *Pvp* in the absence of disease expression. Thus, the aims of this chapter are to characterise the genetic diversity of *Pvp* in New Zealand pea growing regions and to develop a method to detect asymptomatic *Pvp* infection within *Pisum sativum* plants.

2.2 Materials and Method

2.2.1 Sample Collection

Seven sites around Hawke's Bay and Canterbury were surveyed and sampled from during the 2018-2019 growing season (Table 2.1). All South Island sites were sampled personally whereas samples from the North Island were collected by another arable researcher. Each field was sampled by walking from East to West in a 'W' formation, with samples collected from three individual plants along each line of the 'W'. Pea plants infected with downy mildew were distinguished by the characteristic grey-furry mats of mycelia on the underside of the leaves. At the time of collection, plants for sampling had to be podding and have approximately 60% of its pods with visible *Pvp* infection. Twelve plants from each field were sampled from; five pods per plant were collected. Pods were placed into a paper bag which was labelled with the date of collection, site, pea cultivar, and plant number. Random plants exhibiting high levels of disease were collected from Site Two to use for the inoculation experiments (Section 3.2.2) and molecular troubleshooting. As suggested by Plouy (Crites Seeds, personal communication, 2019), the samples were dehydrated in a Contherm oven at 20˚C for five days, then stored within individual centrifuge tubes (15 mL) at 4˚C until required.

Table 2.1 Sites sampled for *Peronospora viciae* f. sp. *pisi* infected plants throughout various pea growing regions in the North and South Islands of New Zealand during the 2018-2019 growing season.

Site #	Region	Latitude	Longitude	Size of area sown (ha)	Cultivar
1	Canterbury (SI)	43°45'38.56"S	171°57'47.50"E	0.9	'Tomahawk'
2	Canterbury (SI)	43°45'35.98"S	171°57'52.66"E	0.42	'Utrillo'
3	Canterbury (SI)	43°45'23.29"S	171°58'18.40"E	0.001	'Prelado'
4	Hawke's Bay (NI)	40° 0'59.91"S	176°21'25.96"E	4.6	'Drummond'
5	Hawke's Bay (NI)	40° 1'49.56"S	176°21'2.28"E	5.95	'Drummond'
6	Hawke's Bay (NI)	39°51'47.37"S	176°28'58.40"E	20.8	'PG King'
7	Hawke's Bay (NI)	39°52'36.38"S	176°40'33.40"E	6.8	'Drumpeel Digit'

*SI and NI refers to the South or North Island of New Zealand

2.2.2 Genomic DNA Extraction

The origin and the type of sample intended for DNA extraction defined the method used for isolation. The samples were referred to as the following: Sample Type One (S1), Sample Type Two (S2), Sample Type Three (S3) and Sample Type Four (S4).

S1 samples derived from the 382 dehydrated field pods (Section 2.2.1). Each pod had two samples taken using a sterile scalpel; one was a 3 mm^2 piece of material from a visibly infected section from the pod, which was typically a mix of pod, *Pvp* and other pathogens (as it was difficult to solely isolate *Pvp*) and the other sample was a 3 mm^2 piece of material from a section of the pod that did not appear to have any *Pvp* present upon visual examination with the naked eye. Individual samples were placed into empty, sterile individual 1.7 mL tubes and stored at 4˚C until further processing.

A subset of pods (S2) were selected by identifying a pod from each plant which had the greatest visible mycelial growth. To ensure a pure sample of *Pvp*, mycelia was scraped from the inside of the pod with a sterile scalpel, placed into empty, sterile individual 1.7 mL tubes and stored at 4˚C until further processing. Some plants were not represented in the subset as their pods did not have mycelia that was easily removed from the pod.

S3 samples were pure sporangial samples, from sporangia present on fresh plant material from visibly infected plants in the field or laboratory growth chamber (Section 3.2.4). Approximately 5

mm^2 of sporangia was lifted from the plants using a sterile needle, placed into empty, sterile individual 1.7 mL tubes and stored at 4˚C for further processing.

S4 samples were derived from asymptomatic plants from experiments in Sections 3.2.3 and 3.2.4. They were taken from the plant by slicing a 5 mm^2 piece from the youngest, fully developed leaf with a sterile scalpel. All samples were placed into empty, sterile individual 1.7 mL tubes and stored at 4˚C until further processing.

For DNA extraction, to each 1.7 mL tube, containing either samples of S1, S2, S3 or S4, a 300 µL aliquot of 10% Chelex (BioRad) solution was added. Tubes were vortexed for 2 seconds, three times, then placed into a block heater (Stuart SBH130D) for 10 minutes at 100˚C. Each tube was then taken out of the heat block and vortexed for another 2 seconds, three times, and returned to the heat block for a further 10 minutes at 100˚C. After a total of 20 minutes in the heat block, the tubes were centrifuged for 10 minutes at 13 000 xg. The supernatant (approximately 200 µL) was aliquoted into 0.6 mL tubes and stored at 4˚C prior to PCR.

2.2.3 Detection Threshold

To investigate the minimum DNA concentration required for successful amplification during PCR, the DNA template from a S3 sample was diluted into a DNA template from healthy pea plant material at the ratios listed in Table 2.1. To create a working solution for dilutions of lower concentrations, 5 µL of 7.7 ng/µL *Pvp* DNA was diluted into 45 µL of pea plant DNA, creating a 0.77 ng/µL working solution (Table 2.2). Concentrations of *Pvp* ranged from 7.70 - 0.231 ng/µL, with the lowest detectable amount being 0.231 ng/µL (231 pg/µL). 1C DNA value of *Pvp* can be presumed to be approximately 0.048 pg, based on the 1C value of the closely related *Peronospora conglomerate* (Voglmayer & Greilhuber 1998; Voglmayer 2008). Therefore, the corresponding approximate number of nuclei, deriving from sporangia or mycelial fragments, required in a DNA extract for detection was calculated (Tables 2.2 and 2.3). Reactions were prepared in a 20 µL volume, containing 10 µL DreamTaq Green PCR Master Mix (Thermofisher), 0.25 µM of both forward (DC6) and reverse (ITS4) primers (Appendix A.1) (Kitz 2008), varying quantities of DNA template, and the remaining volume of H_2O. The cycling parameters were outlined by Kitz (2008) and were as follows: an initial denaturation of 95˚C for 2 minutes, 30 cycles of denaturing at 95˚C for 1 minute, annealing at 55˚C for 1 minute, an extension at 72˚C for 1 minute, and a final extension at 72˚C for 10 minutes. PCR products were separated on a 1% agarose gel, stained with ethidium bromide (0.5 µg/mL), at 100 v for 40 min then visualised under UV light using GelDoc.

Table 2.2 Dilution ratios of pure *Peronospora viciae* f. sp. *pisi* DNA (7.70 ng/µL) into healthy *Pisum sativum* DNA (ng/µL unknown) used to investigate the minimum concentration of *Pvp* DNA required for successful amplification in PCR with primers DC6 and ITS4. Approximate quantities of nuclei in each sample is also indicated.

Pvp DNA (ng/µL)	*Pvp* DNA (µL)	*Pi. sativum* DNA (µL)	Number of nuclei
7.70	2.0	0.0	371
6.93	1.8	0.2	334
6.16	1.6	0.4	297
5.39	1.4	0.6	260
4.62	1.2	0.8	223
3.85	1.0	1.0	186
3.08	0.8	1.2	148
2.31	0.6	1.4	111
1.54	0.4	1.6	74

Table 2.3 Dilution ratios of pure *Peronospora viciae* f. sp. *pisi* DNA (0.77 ng/µL) into healthy *Pisum sativum* DNA (ng/µL unknown) used to investigate the minimum concentration of *Pvp* DNA required for successful amplification in PCR with primers DC6 and ITS4. Approximate quantities of nuclei in each sample is also indicated.

Pvp DNA (ng/µL)	*Pvp* DNA (µL)	*Pi. sativum* DNA (µL)	Number of nuclei
0.770	2.0	0.0	37
0.693	1.8	0.2	33
0.616	1.6	0.4	30
0.539	1.4	0.6	26
0.462	1.2	0.8	22
0.385	1.0	1.0	19
0.308	0.8	1.2	15
0.231	0.6	1.4	11

2.2.4 ITS Region PCR

The internal transcribed spacer (ITS) region was amplified from the DNA extract from the different types of samples (Section 2.2.1) by PCR to determine the presence of *Pvp*. DC6, designed to amplify the ITS region of Peronosporales and Pythiales, was used in combination with the universal primer ITS4 (Appendix A.1) to amplify the entire ITS region (ITS1, 5.8S and ITS2) and partial regions of 18S and 28S rDNA (Fig. 2.1) (Kitz 2008). Reactions were prepared in a 20 µL volume, containing 10 µL DreamTaq Green PCR Master Mix (Thermofisher), varying quantities of primer and DNA, and the remaining volume of H_2O (Table 2.4). A negative control with no DNA template was included so any contaminations or primer annealing issues could be identified. An S3 sample from pure sporangia was also included as a positive control to indicate the success of the reaction. Cycling parameters for all reactions were an initial denaturation at 95˚C for 2 minutes, 30 cycles of denaturing at 95˚C for 20 seconds, annealing at 55˚C for 25 seconds, an extension at 72˚C for 72 seconds, and a final extension

at 72˚C for 10 minutes. PCR products were separated on a 1.5% agarose gel precast with ethidium bromide (0.2 µM), for 45 min at 90 v then visualised under UV light using GelDoc.

Figure 2.1: Primer location in relation to the ITS region. Primers used are circled. Location of primers DM3F and DM3R are approximate. Figure modified from Kitz (2008), modifications are indicated in red.

Table 2.4 Primer concentrations and DNA quantities used in PCR reactions with primers DC6 and ITS4 to amplify *Peronospora viciae* f. sp. *pisi* in different sample types.

#	Sample Type	Primer concentration (µM)	DNA quantity (µL)
1	S3 (Field)	0.250	2
2	S4	0.250	2
3	S4	0.250	3
4	S1	0.250	2
5	S1	0.250	3
6	S1	0.250	4
7	S1	0.375	3
8	S1	0.500	4

Primers DM3F and DM3R (Liu et al. 2013), designed to amplify the ITS region of *P. viciae,* were used together to partially amplify the ITS1, complete 5.8S, and partial ITS2 (~725 bp) of DNA extracted from the different sample types (Section 2.2.2) (Fig. 2.1). Reactions were prepared in a 20 µL volume, containing 10 µL DreamTaq Green PCR Master Mix, varying quantities of primer and DNA, and the remaining volume of H_2O (Table 2.5). Negative and positive controls were also included in each reaction as previously described. Cycling parameters for all reactions involving these primers were an initial denaturation of 94˚C for 5 minutes, 40 cycles of denaturing at 94˚C for 30 seconds, annealing at 55˚C for 1 minute, an extension at 72˚C for 1 minute, and a final extension at 72˚C for 10 minutes. PCR products were separated and visualised as previously described.

S2 PCR products, from Reaction #17, were sequenced at the Lincoln University sequencing facility with primers DM3F and DM3R (Appendix A.1) (Liu et al. 2013) to confirm the presence of *Pvp* and to identify any genetic dissimilarities within the ITS region. Sequences were evaluated to ensure a true

reading was produced and trimmed to the same size using BioEdit, then analysed with MEGA X software (version 10.0.5). Using a neighbour-joining method with 1000 bootstrap replications and a Poisson substitution model, a dendrogram was produced to compare the samples against one another and other known sequences (GenBank accession numbers: AY353910; AY225471). A known partial sequence of *Pvp* (GenBank accession number: DQ078696) was also compared to the samples in the current study, but was not included in the dendrogram analysis due to the low number of base pairs. A contig was not produced for analysis due to the poor amplification of the product with the DM3F primer. Thus, the reverse primer sequence was used in analyses. In a few instances the forward sequence was of higher quality than the reverse and used instead of a reverse complement (Appendix A.3). Due to considerable differences in the quality of sequences produced, those with shorter, unreadable sequences were not included in analyses.

Table 2.5 Primer concentrations and DNA quantities used in PCR reactions with primers DM3F and DM3R to amplify *Peronospora viciae* f. sp. *pisi* in different sample types.

#	Sample Type	Primer concentration (µM)	DNA quantity (µL)
9	S3 (Field)	0.25	2.0
10	S4	0.50	1.5
11	S4	0.25	1.0
12	S4	0.50	1.0
13	S3 (Lab)	0.50	1.0
14	S1	0.25	0.5
15	S1	0.25	1.0
16	S1	0.25	2.0
17	S2	0.50	1.5

As *Pvp* infection levels were potentially low in S1 and S4 samples, primer pairs DC6/ITS4 and DM3F/DM3R were used together in a two-step, nested PCR. The first step used primers DC6 and ITS4 to amplify the ITS region, and an aliquot of extracted *Pvp* DNA, whilst the second step amplified a region within this using the PCR product and primers DM3F and DM3R. Different primer and DNA quantities were tested in 20 µL reactions containing 10 µL DreamTaq Green PCR Master Mix, and the remaining volume of H_2O (Table 2.6). Negative and positive controls were included as previously described. The effect of diluting the DNA template on the clarity of the amplified PCR product was tested. Templates were diluted in nuclease-free H_2O, at a ratio of 1:9, either before the first step, between the first and second steps, or not at all. Cycling parameters, gel electrophoresis and gel visualisation for each primer pair and PCR product were as previously described.

Table 2.6 Primer concentrations, dilutions and DNA quantities used in two-step nested PCR reactions to amplify *Peronospora viciae* f. sp. *pisi* in different sample types. Primers DC6 and ITS4 were used in the first step and primers DM3F and DM3R were used in the second step.

#	Sample Type	First Step		Dilution	Second Step	
		Primer concentration (µM)	DNA quantity (µL)		Primer concentration (µM)	DNA quantity (µL)
18	S3 (Field)	0.075	0.5	Between	0.250	0.5
19	S1	0.250	3.0	None	0.250	3.0
20	S1	0.250	3.0	None	0.250	1.5
21	S1	0.250	1.5	None	0.250	3.0
22	S1	0.250	1.5	None	0.250	1.5
23	S1	0.125	1.0	None	0.125	1.0
24	S1	0.125	0.5	None	0.125	0.5
25	S1	0.125	0.5	Before	0.125	0.5
26	S1	0.250	1.0	Before	0.250	1.0
27	S1	0.125	0.5	Between	0.125	0.5
28	S1	0.250	1.0	Between	0.250	1.0
29	S1	0.125	1.5	None	0.125	1.5
30	S1	0.125	1.5	Between	0.125	1.5
31	S1	0.500	1.5	None	0.500	1.5
32	S1	0.500	1.5	Between	0.500	1.5
33	S1	0.075	0.5	Between	0.125	0.5
34	S4	0.075	0.5	Between	0.125	0.5
35	S4	0.125	0.5	Between	0.125	0.5
36	S4	0.125	1.0	Between	0.125	1.0
37	S4	0.125	1.5	Between	0.125	1.5
38	S4	0.125	2.0	Between	0.125	2.0
39	S4	0.100	0.5	Between	0.125	0.5
40	S4	0.075	0.5	Between	0.125	0.5
41	S4	0.050	0.5	Between	0.125	0.5

Duplicate PCR was trialled with primer pairs DC6/ITS4 and DM3F/DM3R, where the same primer pair was used for both reactions (Tables 2.7 and 2.8). Negative and positive controls were also included to indicate the efficacy of each reaction. The reactions were prepared, the cycling parameters used dependent on the primers are utilised and then visualised on an agarose gel as previously described.

Table 2.7 Primer concentrations, dilutions and DNA quantities used in duplicate PCR reactions with primers DC6 and ITS4 to amplify *Peronospora viciae* f. sp. *pisi* in S1 samples.

#	Sample Type	First Step		Dilution	Second Step	
		Primer concentration (µM)	DNA quantity (µL)		Primer concentration (µM)	DNA quantity (µL)
42	S1	0.25	2	None	0.25	2
43	S1	0.25	2	Between	0.25	2

Table 2.8 Primer concentrations, dilutions and DNA quantities used in duplicate PCR reactions with primers DM3F and DM3R to amplify *Peronospora viciae* f. sp. *pisi* in different sample types.

#	Sample Type	First Step		Dilution	Second Step	
		Primer concentration (µM)	DNA quantity (µL)		Primer concentration (µM)	DNA quantity (µL)
44	S4	0.25	1	None	0.25	1
45	S4	0.25	1	Between	0.25	1
46	S4	0.125	1	None	0.125	1
47	S4	0.125	1	Between	0.125	1
48	S1	0.25	1	None	0.25	1
49	S1	0.25	1	Between	0.25	1
50	S1	0.125	1	None	0.125	1
51	S1	0.125	1	Between	0.125	1

2.2.5 Inspecting Primers for Specificity

Primers DM3F and DM3R were inspected for their specificity and efficacy at amplifying the targeted portion of DNA with Primer-BLAST. The primers were BLASTed against a known *Peronospora viciae* sequence (GenBank accession number: EF174953) and randomly selected sequences from the subset of dehydrated field pod samples.

2.2.6 Restriction Fragment Length Polymorphism (RFLP)

To select enzymes to which could successfully digest *Pvp* within a PCR product, Anza starter pack (Thermofisher), containing enzymes NotI (GC^GGCCGC), BamHI (G^GATCC), EcoRI (G^AATTC), XbaI (T^CTAGA) and HindIII (A^AGCTT), was screened using the PCR product of four sporangial samples (S3) from Reaction #1, Section 2.2.4. The reactions were set up on ice, in a 10 µL volume containing 1 µL of 10x buffer, 1U of enzyme, 5 µL of PCR product and the remaining volume H_2O. Reactions were then placed in a 37˚C water bath for 3 h. Products were visualised as described in Section 2.2.4. Enzymes which successfully digested the product were used to screen the subset of dehydrated pods (S2) with PCR products from Reaction #17, Section 2.2.4. Reactions were set up and visualised using the methods previously described.

2.2.7 *Cox* Region PCR

The *Cox*2 loci (Fig. 2.2) was amplified from the S1 samples with known downy mildew infection by PCR using primers *Cox*2-F and *Cox*2-RC4 (Choi et al. 2015) (Appendix A.1). Reactions were set up in 20 µL volumes, with 10 µL DreamTaq Green PCR Master Mix, varying DNA and primer quantities, and

the remaining volume being made up with H_20 (Table 2.9). Negative and positive controls were included to indicate the efficacy of each reaction. Cycling parameters are transferable between primer pairs, as indicated by Choi et al. (2015), and were as follows: an initial denaturation of 95˚C for 4 minutes, 36 cycles of denaturing at 95˚C for 40 seconds, annealing at 50˚C for 40 seconds, an extension at 72˚C for 1 minute, and a final extension at 72˚C for 5 minutes. PCR products were visualised on an agarose gel as previously described.

Table 2.9 Primer concentrations and DNA quantities used in PCR reactions with primers *Cox*2-F and *Cox*2-RC4 to amplify the *Cox*2 loci of *Peronospora viciae* f. sp. *pisi* in different sample types.

#	Sample Type	Primer concentration (µM)	DNA quantity (µL)
52	S1	0.25	2
53	S1	0.50	3
54	S1	0.50	4
55	S1	0.50	5
56	S1	0.50	6
57	S1	1.25	2
58	S1	1.25	3
59	S1	1.25	5
60	S1	2.50	2
61	S1	2.50	3
62	S2	0.50	4

The entire *cox* region was also amplified, as well as *cox*1 and *cox*2 undergoing a two-step, nested PCR. Primers *Cox*2-F and Oom*Cox*1-levlo (Choi et al. 2015) (Appendix A.1) were selected to amplify the targeted *cox* region (Fig. 2.2) and were prepared in a reaction consisting of 10 µL DreamTaq Green PCR Master Mix, 0.125 µM of each primer, 1 µL DNA, and the remaining volume being made up with H_20. The reaction was cycled and visualised as previously described. The product of this reaction was also used as the first step in both of the following two-step, nested PCR reactions. Oom*Cox*1-levup and Oom*Cox*1-levlo are designed to amplify the *Cox*1 loci and were selected as the second set of primers in the two-step, nested PCR. To amplify the *Cox*2 loci, another reaction was set up using primers *Cox*2-F and *Cox*2-RC4. Both reactions were set up in a 20 µL volume, consisting of 10 µL DreamTaq Green PCR Master Mix, 0.125 µM of each primer, 1 µL PCR product from step one, with the remaining volume being made up with H_2O. Cycling parameters were as previously described. PCR products were visualised on agarose gel as previously described.

Material removed due to copyright compliance

Figure 2.2: Primer location in relation to the *Cox*1 and *Cox*2 loci. Primers *Cox*2-F, *Cox*2-RC4 and Oom*Cox*I-Levlo were used in PCR reactions to amplify the targeted regions (modified from Choi et al. 2015).

2.2.8 Random Amplification of Polymorphic DNA (RAPD) Analysis

The twenty-four samples from the subset of dehydrated field pods (S2) which produced the most distinct bands subsequent to PCR and gel visualisation were selected for RAPD analysis to detect dominant alleles which could indicate genetic dissimilarities between samples. Six primers (DMp4, DMp6, DMp50, DMp51, DMp67 and DMp73) (Appendix A.2) which Liu et al. (2013) identified as most suitable for producing clear and repeatable bands for *Pvp* samples, were screened against each of the 24 S2 samples to evaluate their efficacy. The two primers which produced the clearest and greatest number of polymorphisms were selected to be used in another two reactions using the DNA from the same 24 S2 samples. For each of the two primers, reactions were run with 48 samples; each of the 24 S2 samples were duplicated. All reactions were prepared in 20 µL reactions containing 10 µL DreamTaq Green PCR Master Mix, 0.725 µM, 1.5 µL DNA and the remaining volume of H_2O. Cycling parameters consisted of an initial denaturation at 94˚C for 2 minutes, 45 cycles of denaturing at 94˚C for 30 seconds then annealing at 35˚C for 1 minute followed by an extension at 72˚C for 3 minutes, and a final extension at 72˚C for 10 minutes. Products were visualised on agarose gel as previously described.

In total, 24 S2 samples underwent RAPD PCR however only 21 were analysed as some samples did not amplify consistently and therefore did not produce many polymorphic bands. For inclusion in the analysis, bands had to present in both duplicates. Banding patterns were analysed manually to produce a presence or absence matrix (Appendix A.4) then compared to one another using MEGA X software (version 10.0.5) to produce a dendrogram. An UPGMA method was used in combination with a Poisson substitution method to create the dendrogram.

2.3 Results

2.3.1 Detection Threshold of *Peronospora viciae* f. sp. *pisi*

DNA extracted from pea plant material was spiked with DNA extracted from *Pvp* at known concentrations. In the presence of plant material DNA, *Pvp* was detected at concentrations as low as 0.231 ng/µL (equivalent to 11 nuclei) using primers DC6 and ITS4. Consistent and reproduceable banding was not achieved at concentrations less than 2.31 ng/µL, which equates to approximately 111 sporangia or mycelial fragments containing a nucleus (Fig. 2.3).

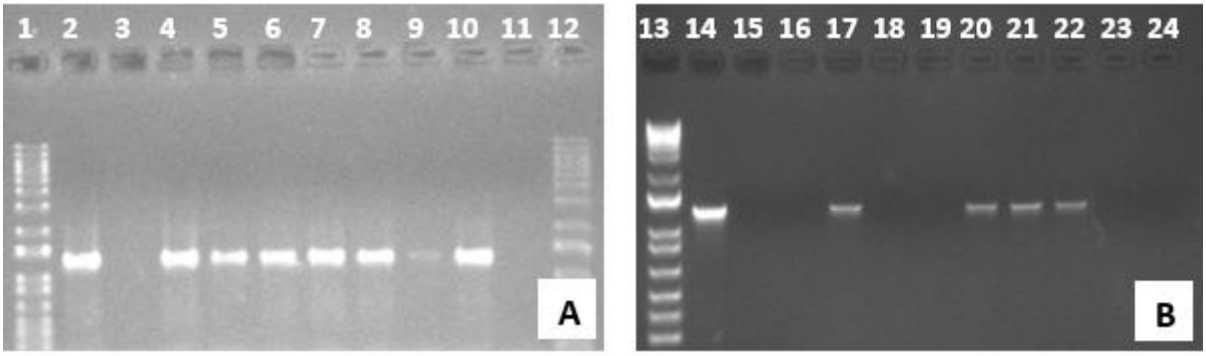

Figure 2.3: PCR products from different concentrations of *Peronospora viciae* f. sp. *pisi* DNA in the presence of plant DNA amplified using DC6 and ITS4 primers. Bands representing a product of approximately 1500 bp indicate positive amplification of *P. viciae* f. sp. *pisi*. **A**. 1% agarose gel. Lanes 1 and 12: 1 kb ladder. Lane 2: Positive control (7.7 ng/µL). Lane 11: Negative control (no DNA template). Lanes 3-10: Decreasing DNA concentrations (ng/µL) left to right: 6.93, 6.16, 5.39, 4.62, 3.85, 3.08, 2.31, and 1.54 ng/µL, respectively. **B**. 1.5% agarose gel. Lane 13: 1 kb+ ladder. Lane 14: Positive control (7.7 ng/µL). Lane 24: Negative control (no DNA template). Lanes 15-23: Decreasing DNA concentrations (ng/µL) left to right: 0.77, 0.693, 0.616, 0.539, 0.462, 0.385, 0.308, 0.231, and 0.154 ng/µL, respectively.

2.3.2 ITS Region PCR

Amplification success was variable depending on the sample type, whether it was S1, S2, S3 or S4. Positive bands occurred in 8 out of 9 S3 (pure sporangia) samples with primers DC6 and ITS4 (Fig. 2.4), 7 out of 9 S3 samples with primers DM3F and DM3R (Fig. 2.5) and 39 out of 52 S2 samples (mycelium from pods) with primers DM3F and DM3R (Fig. 2.6). Sequencing of the PCR product using the corresponding primers confirmed the presence of *Pvp*. For S1 samples (dehydrated field pods), primer pair DC6/ITS4 produced a faint band from a single sample (Fig. 2.7, lane 12) however no other samples gave a positive reaction (Fig.2.7). Primer pair DM3F/DM3R did not detect *Pvp* in S1 samples (Fig. 2.8).

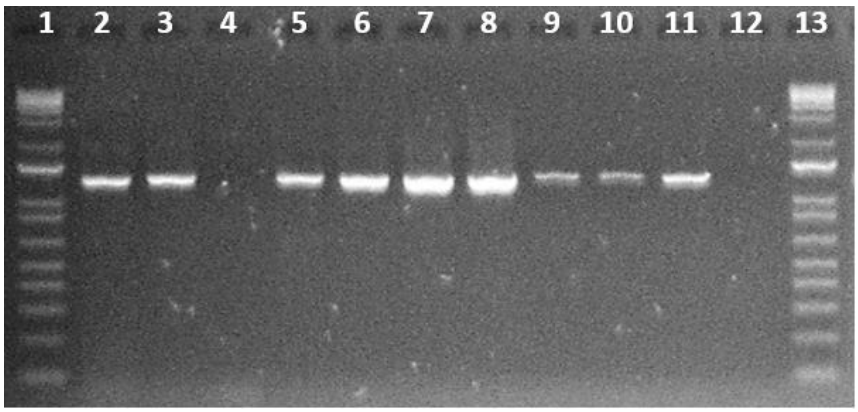

Figure 2.4: Reaction #1. PCR products of approximately 1500 bp of the partial internal transcribed spacer region amplified with primers DC6 and ITS4 from sporangial samples collected in the field (S3 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 13: 1 kb+ ladder. Lane 2: Positive control. Lane 12: Negative control. Lanes 3-11: S3 samples.

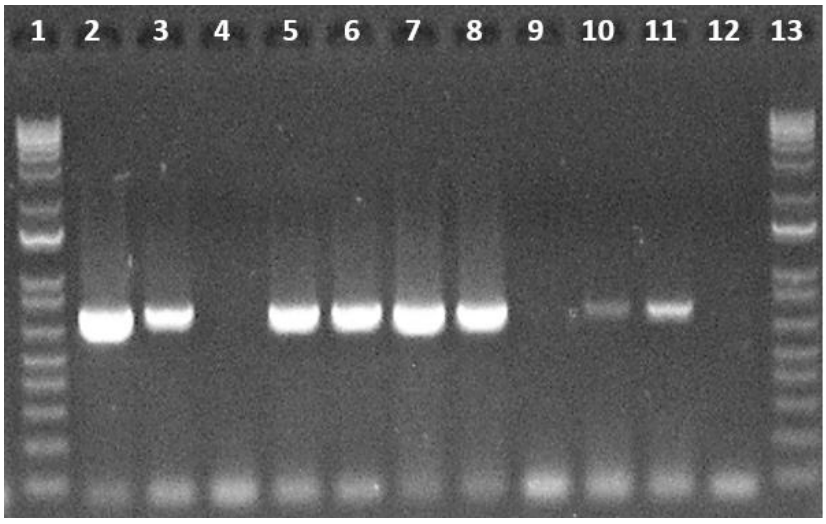

Figure 2.5: Reaction #9. PCR products of approximately 725 bp of the partial internal transcribed spacer region amplified with primers DM3F and DM3R from sporangial samples collected in the field (S3 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 13: 1 kb+ ladder. Lane 2: Positive control. Lane 12: Negative control. Lanes 3-11: S3 samples.

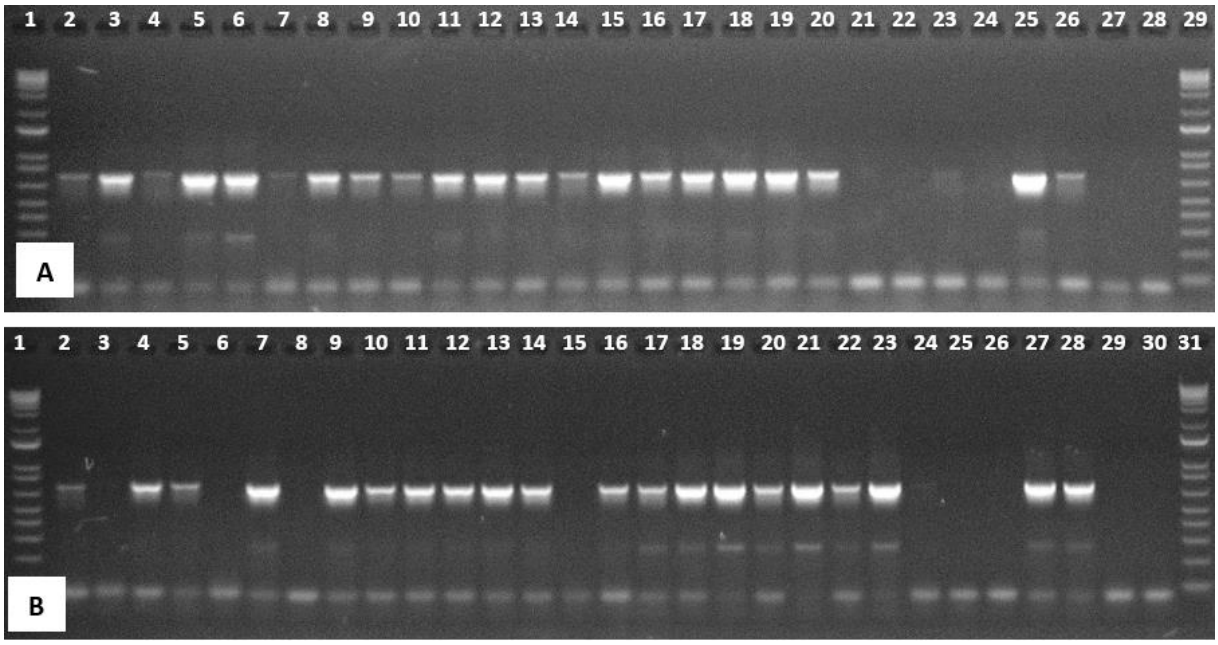

Figure 2.6: Reaction #17. PCR products of approximately 725 bp of the partial internal transcribed spacer region amplified with primers DM3F and DM3R from mycelial samples from field pods (S2 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. A. S2 samples from the North Island (Sites 4, 5, 6 and 7). Lanes 1 and 29: 1kb+ ladder. Lane 2: Positive control. Lane 28: Negative control. Lanes 3-11: S2 samples from Site 4. Lanes 12-16: S2 samples from Site 5. Lanes 17-20: S2 samples from Site 6. Lanes 21-27: S2 samples from Site 7. B. S2 samples from the South Island (Sites 1, 2, and 3). Lanes 1 and 31: 1 kb+ ladder. Lane 2: Positive control. Lane 30: Negative control. Lanes 3-14: S2 samples from Site 1. Lanes 15-22: S2 samples from Site 2. Lanes 23-29: S2 samples from Site 3.

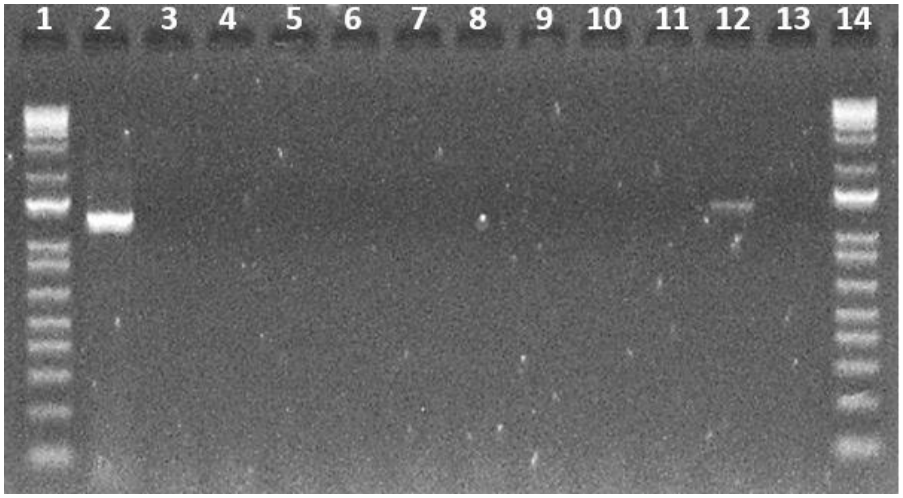

Figure 2.7: Reaction #4. PCR products of approximately 1500 bp of the partial internal transcribed spacer region was amplified using primers DC6 and ITS4 from dehydrated field pod samples (S1 samples. Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: Positive control. Lane 13: Negative control. Lanes 3 -12: Samples of dehydrated field pod samples from South Island Site 1, Plant 1.

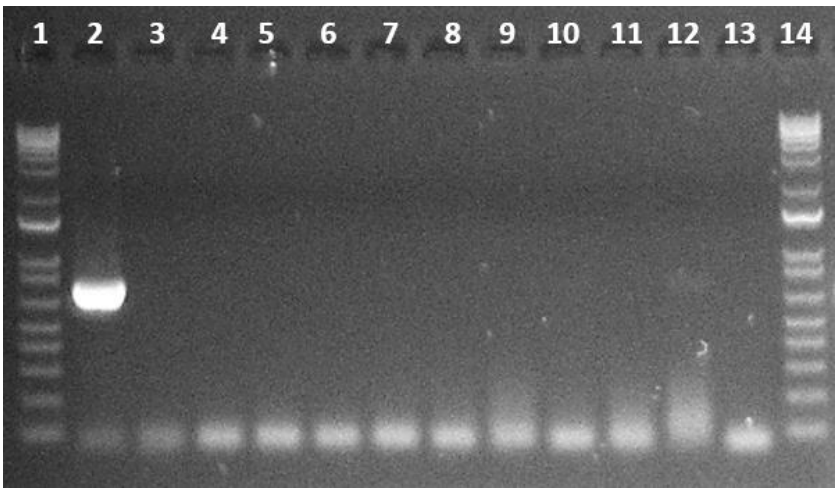

Figure 2.8: Reaction #16. PCR products of approximately 725 bp of the partial internal transcribed spacer region amplified using primers DM3F and DM3R from dehydrated field pod samples (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: Positive control. Lane 13: Negative control. Lanes 3 -12: Samples from dehydrated field pods from South Island Site 1, Plant 1.

Nested PCR led to excessive smearing and non-specific binding. Variations in primer concentrations, DNA quantities and dilutions of the DNA template failed to eliminate non-specific binding for S1 samples, thus nested PCR was excluded as a method to amplify *Pvp* from dehydrated field pods from Sample Type One (Appendix A.7). Comparatively, nested PCR reactions successfully detected *Pvp* in asymptomatic plants grown in the laboratory (S4). Sequencing confirmed the presence of *Pvp*. A full description of the detection of *Pvp* in asymptomatic plants are presented in Sections 3.2.4 and 3.2.5.

Primer pairs DC6/ITS4 and DM3F/DM3R were examined for their efficacy at detecting *Pvp* in S1 samples during duplicate PCR. Neither primer pair detected *Pvp* in any of the tested samples, with the gels using products amplified with primers DC6 and ITS4 appearing highly smeared. A faint, yet highly smeared band was produced for the positive control. Comparatively, a band was not produced for the positive control in any duplicate PCR reactions with primers DM3F and DM3R, yet a bright band was produced for one S1 sample. No smearing occurred although some primer dimers were present (Appendices A.8 and A.9).

2.3.3 ITS Primer BLAST

A Primer-BLAST of primers DM3F and DM3R against a known *P. viciae* sequence (GenBank accession number: EF174953) found the primers amplify a 752 bp product specific to *P. viciae*. Minimal mismatches occurred between unintentional targets of other *Peronospora* and *Phytophthora* species.

2.3.4 Sequencing the Partial ITS Region of *Peronospora viciae* f. sp. *pisi*

Of the 39 S2 field pods which successfully amplified using primers DM3F and DM3R (Section 2.3.2), 28 samples were successfully sequenced. Analysis of the 508 nucleotides of the partial ITS1, entire 5.8S rDNA and partial ITS2 of these samples showed no genetic differences between the S2 field pods samples (Fig. 2.9). A BLAST search was conducted on all sequences and compared to a known sequence of a *P. viciae* isolate deriving from *Pisum sativum* in GenBank (Accession number: AY225471); of which each sequence shared 99% identity, confirming the identification of the samples as *Peronospora viciae* f. sp. *pisi*. Conversely, no similarities were identified when compared to another known partial sequence of a different *Pvp* isolate (GenBank accession number: DQ078696). The branches of the dendrogram indicate no genetic dissimilarities between the samples upon analysis of the sequences of the partial ITS1, entire 5.8S, and partial ITS2 region.

Figure 2.9: Dendrogram presenting a subset of sequenced samples of the partial ITS1, complete 5.8S rDNA gene and partial ITS2 of mycelial samples from dehydrated field pods (S2 samples), a known sequence of a closely related *Peronospora* species (*Peronospora viciae.* GenBank accession number: AY225471) and a known sequence of a genetically dissimilar *Aphanomyces* species (*Aphanomyces euteiches* f. sp. *phaseoli.* GenBank accession number: AY353910). The site number and Island of origin are noted.

2.3.5 RFLP of the Partial ITS Region of *Peronospora viciae* f. sp. *pisi*

A preliminary restriction fragment length polymorphism reaction was performed on the PCR product of four sporangial samples to identify which enzymes would successfully digest the PCR product. One out of the five enzymes (EcoRI) digested the product. EcoRI was then used to screen 52 representative S2 samples for genetic differences, however, no observable differences were detected (Fig. 2.10). The products were digested into the following band sizes; 675 bp and 50 bp, adding up to the original product size of approximately 725 bp.

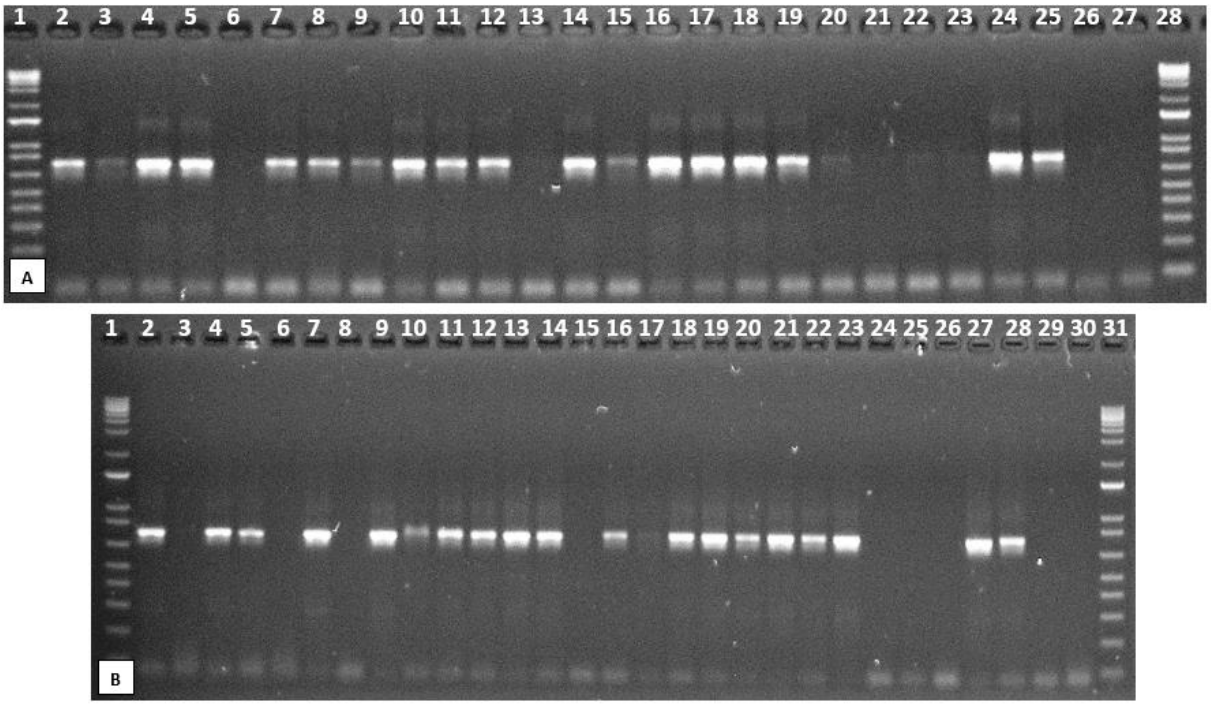

Figure 2.10: Restriction Fragment Length Polymorphism (RFLP) digestion with EcoRI enzyme of 52 S2 samples from seven pea growing regions in New Zealand. **A.** S2 samples from the North Island (Sites 4, 5, 6 and 7). Lanes 1 and 28: 1 kb+ ladder. Lane 27: Negative control. Lanes 2-10: S2 samples from Site 4. Lanes 11-15: S2 samples from Site 5. Lanes 16-19: S2 samples from Site 6. Lanes 20-26: S2 samples from Site 7. **B.** S2 samples from the South Island (Sites 1, 2 and 3). Lanes 1 and 31: 1 kb+ ladder. Lane 2: Positive control. Lane 30: Negative control. Lanes 3-14: S2 samples from Site 1. Lanes 15-22: S2 samples from Site 2. Lanes 23-29: S2 samples from Site 3.

2.3.6 *Cox* Region PCR

Positive bands of approximately 650 bp were detected in 45 out of the 52 S2 samples with primers *Cox*2-F and *Cox*2-RC4 (Fig. 2.11). The same primers failed to amplify any band in the tested S1 samples (Fig. 2.12). Faint bands in were produced for some reactions using primers *Cox*2-F and *Cox*2-RC4 but these were inconsistent.

The entire *Cox* region was targeted with primers *Cox*2-F and Oom*Cox*1-levlo, however, no bands were observed in any of the tested samples, including the positive control. In subsequent nested reactions of the *Cox*1 and *Cox*2 loci using primer pairs Oom*Cox*1-levup/Oom*Cox*1-levlo and *Cox*2-F/*Cox*2-RC4, the positive control successfully amplified however no bands were detected in any of the tested S1 samples.

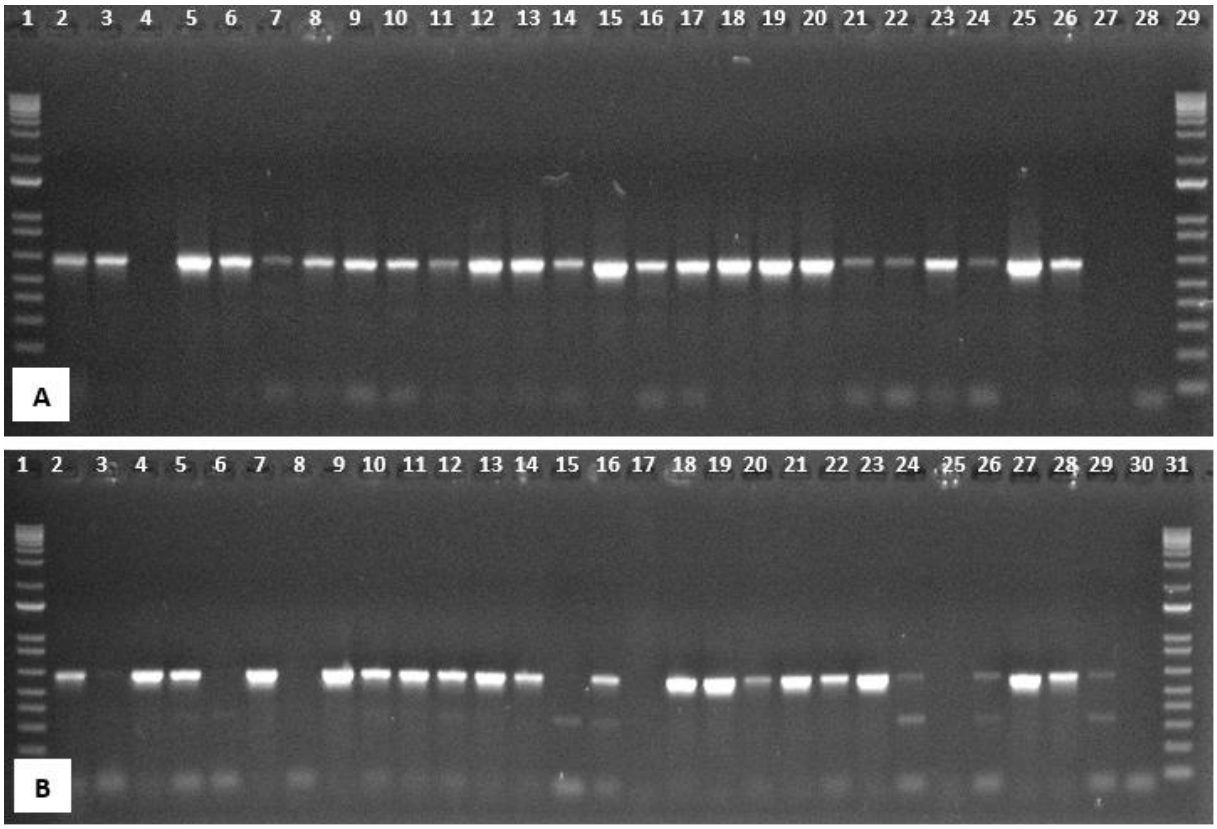

Figure 2.11: Reaction #62. PCR products produced of approximately 650 bp of the *Cox*2 loci using primers *Cox*2-F and *Cox*2-RC4 from mycelial samples from dehydrated field pods (S2 samples) collected from seven sites across pea growing regions in New Zealand. Bands indicate the presence of Oomycetes DNA. **A**. S2 samples from the North Island (Sites 4, 5, 6 and 7). Lanes 1 and 29: 1 kb+ ladder. Lane 2: Positive control. Lane 28: Negative control. Lanes 3-11: S2 samples from Site 4. Lanes 12-16: S2 samples from Site 5. Lanes 17-20: S2 samples from Site 6. Lanes 21-27: S2 samples from Site 7. **B**. S2 samples from the South Island (Sites 1, 2, and 3). Lanes 1 and 31: 1 kb+ ladder. Lane 2: Positive control. Lane 30: Negative control. Lanes 3-14: S2 samples from Site 1. Lanes 15-22: S2 samples from Site 2. Lanes 23-29: S2 samples from Site 3.

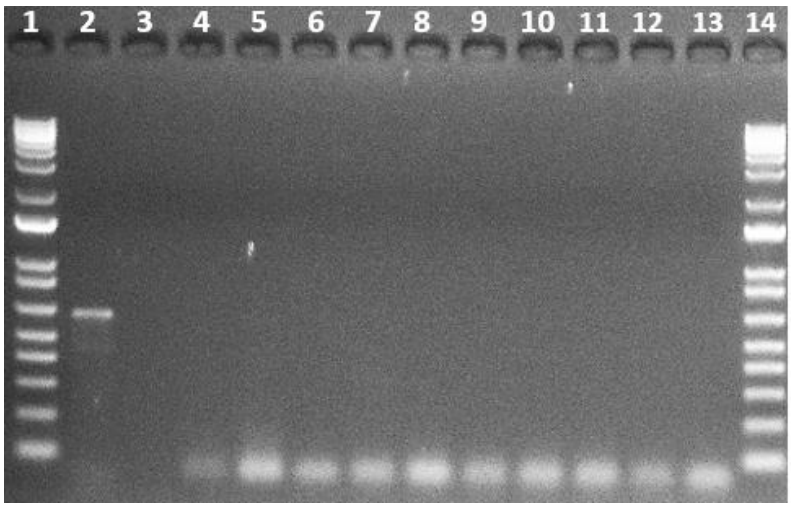

Figure 2.12: Reaction #55. PCR product of approximately 650 bp of the *Cox*2 loci using primers *Cox*2-F and *Cox*2-RC4 from dehydrated field pod samples (S1 samples), collected from Site 1 in the South Island. Bands indicate the presence of Oomycetes DNA. Lanes 1 and 14: 1 kb+ ladder. Lane 2: Positive control. Lane 13: Negative control. Lanes 3 -12: S2 samples from South Island Site 1, Plant 1.

2.3.7 Random Amplification of Polymorphic DNA (RAPD)

Six primers were screened against 24 S2 samples and each produced banding patterns of varying intensities. Two primers (DMp4 and DMp51) produced the clearest and greatest number of polymorphic bands and were used in further RAPD reactions with 24 representative S2 samples. Two samples from North Island Site 7 did not amplify consistently (Fig. 2.13; Lanes 23, 24, 27 and 28) and were not included in the analysis. Primer DMp51 produced more polymorphic bands than DMp4 (Appendix A.13). Overall, the genetic variation was minimal, as indicated by the number of nucleotide substitutions presented in the dendrogram (Fig. 2.14). Of the 22 samples, 11 genotypes were observed, with 7 represented by more than one individual sample. DMp51 identified two dominant alleles present in all samples, (excluding the two Site 7 samples) at approximately 2000 and 650 bp. The samples from the South Island grouped separately from those from the North Island, with the samples from the North Island sites appearing to more diverse than those from the South Island sites, shown by the number of clades and nucleotide substitutions.

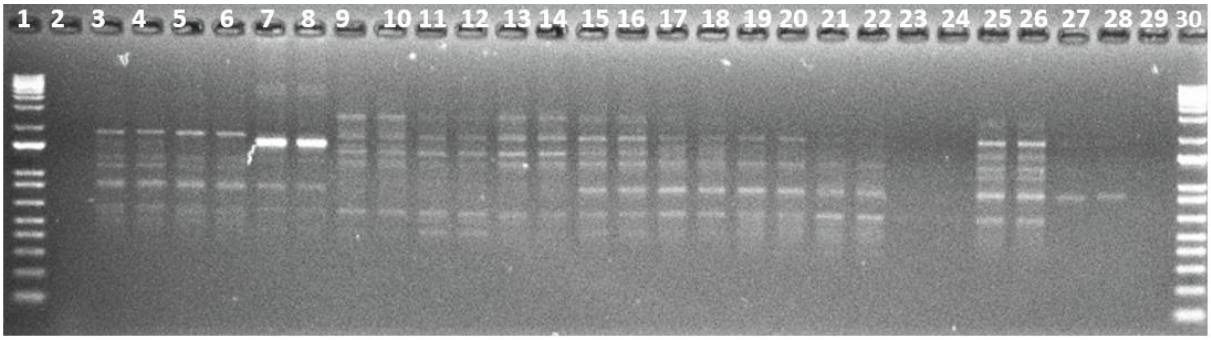

Figure 2.13: Polymorphic bands produced with RAPD primer DMp51 for 13 *Peronospora viciae* f. sp. *pisi* mycelial samples from dehydrated field pods (S2 samples) from the North Island (Sites 4, 5, 6 and 7). Lanes 1 and 30: 1 kb+ ladder. Lanes 2 and 29: Negative controls. Lanes 3 and 4: Site 4, Plant 3 duplicate 1 and 2. Lanes 5 and 6: Site 4, Plant 4 duplicate 1 and 2. Lanes 7 and 8: Site 4, Plant Seven duplicate 1 and 2. Lanes 9 and 10: Site 5, Plant 3 duplicate 1 and 2. Lanes 11 and 12: Site 5, Plant 7 duplicate 1 and 2. Lanes 13 and 14: Site 5, Plant 10 duplicate 1 and 2. Lanes 15 and 16: Site 6, Plant 1 duplicate 1 and 2. Lanes 17 and 18: Site 6, Plant 2 duplicate 1 and 2. Lanes 19 and 20: Site 6, Plant 3 duplicate 1 and 2. Lanes 21 and 22: Site 6, Plant 5 duplicate 1 and 2. Lanes 23 and 24: Site 7, Plant 5 duplicate 1 and 2. Lanes 25 and 26: Site 7, Plant 8 duplicate 1 and 2. Lanes 27 and 28: Site 7, Plant 9 duplicate 1 and 2.

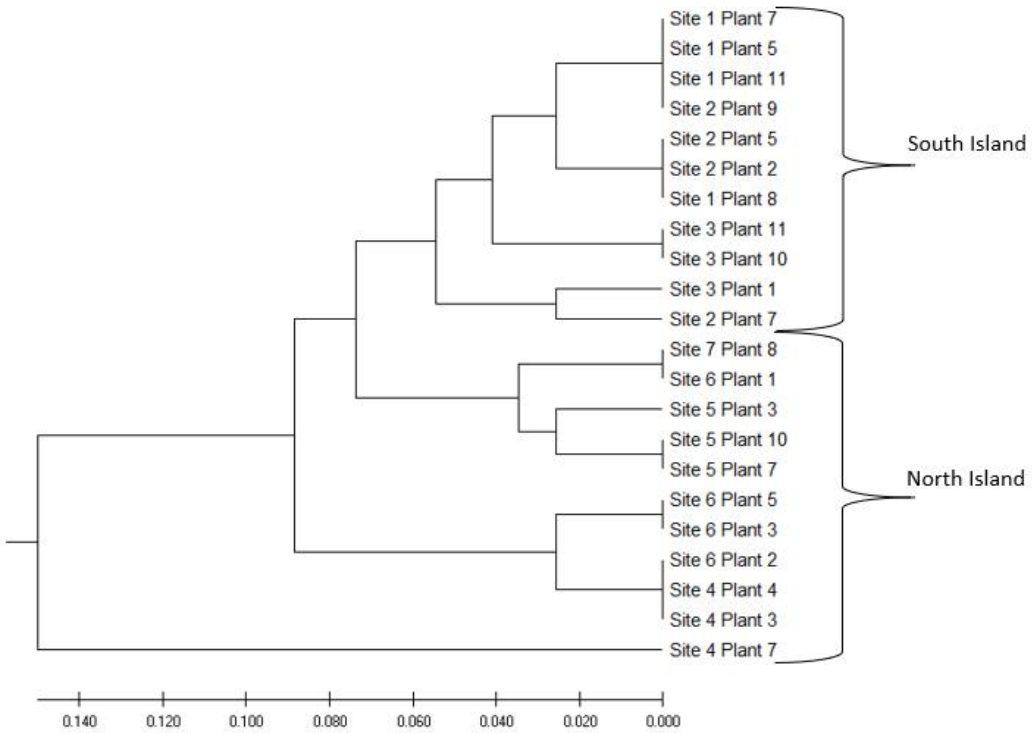

Figure 2.14: Dendrogram of RAPD fingerprints generated using primers Dmp4 and DMp51 from *Peronospora viciae* f. sp. *pisi* mycelial samples from dehydrated field pods (S2 samples) from North and South Island pea growing regions in New Zealand. Scale represents the evolutionary distance using the Poisson model and are in units of nucleotide substitutions.

2.4 Discussion

Peronospora viciae f. sp. *pisi* isolates were collected from seven different pea growing sites across New Zealand during the 2018-2019 season. Representative samples were selected and their partial ITS1, entire 5.8s and partial ITS2 region and entire genome was examined for genetic variation by sequencing, RFLP and RAPD analyses. Sequencing of the partial ITS region confirmed *Pvp* as the casual organism for downy mildew on peas in New Zealand pea growing regions. There was no genetic variation observed between sequences of the selected samples or after RFLP of the PCR product. RAPD analysis however indicated variation within the population. Variation appears to be geographical with notable differences between sites, particularly in the North Island. In the current study, *Pvp* was not detected in any samples from dehydrated field pods (S1) yet was detected from mycelial samples from the subset of dehydrated field pods (S2), fresh sporangial samples (S3) and asymptomatic leaf samples from plants growth in a laboratory growth chamber (S4).

2.4.1 Genetic Analyses

Fifty-two dehydrated field pods with more visible *Pvp* infection were selected to represent the seven sampled sites in the genetic analyses. *Peronospora viciae* f. sp. *pisi* was detected in 39 of the 52 samples using primers DM3F and DM3R. Sequencing of approximately 508 bp of the partial ITS1, entire 5.8S rDNA and partial ITS2 region indicated no genetic variation in the representative New Zealand population. Additionally, an RFLP analysis was conducted on the partial ITS PCR product of the 52 mycelial samples from the dehydrated field pods (S2 samples) which also indicated no genetic variation. A BLAST against a known sequence of a *P. viciae* isolate obtained from a *Pi. sativum* plant (GenBank accession number: AY225471) indicated 99% identitydomi with only two nucleotide substitutions, thus the casual organism for downy mildew on peas in New Zealand pea growing regions was identified as *Peronospora viciae* f. sp. *pisi*. Sequences were then compared to a known sequence from *Pvp* of approximately 231 bp of the partial 18S, complete ITS1 and partial 5.8S region (GenBank accession number: DQ078696) however no similarities were identified. As there is little overlap between the sequenced regions and the number of base pairs between the sequences are considerably different, the absence of similarities is unexpected. However, for an unequivocal identification it is suggested that future studies examine the entire ITS region as well as the *Cox* loci. Although the 5.8S rRNA gene tends to remain relatively conservative within a species (Cooke et al. 2000), Liu et al. (2013) reported the greatest variability between their isolates within that region. Comparative to the current study, using the same DM3F and DM3R primers, Liu et al. (2013) identified 8 of the 30 isolates as genetically different from one another. However, the sequences used in their analyses were longer (approximately 725 bp) than those in the current study (approximately 508 bp), allowing for a greater possibility of detecting variations between samples. Upon closer inspection, Liu et al. (2013) compared their samples to *P. viciae* isolates deriving from

common vetch (*Vicia augustifolia*) (GenBank accession numbers: EF174952 and AY198230), therefore it is unclear whether the *P. viciae* identified in their study is conclusively *Pvp* or is *P. viciae* which has transferred from nearby vetch plants. If the latter is true, the further research is needed to investigate the potential for related Fabaceae weeds to act as potential green bridges, or alternative hosts, for *Pvp* to survive between pea crops. García-Blázquez et al. (2007) examined the phylogenetic relationship between *Peronospora* spp. of Fabaceae plants and placed *Pvp* and *P. viciae* of vetch within the same clade, therefore genetic cross-over may be possible and potential green bridges should not be discounted.

The RAPD analysis of the 24 samples in the current study using primers DMp4 and DMp51 indicated genetic diversity within and between the sampled pea growing sites in New Zealand. From the 22 *Pvp* samples which produced a suitable number of polymorphic bands for analyses, 11 distinct genotypes were observed, with 7 representing more than one individual. In contrast, Liu et al. (2013) identified 27 genotypes between 30 isolates. However, Liu et al. (2013) sampled from 24 fields over 2 seasons, then analysed their samples with 6 RAPD primers. Thus, further variation may be detected within the New Zealand population should further sampling occur. A comparison of banding patterns produced with primers DMp4 and DMp51 indicated genetic diversity within and between sites. Greater genetic variation was seen for samples from the North Island sites, with a maximum of approximately 15 nucleotide substitutions, whilst the South Island sites appear to be more conservative (~5 substitutions). Variations could be attributed to the regional location of sites; South Island sites were in close proximity to one another, whereas the North Island sites were more widely spread. Without further population structure analyses, it can only be speculated as to how this apparent regional variation arose. As *Pvp* is homothallic and variations were not detected in the partial ITS region, it is hypothesized that variations occurred due to simple mutations within the genome. Minor isolate differences, such as the geographically isolated North Island sites, have the potential to strengthen and develop into distinct pathotypes, causing potential issues for pea growers and breeders (Groβinsky et al. 2015). It is unknown as to what effect the host plant cultivars had on the observed population structure and whether their varying tolerances influences isolate patterns. To enable the evaluation of the pathogenicity of the different genotypes to different cultivars, a rapid and robust screening method is required. Until such studies are conducted, it is recommended that growers remain vigilant and work to minimise the likelihood of transporting *Pvp* between sites. The most simple and effective method to reduce human influenced inoculum transport is to maintain standard cultural sanitary practises. Cleaning vehicles, machinery, equipment and work boots will decrease the possibility of moving potential sources of inoculum, which may host an unintroduced virulent isolate, between sites (Biddle 2017). This is important for all sites, however, care should be taken when travelling or transporting goods between more geographically isolated sites, as sporangia are less

likely to successfully spread between sites and survive when travelling by natural means such as wind currents. Sampling sites and comparing their genetic variation over a number of growing seasons could provide insight to isolate movement and virulence, and aid in predicting genetic shift. Genotype separation appears between the North and South Island, with no cross-over, which suggests that the pathogen is not being transmitted between the two islands.

2.4.2 Polymerase Chain Reaction

No *Pvp* DNA was detected by PCR from any of the dehydrated field pods (S1 samples). Some reactions produced a faint band for a single sample however, this was not consistent nor reflective of the expected results. Since some of the samples were taken from visibly infected and sections of the pods with no visible signs of disease, it was expected that these would be more likely to produce a positive amplification for *Pvp*, however this did not occur. The failure of these reactions could be attributed to inhibitory compounds within the plant material or degradation of the DNA in the dehydrated pod samples. Schrader et al. (2012) identified polyphenols, polysaccharides, pectin and xylan as the main PCR inhibitors found in plant material. Such compounds can interfere with many stages of PCR, particularly primer annealing. Competitive binding of the inhibitor to the template can prevent the primer annealing to the targeted portion of DNA (Schrader et al. 2012). Universal primer ITS4 was used in many reactions, therefore it is possible that ITS4 annealed to inhibitors within the plant instead of *Pvp,* despite the use of forward primer DC6. However, since these primers were successful in amplification of *Pvp* from asymptomatic plant samples (S4 samples) this is unlikely to be the reason for the inefficacy of these reactions. The lack of successful amplification is possibly due to the degradation of the DNA. Subsequent to removal from its natural substrate, DNA begins to degrade, with rapid degradation observed after as little as 72 h (Abu Almakarem et al. 2012). In this study, *Pvp* from the S1 samples was dehydrated, stored for 4 months and was not isolated from the plant before undergoing DNA extraction. Therefore, it is presumed that a combination of inhibitory compounds and DNA degradation led to the failed PCR reactions.

Peronospora viciae f. sp. *pisi* was detected in mycelium isolated from dehydrated field pods, pure sporangia and asymptomatically infected plant samples (S2, S3 and S4 samples, respectively). Despite the dehydration and long-term storage of S2 samples, *Pvp* was detected in the conventional PCR using primers DM3F and DM3R. The isolation method for S2 samples ensured DNA was extracted from a pure sample of *Pvp* whereas S1 did not, with the DNA extracted from the infected pod material. Whilst the DNA was potentially degraded, inhibitory compounds deriving from the plant would have been eliminated, considerably reducing PCR interference. In contrast however, S4 samples consisted largely of plant material yet *Pvp* was positively identified using a reaction which had previously failed with S1 samples. S4 samples were taken from the youngest leaf which naturally

have lower quantities of polyphenols and polysaccharides (Sahu et al. 2012), minimising the number of inhibitory compounds entering the reaction. Samples also underwent DNA extraction immediately, resulting in DNA being extracted from viable *Pvp* and therefore less likely to have degraded (Abu Almakarem et al. 2012). The success of PCR reactions using S2, S3 and S4 samples, regardless of whether primer pair DC6/ITS4 or DM3F/DM3R was used, indicates that the PCR success in this study was determined by the quality of DNA. For future studies, it is recommended that DNA extracted from pure *Pvp* is used where possible to reduce the interference of inhibitory compounds, and in cases where plant material is tested for asymptomatic infection, DNA extraction should occur immediately to minimise DNA degradation.

Primer specificity is increasingly important for PCR, particularly for detection of oomycetes or fungi, in plant material (Ward et al. 2016). Primer DM3F consistently produced lower quality, shorter sequences, thus sequences produced with primer DM3R were used for analyses. All sequences had to be trimmed of unclear nucleotide readings for analysis, eliminating the possibility of detecting the primer sequences within the sequence when subjected to a Primer-BLAST. As not all of the S2 samples were able to be sequenced due to failure to amplify (13 of the 52 samples) or poor sequence results (11 of the 52 samples), and false banding occurring in two-step nested PCR reactions, primers DM3F and DM3R were subjected to a Primer-BLAST against the GenBank database to check for specificity. The possibility of reagent contamination was rejected subsequent to reaction repetitions and both primers were identified as specific to *P. viciae,* therefore it was concluded that there were issues with primer annealing or the Mg^{2+} content of the Taq polymerase. Adjusting the annealing temperature and time or changing the Taq could have eliminated issues associated with primer annealing or Mg^{2+} concentration, however, future work would have to confirm this (McPherson & MØller 2000). As the entire ITS region was not amplified or sequenced, the sequences could not be checked to ensure the primer sequences align with a portion of the target DNA and therefore could anneal to the template. It is possible that the primers were designed with such specificity that new isolates of *Pvp* with some nucleotide substitutions would not allow the primer to anneal properly. However, to determine this the entire ITS region of samples from this study would need to be compared against other known *Pvp* sequences.

S1 samples from the dehydrated infected field pods collected for genetic analyses were a combination of plant and *Pvp* DNA, therefore spectrophotometer readings were not included in the current study. PCR reactions were performed with primers DC6 and ITS4, and varying concentrations of pure *Pvp* DNA mixed with DNA extracted from pure plant material to determine the sensitivity of the PCR was with regards to detecting *Pvp in planta*. Consistent amplification occurred with DNA concentrations up to approximately 2.31 ng/µL, or approximately 111 nuclei, however, as reaction replications were not conducted it is unknown how reproducible these results are. Compared to

other studies, the reaction used for detecting *Pvp in planta* in this this study appears to be much less sensitive; possibly due to DNA degradation or inhibitory compounds. Using a standard PCR protocol, Landa et al. (2007) were able to detect 0.1-10 pg of *P. arborescens* in asymptomatic poppy plants, dependant on which primers were used, whilst Herath Mudiyanselage (2015) detected 0.4 ng of *P. sparsa* DNA in boysenberry. Nested PCR reactions have been proven to be far more sensitive with Jamali & Banihashemi (2013) reporting the detection of as little as 0.2 pg of *P. juniperi* DNA in *Helianthemum ledifolium* root stocks and Herath Mudiyanselage (2015) reporting the detection of 0.4 pg of *P. sparsa* in boysenberry. It is highly probable that the detection threshold determined in the current study will not be reflective thresholds for S1, S2, S3 and S4, as each sample type has a different composition due to the methods in which they were sampled and how the DNA was extracted. Comparative to the DNA used in detection threshold reactions which was from pure *Pvp* sporangia, the DNA extracted from S1 and S4 sampled consisted of both plant and *Pvp* DNA, therefore, their actual detection threshold is likely to be greater than 111 nuclei. The detection threshold in S3 samples is expected to be considerably lower as the DNA was extracted from pure samples and not diluted with any plant DNA prior to PCR. Although S2 samples were pure *Pvp*, the degradation of the DNA would likely influence the number of nuclei required within a sample for successful amplification. Detection thresholds of DM3F and DM3R were not examined, however, the required number of sporangia or mycelial fragments containing nuclei would be again be expected to vary.

2.5 Limitations and Further Research

The greatest limitation, which consistently arose throughout genetic analyses and PCR reactions, was the method as to which the initial S1 samples were collected and extracted. The 382 pods were initially collected and subsequently dehydrated. For DNA extraction, *Pvp* was not isolated from the pod, with pieces of dehydrated pods consisting of both *Pvp* mycelium and pod tissue being used for DNA extraction, resulting in an extract likely consisting of degraded DNA and inhibitory compounds. All PCR reactions failed to amplify *Pvp* from S1 samples. For subsequent reactions *Pvp* was isolated from the pod prior to extraction, which allowed the successful amplification of *Pvp* DNA, despite the likely large amount of DNA degradation. Due to the difficulties in isolating *Pvp* from the pods, only 20% of the initial samples were subjected to analyses of the partial ITS1, complete 5.8S, and partial ITS2. Whilst the ITS region is the most commonly used region for inter species analyses, its success for examining genetic variability within a species is reportedly unreliable (Choi et al. 2007; Robideau et al. 2011; Liu et al. 2013; Sapkota & Nicholaisen 2015). The lack of variability within the ITS region has been acknowledged and the *Cox*2 loci has been suggested as an alternative (Choi et al. 2007;

Robideau et al. 2011). Similarly to the ITS region, the *Cox*2 loci is best suited for identification between related Oomycetes, rather than isolates within a species (Robideau et al. 2011). In this study, the *Cox*2 loci was successfully amplified in S2 samples but the product was not sequenced, nor subjected to RFLP due to time constraints. It is possible that further analyses of the *Cox*2 loci could have identified variation between samples, but this is only speculative. Variation within *Pvp* is well recognised, with 14 pathotypes reported in different pea growing regions throughout numerous countries (Stegmark 1990; Davidson et al. 2011; Liu et al. 2013), therefore results yielded from examining the ITS region alone should not be considered conclusive and further molecular analyses should be performed.

Only 9% of the samples initially collected underwent RAPD analysis. It is probable that if all 382 samples were processed, patterns within and between sites would arise and more conclusive conclusions on *Pvp* populations within New Zealand pea growing regions could be drawn. Due to time and resource constraints, RAPD reactions were not repeated. Banding patterns produced during RAPD analyses are known to be difficult to reproduce between and within laboratories (Arif et al. 2010), thus it is unknown how reproducible these results are. Future work whereby DNA is extracted from fresh infected pod or plant samples is required to determine the genetic diversity of the New Zealand *Pvp* populations in the main pea growing regions. As well as analysing these using the RAPD method tested here, other methods such as microsatellite (or simple sequence repeats (SSR)) markers could be used to determine the genetic diversity of the New Zealand *Pvp* population. Microsatellite markers have been identified in other *Peronospora* spp. (Trigiano et al. 2011; Feng et al. 2018) however, to date, no markers have been identified for *Pvp*. Perumal et al. (2008) identified and developed microsatellite primer sets from *Peronosclerospora sorghi* which proved to be useful for other downy mildew species, including *P. sparsa*, thus future studies analysing *Pvp* with microsatellites may consider trialling those identified in the 2008 study. Microsatellite loci have been used successfully to examine the genetic variation of *Phytophthora ramorum* populations within and between continents (Ivors et al. 2006), thus, when identified, markers could be used to compare *Pvp* diversity between local and global pea growing regions, linking New Zealand populations of *Pvp* to described pathotypes.

Further molecular studies could develop a qPCR (quantitative polymerase chain reaction) protocol to investigate the concentrations of *Pvp* within different plant tissues and locations throughout the plant. Conventional PCR gives an indication of presence or absence of *Pvp in planta* but is only reflective of the sampled section of the plant and if the concentration of *Pvp* is high enough to detect (approximately 111 sporangia or mycelial fragments containing a nuclei) (Garibyan & Avashia 2013). Understanding how the pathogen systemically spreads through the plant and where the pathogen is

likely to be most concentrated would allow the researcher to conduct a standard, or nested PCR on a section of the plant where *Pvp* DNA would be most likely detected.

2.6 Conclusion

Peronospora viciae f. sp. *pisi* was unable to be detected in any PCR reaction using dehydrated field pods (S1 samples) but was detected from pure mycelial/sporangial samples and asymptomatic plants from S2, S3 and S4 samples. In the current study, it is concluded that the initial methods of sampling, storage and DNA extraction were inefficient and severely impacted the ability to screen the samples for genetic variability. Partial ITS sequencing and RFLP indicated no genetic variability between any of the samples which were screened. When a larger portion of the genome was examined with RAPD, genetic dissimilarities within and between different sites became apparent. For future studies examining the genetic variability it is recommended that *Pvp* is isolated from the plant and DNA is extracted immediately. In addition, the use of more RAPD primers or microsatellite markers would provide supplementary information on the genetic diversity of the New Zealand *Pvp* population. However, to determine whether the different genotypes identified within the population represent different pathotypes a rapid and robust screening method is required, which will be investigated in the next chapter.

Chapter 3

Evaluation of Bioassays for Inoculation of *Pisum sativum* with *Peronospora viciae* f. sp. *pisi*

3.1 Introduction

Downy mildew diseases are commonly found on both horticultural and ornamental crops and can be caused by many different species belonging to nineteen genera of oomycetes. The genus *Peronospora* includes the greatest number of species; estimated at approximately 500 (Fletcher et al. 2018). *Peronospora viciae* f. sp. *pisi* (*Pvp*) is the pathogen responsible for causing downy mildew on peas, which causes grey, cotton-like mycelial growth on the underside of the leaves and overall stunted growth and distortion of plants (Stegmark 1994). The impact *Pvp* can have on a crop varies considerably due to many influencing factors; how the crop came to be infected, the local climate, and the cultivation practises implemented by the grower.

Once *Pvp* has entered a cropping system, it is extremely difficult to eliminate. Fungicides are often considered the 'go to' option for disease control, however Oomycetes like *Pvp* tend to only be susceptible to a narrow range of chemical groups. The New Zealand NovaChem Agrichemical Manual currently lists coppers, QoI-fungicides, and phenylamide seed treatments as registered products for *Pvp*. Chemicals play a valuable role in crop protection, but it is important that they are not exclusively relied on. If the use of chemicals cannot be avoided, it is advised that they are incorporated into integrated pest management strategies which are heavily influenced by cultural practices such as crop rotation, healthy seeds, and the use of tolerant cultivars (Biddle 2017).

Management practises aimed at preventing disease incidence are desired and encouraged. The simplest way to prevent disease within the crop is to sow cultivars which are highly tolerant to *Pvp* (Biddle 2017). Breeding pea lines for resistance towards *Pvp* has occurred since the 1980's and prior to that, research was being conducted on screening cultivars against the pathogen to evaluate the level of tolerance within the plant (Jermyn 1987; Stegmark 1994). Studies that assessed resistance of pea cultivars towards downy mildew identified virulence variation and physiological specialization within the *Pvp* species, which led to the informal identification of genetically dissimilar pathotypes of *Pvp* (Taylor et al. 1989; Stegmark 1994; Davidson et al. 2011; Liu et al. 2013). Liu et al. (2013) conducted the first molecular study on the genetic diversity of *Pvp* and they confirmed the existence of genetic dissimilarities between isolates.

Understanding the genetic diversity of *Pvp* is crucial when evaluating the tolerance of a pea cultivar against the pathogen. There are many known methods used to successfully screen pea lines against downy mildew and to maintain the pathogen in a laboratory setting. The most common approach uses a spore suspension created from vortexing fresh sporangia in sterile water and applying the suspension over young host plants (Taylor et al. 1989; Stegmark 1990; Danielsen & Ames 2000; Davidson et al. 2004; Liu et al. 2013). Another known inoculation method is to collect soil from a field with a known history of hosting *Pvp* infected pea plants and to add the soil to trays containing potting soil and pea seeds (Davidson et al. 2011). It has also been suggested that burying a piece of diseased plant material with the seed at the time of planting can also lead to *Pvp* infection (Alexis Plouy, Crites Seeds, Personal Communication, 2019).

Biotrophic fungi are notoriously difficult to maintain in a laboratory setting (Danielsen & Ames 2000). Most often, infected material is collected and immediately placed in -80˚C storage until required (Davidson et al. 2011; Liu et al. 2013). Maintaining a living source of inoculum and pathogen viability will often mean the researcher is limited to successive transfers between infected and healthy plants. However, it has been suggested that pipetting a spore suspension onto water agar or filter paper and placing a pea leaf on top can maintain a source of *Peronospora* inoculum (Danielsen & Ames 2000).

Having a successful method to screen pea lines against *Pvp* in a laboratory setting is invaluable as field screening can often produce unreliable results. Local weather conditions, the reliance on natural downy mildew outbreaks, and the possibility of infection from other pests or pathogens means there are many outside influences that may impact the results of field experiments (Davidson et al. 2004). The aim of this chapter was to develop a method to screen pea cultivars against *Pvp* which could be applicable to global pea growing regions.

3.2 Materials and Method

3.2.1 Growth Media Trials

To attempt to propagate *Pvp*, three separate experiments were conducted on different media; water agar, 10% potato dextrose agar (PDA; Difco, Becton, Dickinson and Company), and sterile filter paper (Whatman Filter paper No 1) which had been dampened with sterile water. Ten repetitions were set up for each media. Using fresh, infected leaf material collected at the time of initial sampling (Section 2.2.1), three sections with dense sporangial growth were cut from the leaves and placed on each plate. Plates were incubated in a growth chamber with a 16 h photoperiod, at 20˚C and monitored for signs of aseptate mycelial growth and fresh sporangia which could indicate new *Pvp* growth. After 72 hours the plates were removed and examined under a stereo microscope for evidence of fresh

Pvp growth. Any fresh *Pvp* mycelia or sporangia would be continuously transferred onto a new plate of the most suitable medium to be maintained in a controlled environment, in the absence of a living host.

3.2.2 Bioassays

A range of bioassays were set up to identify the most successful way to cause *Pvp* infection on pea plants. Four pea cultivars ('CS480-AF', 'Bolero', 'Rondo', and 'Utrillo'), provided by Townsend Seeds, each with varying tolerance to *Pvp* were used as differential hosts. For each treatment, three types of inoculum were used, described as either fresh, dried or soil. Dried inoculum was derived from the additional infected pods which had previously been collected and dehydrated (Section 2.2.1). Pods were individually ground into a fine powder using an electric herb grinder (Sunbeam Multigrinder II). Fresh inoculum consisted of sporangia which had been carefully lifted from live plant material with a needle. Soil was also collected from fields with a known history of *Pvp* infection and was used as an inoculation method.

Experiment A

Five seeds of three different cultivars ('Bolero', 'Rondo' and 'Utrillo') were sown individually into single, 0.2 L, seed-raising pots with potting mix (4:1 bark to pumice media, Osmocote® 3-4 month controlled release fertiliser, lime, and hydroflo). Approximately 0.2 grams of dried inoculum was added on top of the potting mix, directly above each seed. The plants were watered with reverse osmosis (RO) water when required and incubated for 4 weeks in a cycle of 16 h of light at 15˚C and 8 h of dark at 8˚C. After 7 days, all seed-raising pots and plants were placed into a single plastic bag to maintain a high relative humidity (RH). Plants were misted within the plastic bag with a hand-held spray bottle containing RO water when required to maintain a high RH, indicated by the presence of condensation on the inside of the bag. Throughout incubation, all plants were monitored for signs of disease expression characteristic to *Pvp*, such as sporangia on leaves and stems. Upon detection, a sample of the pathogen was lifted from the plant with a sterile needle and examined under a compound microscope to check for distinctive *Pvp* structures like aseptate hyphae and sporangia. Any confirmed fresh *Pvp* mycelia or sporangia were collected with a sterile needle and used for further inoculation experiments.

Experiment B

Three seeds from three different cultivars ('Bolero', 'Rondo' and 'Utrillo') were sown into individual pots containing potting mix, as previously described, and grown under glasshouse conditions until the first three sets of leaves had formed. Leaves were removed from each plant and divided by cultivar, then placed into three 2L plastic boxes, lined with sterlie paper towels. All of the leaves were laid flat; approximately half of the leaves from each cultivar had either their abaxial or adaxial surface

exposed. The leaves were misted with RO water until run off and the paper towels were soaked. Per container, 0.5 grams of a dried inoculum sample was sprinkled evenly over the leaves. The leaves were lightly misted with RO water again and then incubated for 4 weeks in a cycle of 16 h of light at 15˚C and 8 h of dark at 8˚C. Throughout incubation, all plants were monitored and examined for characteristic *Pvp* structures such as aseptate hyphae and sporangia as previously described. Any confirmed fresh *Pvp* mycelia or sporangia were collected with a sterile needle and used for further inoculation experiments.

Experiment C

Five seeds of three different cultivars ('Bolero', 'Rondo' and 'Utrillo') were sown into individual pots containing potting mix as previously described and grown under glasshouse conditions until the first three sets of leaves had formed. A liquid sample of inoculum was prepared by finely grinding a heavily infected, dried pea pod in a herb grinder and mixed with 100 mL of sterile water. The plants were inoculated by injecting approximately 1 µL of the liquid sample into the meristem, directly below the first node. Plants were misted with RO water and incubated for 4 weeks in a growth chamber with a cycle of 16 h of light at 15˚C and 8 h of dark at 8˚C. After 7 days, the plants were placed into a plastic bag to maintain a high RH. Plants were misted within the plastic bag with a hand-held spray bottle containing RO water when required to maintain a high RH, indicated by the amount of condensation on the inside of the bag. Throughout the incubation period, all plants were monitored and examined for characteristic *Pvp* structures such as aseptate hyphae and sporangia as previously described. Any confirmed fresh *Pvp* mycelia or sporangia were collected with a sterile needle and used for further inoculation experiments.

Experiment D

Five seeds of three different cultivars ('Bolero', 'Rondo' and 'Utrillo') were sown into individual pots containing potting mix as previously described and grown under glasshouse conditions until the first three sets of leaves had formed. The remaining liquid sample prepared during the set up for Experiment Three was used for inoculation; approximately 10 µL was pipetted over the apical tip of each plant. Plants were misted with RO water and incubated for 4 weeks in a cycle of 16 h of light at 15˚C and 8 h of dark at 8˚C. After 7 days, the plants were placed into a plastic bag to maintain a high RH. Plants were misted within the plastic bag with a hand-held spray bottle containing RO water when required to maintain a RH, indicated by the amount of condensation on the inside of the bag. Throughout the incubation period, all plants were monitored and examined for characteristic *Pvp* structures such as aseptate hyphae and sporangia as previously described. Any confirmed fresh *Pvp* mycelia or sporangia were collected with a sterile needle and used for further inoculation experiments.

Experiment E

Eight seeds of three different cultivars ('Bolero', 'Rondo' and 'Utrillo') were sown in individual pots. Prior to covering the seed in potting mix, approximately 0.2 grams of a dried sample was added on top of the seed. The plants were incubated at 10˚C until the first sign of seedling emergence was observed. The plants were then incubated for 4 weeks in a cycle of 16 h of light at 17˚C and 8 h of dark at 15˚C. After 7 days, the plants were placed into a plastic bag to maintain a high RH. Throughout incubation, all plants were monitored and examined for characteristic *Pvp* structures such as aseptate hyphae and sporangia as previously described. Any confirmed fresh *Pvp* mycelia or sporangia were collected with a sterile needle and used for further inoculation experiments.

PCR Verification

After 4 weeks of incubation, one plant of each cultivar without any visible symptoms, from experiments A, C, D, and E underwent a polymerase chain reaction (PCR) to check for asymptomatic infection. Experiment B and 'Utrillo' plants from Experiment A were not included due to low germination and the presence of saprophytic fungi. Each plant that was selected for PCR analysis had a 5 mm^2 piece removed from the centre of the youngest, fully formed leaf with a sterile scalpel. Samples were then placed into individual 1.7 mL tubes and the DNA extracted using the Chelex 100 (BioRad) DNA extraction method described in Section 2.2.2. The DNA was subjected to PCR to indicate the presence or absence of *Pvp in planta* using primers DC6 and ITS4. PCR reactions were prepared in a 20 µL volume which contained 10 µL DreamTaq Green PCR Master Mix, 0.25 µM of each primer, 2 µL of DNA template, and the remaining volume H_2O. A full description of the reaction preparation, cycling parameters, and gel visualisation can be found in Section 2.2.4, Reaction #2. To confirm the presence of *Pvp* asymptomatic infection, any samples amplifying a band positive for *Pvp* (approximately 1500 bp) were sequenced at the Lincoln University sequencing facility.

3.2.3 Seed Treatment Experiments

Experiments were set up to assess if seed manipulation would increase the likelihood of achieving *Pvp* infection. Three seed treatment methods were applied to seeds; soaking, slicing the seed coat and an untreated control. For each of the three treatments, ten seeds of four different cultivars ('CS480-AF', 'Bolero', 'Rondo' and 'Utrillo') were used. For treatment one, seeds were soaked by being placed in a Petri dish, submerged in RO water and incubated for 3 h at 24˚C, under lights (Fig. 3.1A). For treatment two, seeds had a 2 mm long scratch sliced into the seed coat. The control seeds were left untreated. After the treatment had been applied, the seeds were folded within sterile paper towels and placed into a single lidded plastic container (14 H x 58 W x 36 D cm). The container had three dividers to separate cultivars. Seeds of each cultivar were grouped by treatment (Fig. 3.1B). The paper towels were misted with 100 mL of sterile water until soaked and then incubated in

a cycle of 16 h of light at 24˚C and 8 h of dark at 18˚C. Every 24 h germination was recorded, and another 100 mL of RO water was misted into each container to prevent the paper towels from drying out. Germination was defined as any seed with a radicle 1 cm or longer. After 72 h, seeds were grouped by treatment and cultivar and placed into nine separate Petri dishes. The dishes contained RO water and approximately 0.2 g of a ground-up, infected, dehydrated pea pod, which had been ground up in a herb grinder, was sprinkled over the seeds. Dishes were wrapped with tin foil and incubated for 100 h in a cycle of 17˚C for 16 h and 14˚C for 8 h in the dark. The seeds were then sown into individual pots containing potting mix as previously described in Section 3.2.2 and watered accordingly. The pots and plants were placed into a plastic bag to maintain a high RH and incubated at 10˚C for 3 days, then incubated for 4 weeks in a cycle of 16 h of light at 17˚C and 8 h of dark at 15˚C. To maintain a high RH, plants were misted with RO water when required, whilst remaining in the plastic bag. The plants were assessed for disease expression as previously described in Section 3.2.2.

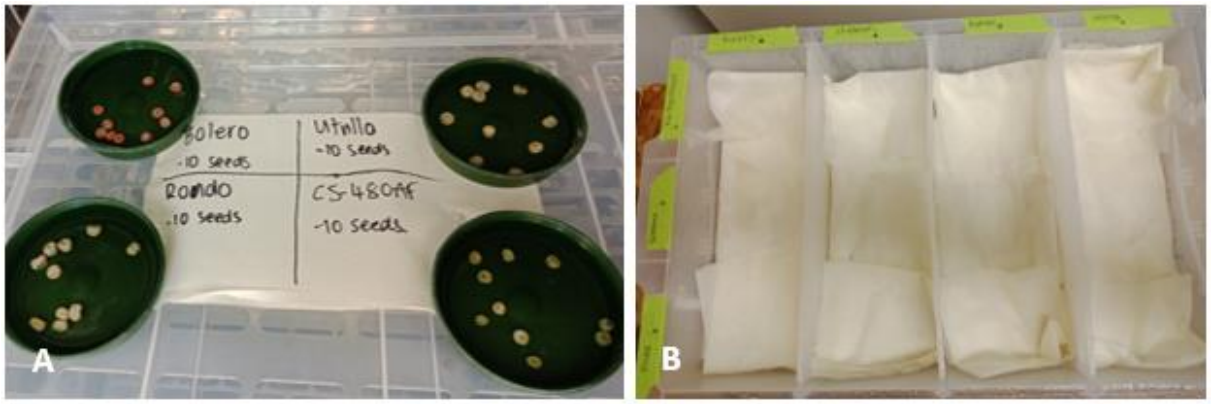

Figure 3.1: Seed treatment experiments **A**. Seeds soaking in RO water. **B**. Seeds wrapped in damp, sterile paper towels. Labels of the tape show seed groupings of cultivars and treatments. The four labels running horizontally at the top of the image reads as: 'Bolero', 'CS480-AF', 'Rondo' and 'Utrillo'. The three labels running vertically on the left of the image reads as: 'no treatment', 'soaked' and 'sliced'.

PCR Verification

After 4 weeks of incubation, one plant of each cultivar and treatment without any visible disease symptoms was removed and the DNA was extracted using the Chelex 100 method, as described in Section 2.2.2. The sample underwent PCR to check for asymptomatic infection, as described in Section 3.2.2. A full description of the primers used, cycling parameters, and gel visualisation can be found in Section 2.2.4, Reaction #3. To confirm the presence of asymptomatic *Pvp* infection, and samples which produced a positive band (approximately 1500 bp) for *Pvp* were sequenced at the Lincoln University sequencing facility.

3.2.4 Fresh Inoculum Experiments

Experiment F

This inoculation method was based on the hypothesis that oospores within the soil would provide a fresh source of primary inoculum to cause systemic infection within the plants. Twenty-five samples were collected from the top 10-15 cm of soil from field Site Two, using a soil corer (approximately 2 cm diameter). Samples were collected in a grid formation, 1x1 m apart within a 5 m^2 plot to result in 25 subplots. For each of the 25 samples, four soil core samples (approximately 540 g/sample) were combined and then placed into a plastic bag. Any stones or grass remnants were removed from the samples and any larger clumps of soil were manually broken apart. Samples were refrigerated at 4°C until required (for approximately 5 days). The soil samples were placed into individual, 0.2 L seed-raising pots and then had two 'Utrillo' seeds sown into each (50 seeds total). The pots were watered, placed in a plastic bag to maintain a high RH and incubated for 6 weeks in a cycle of 16 h of light at 17˚C and 8 h of dark at 15˚C. To maintain a high RH, plants were misted with RO water when required, whilst remaining in the plastic bag. The plants were monitored and assessed for disease expression as previously described in Section 3.2.2.

Experiment G

At the time of the soil sample collection, Site Two was also examined for signs of fresh *Pvp* infection. Despite it being winter with only volunteer 'Utrillo' pea plants remaining in the field, two 'Utrillo' plants with fresh sporangia were collected and returned to the Lincoln University laboratory for inoculation treatments. To prepare plants for the two inoculation treatments, twenty 'Utrillo' seeds were sown in individual pots with potting mix, as previously described in Section 3.2.2. They were grown in an incubator at 15˚C with a 16 h photoperiod, until the three sets of leaves had formed. Prior to inoculation, the plants were sprayed with RO water containing two drops of Tween 20 (LabChem)/500 mL until run-off. Fresh sporangia were identified with a compound microscope and were removed from the volunteer field plants with a sterile needle for inoculation. Ten plants underwent treatment one, which involved an approximate 2 mm^2 cluster of fresh sporangia being placed directly on the abaxial side of each of the youngest set of leaves with a sterile needle. For treatment two, another ten plants had an approximate 2 mm^2 cluster of fresh sporangia placed directly onto their apical tip, using a sterile needle. The soil was then watered with RO water and plants were placed in a plastic bag to maintain a high RH and incubated for 6 weeks in a cycle of 16 h of light at 17˚C and 8 h of dark at 15˚C. To maintain a high RH, plants were misted with RO water when required, whilst remaining in the plastic bag. The plants were monitored and assessed for disease expression as previously described in Section 3.2.2.

Experiment H

Six 'Utrillo' seeds were soaked in a Petri dish which held approximately 25 mL of RO water and 3-4 sections of volunteer 'Utrillo' leaves which were covered heavily in fresh sporangia (Fig. 3.2A). After being soaked for 24 h in a growth chamber with 16 h of light at 17˚C and 8 h of dark at 15˚C, the seeds were sown into individual pots with potting mix as described in Section 3.2.2. Prior to being covered in potting mix, a 5 mm^2 piece of leaf tissue with fresh sporangia was placed on top of each seed. Another six 'Utrillo' seeds were not soaked and were then sown into individual pots containing potting mix, as previously described. Consistent with the other six seeds, a 5 mm^2 piece of leaf tissue with fresh sporangia was placed on top of each non-soaked seed (Fig. 3.2B). The potting mix was then watered, and all pots were placed in a single plastic bag to maintain a high RH. The seeds were incubated for 6 weeks in a cycle of 16 h of light at 17˚C and 8 h of dark at 15˚C. To maintain a high RH, plants were misted with RO water when required, whilst remaining in the plastic bag. The plants were monitored and assessed for disease expression as previously described in Section 3.2.2.

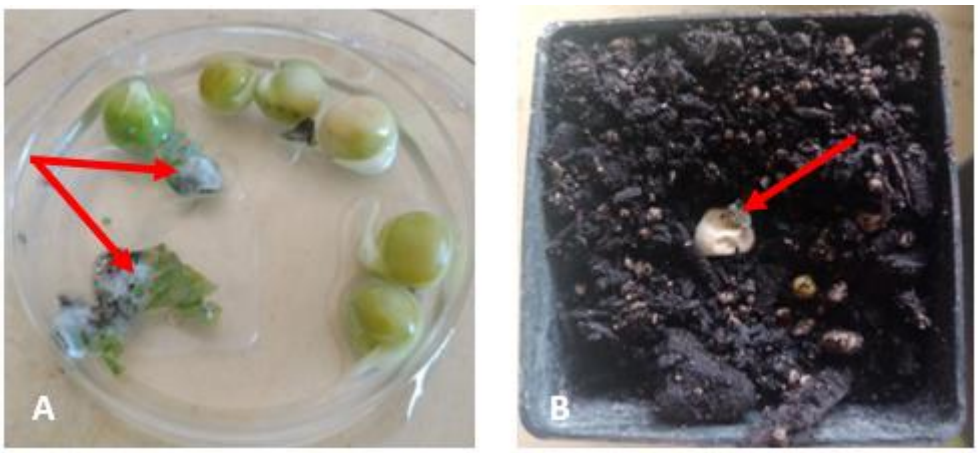

Figure 3.2: Experiment H. **A**. Seeds soaking in RO water containing *Peronospora viciae* f. sp. *pisi* infected leaf material. **B**. An untreated seed being buried with a piece of *Peronospora viciae* f. sp. *pisi* infected leaf material. Arrows indicate infected leaf material.

PCR Verification

After 6 weeks of incubation, each plant underwent a nested PCR to check for asymptomatic infection, irrespective of whether visible disease symptoms were observed. Each plant had a 5mm^2 piece sliced from the youngest, fully formed leaf with a sterile scalpel and the DNA was extracted using the Chelex 100 DNA extraction method, as described in Section 2.2.2. One plant from Experiment H that had no prior treatment was not included in PCR analysis due to a heavy presence of *Mucor* sp. on the leaf surface. The DNA was subjected to a two-step nested PCR to determine the presence or absence of *Pvp in planta*. The first step used primers DC6 and ITS4 whilst the second step used DM3F and DM3R. PCR reactions were prepared in a 20 µL volume which contained 10 µL DreamTaq Green PCR Master Mix, 0.125 µM of each primer, 0.5 µL of DNA template, and the

remaining volume H_2O. A full description of the PCR preparation, cycling parameters, and gel visualisation can be found in Section 2.2.4, Reaction #35. To confirm the presence of *Pvp* asymptomatic infection, the samples which produced the brightest positive bands (approximately 725 bp) for *Pvp,* for each experiment, were selected then sequenced at the Lincoln University sequencing facility.

3.2.5 Soil Experiments

Soil samples were collected from field Sites One, and Two (Chapter 2) by walking from East to West in a 'W' formation, collecting samples along each line of the 'W'. The number of samples collected was reflective of the size of the field: twelve samples from Site One and sixteen samples from Site Two. As Site Three was considerably smaller than the other two sites, Site Three was sampled by taking three, evenly spaced samples from East to West of the site. In total, 31 samples were collected. For each of the thirty-one samples, four soil corer samples were collected as described in Section 3.2.4 (Experiment F) and then placed into a plastic bag. Samples were refrigerated until required. The soil samples were thoroughly mixed and placed into single, 0.2 L seed-raising pots. In total, there were twelve pots for Site One, sixteen pots for Site Two and three pots for Site Three. One seed from each of the following cultivars; 'CS480-AF', 'Rondo', and 'Utrillo' was placed into each pot. The pots were watered, and placed in three plastic bags, grouped by cultivar, to maintain a high RH and incubated for 6 weeks at 15˚C with a 16 h photoperiod. To maintain a high RH, plants were misted with RO water when required, whilst remaining in the plastic bag. The plants were monitored and assessed for disease expression as previously described in Section 3.2.2.

PCR Verification

After 6 weeks of incubation, each plant was visually assessed for signs of disease and had a sample taken to verify the presence or absence of *Pvp in planta*. Samples were taken by one of two methods depending on whether the plant was visibly infected or not. Plants which were visibly infected were examined using a stereo and compound microscope to identify aseptate hyphae and characteristic sporangia which could confirm the presence of *Peronospora* spp. For the plant which had the most sporulation, a 3mm^2 section of sporangia was removed from the leaf using a sterile needle and placed into a 1.7 mL tube for DNA extraction. From the same visibly infected plant, an adjacent leaf which did not have visible signs of disease had a 5mm^2 piece dissected from its centre with a sterile scalpel and placed into another 1.7 mL tube for DNA extraction. Further, a 5 mm^2 piece was removed from the centre of the youngest, fully formed leaf of plants which were not visibly infected with a sterile scalpel and were placed into individual 1.7 mL tubes for DNA extraction.

All samples had their DNA extracted using the Chelex 100 DNA extraction method as described in Section 2.2.2. The DNA was then subjected to PCR to determine the presence or absence of *Pvp in*

planta. A full description of the PCR preparation, cycling parameters, and gel visualisation can be found in Section 2.2.4, Reactions #2, #12, and #13. Due to time and resource constraints, samples which gave a positive band (approximately 725 bp) for *Pvp* were not sequenced to confirm the presence of *Pvp.*

3.3 Results

3.3.1 Growth Media

There was no *Pvp* growth detected on any of the tested media. All media supported the growth of many saprophytic organisms however, the most contamination was observed on the PDA (Fig. 3.3).

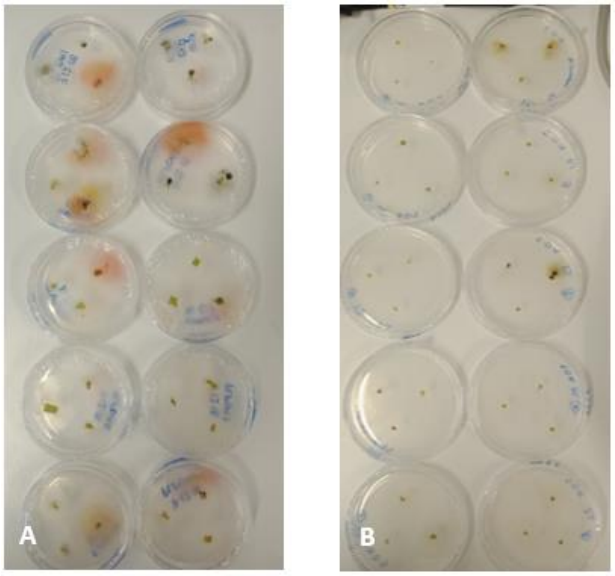

Figure 3.3: Media containing three pieces of *Peronospora viciae* f. sp. *pisi* infected leaf material, after 72 h incubation at 20 ˚C. All media support saprophytic fungal growth. **A**. 10% Potato dextrose agar. **B**. Water agar.

3.3.2 Bioassays

No disease symptoms were observed on any plants from any of the experiments. One plant of each cultivar from experiments A, C, D, and E underwent PCR to test for asymptomatic infection (Fig. 3.4). No 'Utrillo' plants from Experiment A germinated, with exception of the control, thus they were not included in the molecular assessment. Samples from experiment B were discarded and not included in the molecular assessment due to a high presence of saprophytic fungi (Fig. 3.5).

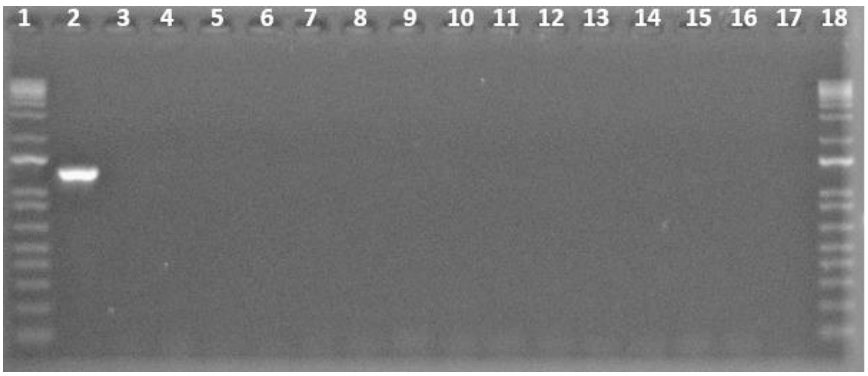

Figure 3.4: Reaction #2, Section 2.2.4. A product of approximately 1100 bp of a partial internal transcribed spacer region was amplified using primers DC6 and ITS4. Samples are from pea plants with no visible signs of disease from Experiments A, C, D and E. Bands show the possible presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 18 1 kb+ ladder. Lane 2 positive control. Lane 17 negative control. Lanes 3-16, Experiment E 'Bolero', 'Rondo' 'Utrillo', Experiment D 'Bolero', 'Rondo', 'Utrillo', Experiment A 'Bolero', 'Rondo', Experiment C 'Bolero', 'Rondo', 'Utrillo', Experiment A controls 'Bolero', 'Rondo', 'Utrillo'.

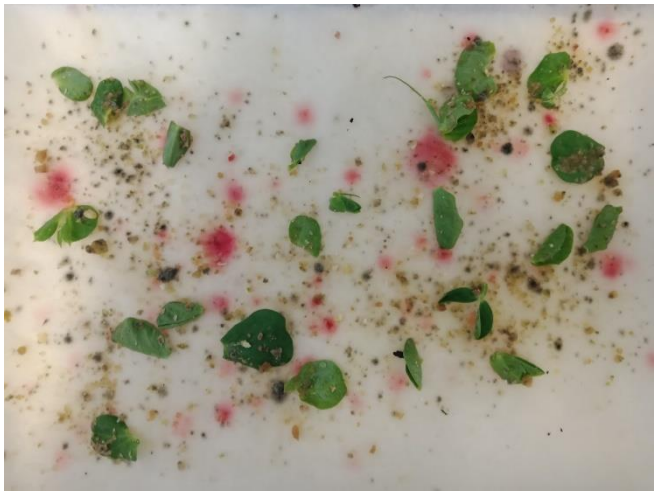

Figure 3.5: Detached leaf assay (Experiment B) after 4 weeks of incubation with abundant saprophytic fungi.

3.3.3 Seed Treatments

After 72 h of being wrapped in a damp paper towel, only six out of the forty seeds that were initially soaked (treatment one) had germinated; three 'Utrillo' seeds, two 'Bolero' seeds and one 'Rondo' seed. No seeds which had their seed coat sliced or had no treatment applied had germinated after 72 h. Upon examination after the 4-week growth period, there were no visible signs of *Pvp* infection observed on any plants from any of the seed treatments. One plant of each cultivar from each of the three treatments underwent PCR to test for asymptomatic infection (Fig. 3.6). No *Pvp* was detected in any of the tested plants.

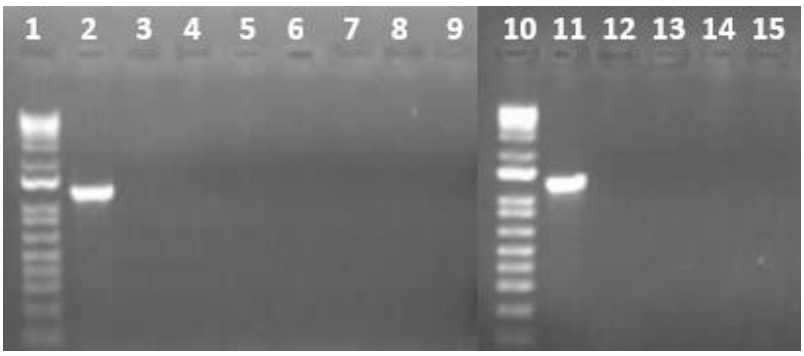

Figure 3.6: Reaction #3, Section 2.2.4. A product of approximately 1100 bp of a partial internal transcribed spacer region was amplified using primers DC6 and ITS4 from samples of *Pisum sativum* plants which had no visible signs of disease, that underwent seed treatments prior to sowing. Bands indicate the possible presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 10: 1 kb+ ladder. Lanes 2 and 11: positive control. Lanes 9 and 15: negative control. Lanes 3-5: controls 'Utrillo', 'CS480-AF', 'Rondo', lanes 6-8: soaked 'Utrillo', 'CS480-AF', 'Rondo', lanes 12-14: sliced 'Utrillo', 'CS480-AF', 'Rondo'.

3.3.4 Fresh Inoculum Experiments

Experiment F

Of the 50 seeds that were planted in the soil collected from Site 2, 15 plants grew and survived the 6-week growth period. There were no visual disease symptoms on any of the surviving plants. A nested PCR analysis identified asymptomatic infection in 7 plants (Fig. 3.7). Infection was confirmed by DNA sequencing of two representative samples.

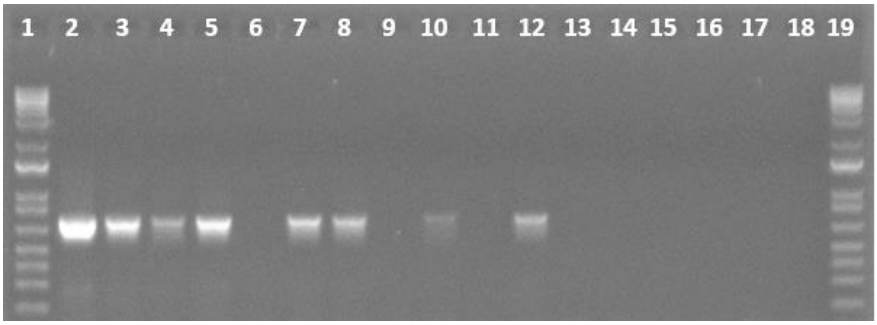

Figure 3.7: Reaction #35, Section 2.2.4. A product of approximately 725 bp of a partial internal transcribed spacer region was amplified with primers DM3F and DM3R. Samples are from plants with no visible signs of disease which were originally sown in soil collected from a site with a history of hosting downy mildew infected pea plants. Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 19: 1 kb+ ladder. Lane 2: positive control. Lane 18: negative control. Lanes 3-17: asymptomatic plants from Experiment F. Samples from lanes 3 and 5 were sequenced.

Experiment G

Upon visual inspection of the plants, there were no signs of *Pvp* expression. Nineteen of the 20 plants initially planted survived the 6-week growth period. A nested PCR analysis indicated asymptomatic infection in 10 out of the 19 plants. Placing sporangia on the apical tip (treatment two) resulted in 7

out of 10 producing positive bands. Comparatively, 3 out of the 9 surviving plants that had sporangia placed on the abaxial side of two leaves (treatment one) produced positive bands indicating the presence of *Pvp* (Fig. 3.8). *Peronospora viciae* f. sp. *pisi* infection was confirmed by DNA sequencing two representative samples.

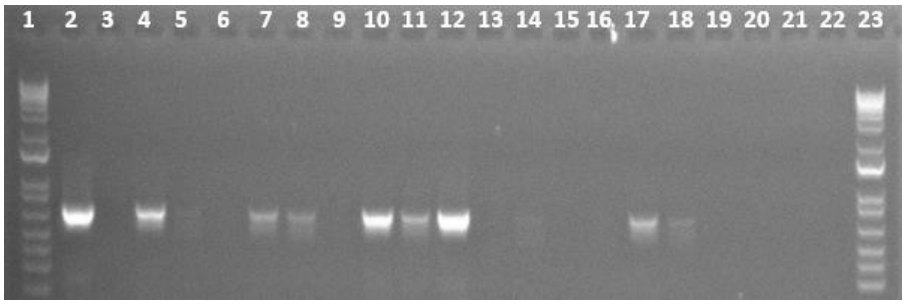

Figure 3.8: Reaction #35, Section 2.2.4. A product approximately 725 bp of a partial internal transcribed spacer region was amplified with primers DM3F and DM3R from samples of *Pisum sativum* plants with no visible signs of disease, from Experiment G. Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 23: 1 kb+ ladder. Lane 2: positive control. Lane 22: negative control. Lanes 3-12: asymptomatic plants from Experiment G that had sporangia placed on the apical tips (treatment two). Lanes 13-21: surviving plants from Experiment G that had sporangia placed on the underside of their youngest leaves (treatment one).

Experiment H

No plants expressed signs of *Pvp* infection. Of the six seeds which were initially soaked in RO water containing a piece of leaf tissue with *Pvp* sporulation then planted with another piece of sporulating leaf tissue, only one survived the 6-week growth period. Comparatively, five out of the six seeds which had no treatment prior to being sown along with a piece of leaf tissue with *Pvp* sporulation survived the growth period. A nested PCR analysis was performed on five out of the six surviving plants and positively identified the five seedlings as having asymptomatic *Pvp* infection. Two samples were sequenced which confirmed *Pvp* infection (Fig. 3.9).

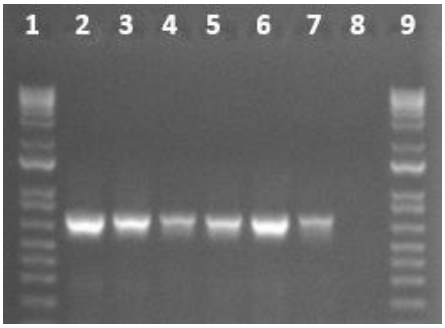

Figure 3.9: Reaction #35, Section 2.2.4. A product approximately 725 bp of a partial internal transcribed spacer region was amplified with primers DM3F and DM3R from samples of *Pisum sativum* plants with no visible signs of disease from Experiment H. Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 9: 1 kb+ ladder. Lane 2: positive control. Lane 8: negative control. Lanes 3-7: asymptomatic plants from Experiment G. Samples from lanes 3 and 6 were sequenced.

3.3.5 Soil Experiments

Of the 93 seeds which were planted, 68 germinated and survived the 6-week growth period; 27 out of 36 of plants grown in soil from Site One, 33 out of 48 from Site Two, and 9 out of 9 from Site Three. There was no notable difference in germination and survival between the different cultivars. Subsequent to PCR analysis, one 'Rondo' plant was indicated to have asymptomatic *Pvp* infection (Fig. 3.10; Fig. 3.11; Fig. 3.12). Two plants, one 'Utrillo' and one 'CS480-AF', experienced *Pvp* sporulation on a leaf and stem (Fig. 3.13A and B). *Peronospora viciae* f. sp. *pisi* infection was confirmed following sequencing. From the 'Utrillo' plant which was visibly infected, two samples were taken; one from the centre of a leaf with no visible signs of disease and the other was *Pvp* sporangia that was harvested directly from the leaf. Only the sporangia sample gave a positive band subsequent to PCR and gel visualisation (Fig. 3.14).

Figure 3.10: Reaction #2, Section 2.2.4. A product approximately 1100 bp of a partial internal transcribed spacer region was amplified using primers DC6 and ITS4, from samples of *Pisum sativum* plants with no visible signs of disease, grown in soil collected from a site with a history of hosting downy mildew infected pea plants. Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 31: 1 kb+ ladder. Lane 2: positive control. Lane 30: negative control. Lanes 3-29: plants grown in soil collected from Site One; lanes 3-10: 'Rondo', 11-22: 'CS480-AF', 23-29: 'Utrillo'.

Figure 3.11: Reaction #2, Section 2.2.4. A product approximately 1100 bp of a partial internal transcribed spacer region was amplified using primers DC6 and ITS4, from samples of *Pisum sativum* plants with no visible signs of disease, grown in soil collected from a site with a history of hosting downy mildew infected pea plants. Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 37: 1 kb+ ladder. Lane 2: positive control. Lane 36: negative control. Lanes 3-35: plants grown in soil collected from Site Two; lanes 3-12: 'Rondo', 13-27: 'CS480-AF', 28-35: 'Utrillo'.

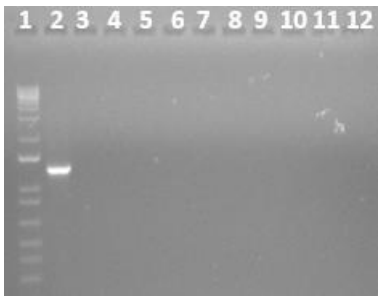

Figure 3.12: Reaction #2, Section 2.2.4. A product approximately 1100 bp of a partial internal transcribed spacer region was amplified using primers DC6 and ITS4, from samples of *Pisum sativum* plants with no visible signs of disease, grown in soil collected from a site with a history of hosting downy mildew infected pea plants. Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lane 1: 1 kb+ ladder. Lane 2: positive control. Lane 12: negative control. Lanes 3-11: plants grown in soil samples from Site 3; lanes 3-5: 'Rondo', lanes 6-8: 'CS480-AF', lanes 9-11: 'Utrillo'.

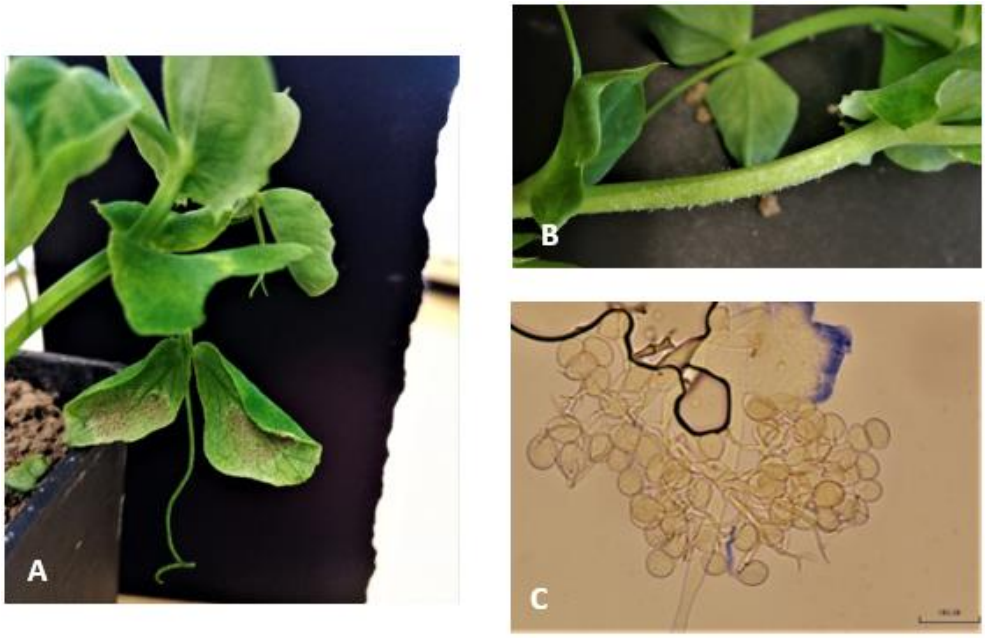

Figure 3.13: A. *Peronospora viciae* f. sp. *pisi* sporangia growing on the underside of leaves from a 'Utrillo' pea plant grown in soil collected from Site Two. **B.** *Peronospora viciae* f. sp. *pisi* sporangia growing from the stem of a 'CS480-AF' pea plant grown in soil collected from Site Two. **C.** Microscopic observation of a *Peronospora viciae* f. sp. *pisi* sporangiophore sampled from a 'CS480-AF' pea plant. Bar represents 100 µm.

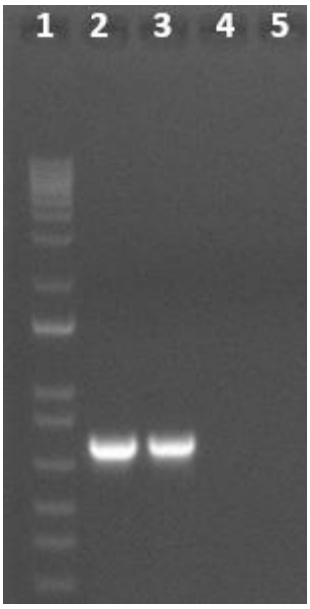

Figure 3.14: Reactions #13 and #13, Section 2.2.4. A product approximately 725 bp of a partial internal transcribed spacer region, amplified with primers DM3F and DM3R. Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lane 1: 1 kb+ ladder. Lane 2: positive control. Lane 5: negative control. Lane 3: DNA from a sporangia sample from a visibly infected pea plant. Lane 4: DNA extracted from a section of leaf sample with no visible infection, from the same pea plant.

3.4 Discussion

The overall aim of this body of research in this Chapter was to develop a reliable method for screening pea plants against *Pvp* under controlled environmental conditions. The use of fresh inoculum consisting of either sporangia or inoculum within soil, resulted in asymptomatic infection of pea plants. In contrast, the use of dehydrated, infected field pods as inoculum was not successful at inducing infection of the plants. Furthermore, although the use of fresh inoculum resulted in asymptomatic infection, limited disease expression was observed, with symptoms consisting of sporulation seen on only two plants across all the experiments in this study.

The use of fresh inoculum derived from soil or sporangia resulted in asymptomatic infection in twenty plants across the experiments in this study (Experiments F, G and H.) In the experiment where seeds were planted in soil collected from sites with a known history of *Pvp* (Experiment F), 15 of the 50 sown seeds, germinated and survived. This may potentially be due to other pathogens such as *Aphanomyces euteiches* or *Pythium* spp. present in the pea cropping soil reducing germination and subsequent emergence. Of these fifteen plants, seven were identified to be infected with *Pvp* by PCR. Stegmark (1991) also reported low frequencies of infected seedlings subsequent to planting seeds in naturally infested soil, however, plants in the reported study were noted as symptomatic with sporulation. Similar to the current study, Stegmark (1991) incubated plants at temperatures between 12-15˚C and at 100% relative humidity. The success of using soil as natural source of

inoculum to bulk inoculum and achieve *Pvp* infection was reported by Davidson et al. (2011). Comparatively, the 2011 study placed pea seeds on a tray potting mix and added a 2 cm layer of collected soil on top of the seeds. This slight difference in methodology between the two studies is unlikely to explain the difference in success in obtaining disease expression in the current study; variations between incubation conditions is most probable. Another possible reason for the lack of disease expression is the level of inoculum in the soil. Although Stegmark (1991) and Davidson et al. (2011) did not report on the quantity of inoculum in the soil in their research, it would have likely influenced their results. To determine the level of inoculum in the soil required for disease expression, a qPCR protocol could be developed to establish what the threshold is for infection development in seedlings. Once a threshold is known, then it could be used to test field soil to provide information to growers regarding potential risk of *Pvp* infection. Such thresholds have been developed for *Aphanomyces euteiches* and have proven to be of great value to growers (Chan & Close 1987).

To maintain a biotroph like *Pvp* in a laboratory, it is recommended that healthy hosts are continuously inoculated with fresh sporangia (Satou & Fukumoto 1993; Danielsen & Ames 2000; Gill & Davidson 2005). Experiment G used fresh sporangia to directly inoculate pea plants by placing spores on the abaxial of the youngest set of leaves (treatment one) or on the apical tip (treatment two). Nineteen of the twenty plants initially planted survived the 6-week growth period. PCR results indicated that treatment two was more successful in achieving *Pvp* infection; 70% plants were positive for asymptomatic infection compared with 33% asymptomatic infection with treatment one. As the plants were inoculated by placing sporangia directly on the leaves or apical tip and DNA extraction was conducted on a section of leaf material it cannot be entirely excluded that remnants of sporangia were being detected in PCR instead of true infection. However, this is unlikely as plants were inoculated and sampled from the youngest set of leaves. For remnant *Pvp* to be detected from the applied sample, it would mean the plant did not produce any new leaves over the growth period. As important as it is to consider, sporangia remnants are not likely to have influenced the PCR results. Future work involving *in planta* DNA extraction subsequent to direct inoculation should consider treatments to exclude relic DNA of non-target propagules, or surface sterilisation of the leaf tissue to remove any potential contamination from the outer surface of the plant. In contrast to this study, Mence & Pegg (1971) obtained symptomatic, systemic infection in 90% of their seedlings by pipetting a sporangial suspension on the apical tip. Future studies could compare direct placement of sporangia and the use of a spore suspension to assess if inoculum type effects infection success.

Experiment H involved placing leaf material with dense sporangial growth on top of the seed before being covered in potting mix. Half of the seeds were soaked in a spore suspension prior to sowing, however, only one seed germinated and survived the growth period. In comparison, all seeds, except

one, germinated after no soaking. PCR indicated that all plants which survived the growth period were experiencing asymptomatic infection. The reason for this contrast in seed survival is unknown and can only be speculated. The Processors and Growers Research Organisation (PGRO) have used a variation of Experiment H to screen pea lines against *Pvp* isolates (PGRO 2016). In their study, seeds were germinated on agar for 3-5 days then sown with a piece of vigorously sporulating *Pvp* placed on the hypocotyl and young root. The researchers concluded that this method of inoculation provided reliable and consistent results which is reflected in the PCR gel visualisation from this experiment. Hickey & Coffey (1977) achieved systemic infection by germinating seeds for 3 days on damp filter paper then soaking seeds in a spore suspension for 30 min prior to sowing. In comparison to the 1977 study, seeds in Experiment H were soaked for considerably longer (24 h), perhaps influencing the likelihood of survival. As successive replicates of Experiment H were not conducted, conclusions cannot be drawn on the efficacy of soaking seeds in water with sporulating leaf material prior to sowing.

The lack of disease symptoms across Experiments F, G and H could be due to nonconducive incubator conditions. Latent infection is described as pathogen infection within a live host, without causing visual disease symptoms until appropriate conditions trigger sporulation (Ngah et al. 2018). *Peronospora sparsa* was shown by Herath Mudiyanselage (2015) to grow asymptomatically in boysenberry plants, only sporulating when conditions were conducive; approximately 15˚C and 100% relative humidity. Latency is commonly observed in pathogen-host interactions such as wheat powdery mildew (*Blumaeria graminis* f. sp. *tritici*), *Botryis cinerea*, and basil downy mildew (*Ocimum basilisum*) (Zeng et al. 2010; Farahani et al. 2012; Ngah et al. 2018). Often field infections of *Pvp* are symptomless until sporulation occurs, indicating a latency period subsequent to the host becoming infected and prior to suitable environmental conditions (Clark & Spencer-Phillips 2011). In the current study, the level of leaf wetness was not considered and therefore it is unknown if leaf wetness conditions influenced the lack of sporulation. It was unclear whether sporulation of *P. sparsa* under humid conditions is enhanced by leaf moisture or a relative humidity close to saturation. Maximum sporulation of a related downy mildew pathogen *P. trifoliorum,* on alfalfa was at a relative humidity below 100%. Also, free leaf moisture has been known to inhibit sporulation for *P. tabacina* on tobacco and *Pseudoperonospora cubensis* on cumcumber (Fried et al. 1977; cited in Roten et al. 1978). The confirmation of asymptomatic infection with PCR as well as the success of experiment variations within the literature suggest that inoculation methods tested in Experiments F, G and H can be used to achieve infection and disease expression. However, further experiments would need to be conducted to identify the incubator conditions required to break latency and cause disease expression.

Disease expression occurred when the temperature was maintained at 15˚C throughout a 16 h photoperiod. This result was not repeatable therefore it cannot be concluded that those conditions are optimal for disease expression. Temperatures that *Pvp* has been successfully incubated at varies between studies, however the conditions tested in this study either fall within or replicate suggested parameters (Pegg & Mence 1970; Liu et al. 2013; Alexis Plouy, Crites Seeds, Personal communication, 2019). Regardless of temperature variations between previously published literature, the need for a high humidity remains absolute (Pegg & Mence 1971; Stegmark 1994; Danielsen & Ames 2000; Liu et al. 2013). A high RH was managed by incubating the plants within plastic bags, however the humidity was not measured within the bags. Additional research would have to be conducted to assess all laboratory conditions relating to *Pvp* expression for more accurate conclusions to be drawn.

Due to the success of getting infection in Experiment F, another experiment using soil collected from three pea cropping sites was initiated. PCR analysis identified one plant as being infected with *Pvp* despite not expressing signs of disease. In addition, two plants expressed signs of disease ('Utrillo' and 'CS480-AF') although the PCR results did not indicate infection. This inconsistency was investigated further by taking two separate samples from the 'Utrillo' plant; one of pure sporangia, the other a section from an adjacent non-symptomatic leaf. The sporangial sample was positive for *Pvp,* but the non-symptomatic leaf sample was not. There are few potential reasons for this discrepancy, including that that the concentration of *Pvp* DNA was minimal in comparison to the plant material or *Pvp* may not be present in the section of plant sampled (Hickey & Coffey 1977). Results obtained in Chapter 2 (Section 2.3.1) indicated that approximately 111 sporangia or mycelial fragments containing nuclei are required in a plant/pathogen mixed DNA sample to be detected in PCR. As this is not a particularly sensitive PCR reaction, intercellular mycelia would need to be abundant within the sample to be detected. Based on inconsistency between PCR and fluorescent microscopy results, *P. sparsa* infection of boysenberry was also indicated to be discontinuous throughout the plant with Herath Mudiyanselage (2015) suggesting replicate plant tissue should be assessed by PCR to overcome variability in detection. Testing subsamples from different sections of the pea plant and analysing these separately is suggested in further research to enable a more accurate determination of the infection status within an entire plant. Whilst this experiment did not yield results for which reliable conclusions can be drawn from, it has provided valuable information regarding future molecular analyses on asymptomatic plants.

Experiments in which dehydrated, infected pea pods were used as inoculum resulted in no infection. A previous study by Ryan (1971) used ground-up, dehydrated, infected pods to infect pea seeds, obtaining a 90% systemic infection rate. Their method was most comparable to Experiment E however, rather than placing the material adjacent to the seed, Ryan (1971) incorporated it into the compost in which the seeds were sown. In the current study (Experiment E) 0.2 g of ground-up,

infected, dehydrated material was added to the soil whilst the 1971 study used a larger amount of 2 g. The difference in quantities used was unlikely to influence the results yielded from this study as Ryan (1971) explained that oospores were confirmed to be present within the pods. Oospores are the survival structures of the pathogen and are typically produced when environmental conditions are no longer favourable for sporangia development. At the time of collection in the current study, pods were infected with *Pvp* however, as conditions were conducive for sporangia production and potential hosts were readily available the pathogen may have not produced the resting oospore propagules in the pod tissue. It is impossible to conclude whether inoculation would have been achieved if sample collection occurred later in the season when oospore production would have likely been initiated by environmental triggers, therefore, more experiments would need to be conducted to further investigate the possible use of dried pods as a source of inoculum.

As an obligate biotroph, *Pvp* can be extremely difficult to manage in a laboratory as it can only sustain itself on a living host (Danielsen & Ames 2000). In this study, an *in vitro* method to enable the maintenance of *Pvp* cultures or spore production on infected detached lea leaves incubated on agar or moistened filter paper were unsuccessful in promoting further sporulation. A similar method has been suggested as a means for short-term inoculum bulking by Danielsen & Ames (2000). Similar to the method used in the current study, the authors suggest placing leaves with recent signs of sporulation onto filter paper or water agar to increase sporangia production. However, in contrast to the present study where sections of the leaf with dense sporulation were used, Danielsen & Ames (2000) advised using leaves with recent, or limited sporulation as the pathogen could continue to develop until sporangia encompass the leaf surface area. Leaves with dense sporangial growth used in the current study are likely to not provide the nutrients required for pathogen maintenance and development. Detached leaves maintained on water agar have reportedly been successful; Kitz (2008) maintained an isolate of *P. farinosa* on detached quinoa leaves on water agar whilst incubated at 20˚C with lights on and 16˚C with the lights off, in a 12 h photoperiod. Relative humidity was not reported. Despite differences in temperatures between in the 2008 study and the current study, sporangial development and production would not likely to have been observed due to the prior density of sporulation. Hearth Mudiyanselage (2015) also reported *P. sparsa* sporulation from symptomatic boysenberry leaves with limited or no initial sporulation to occur after 14 days incubation in plastic containers when maintained at 90-100% RH or on water agar. Future work using leaves with no or limited sporulation should be carried out to determine whether this method could be used to increase spore production for use in subsequent infection studies.

In experiment B, which tested whether *Pvp* could be maintained on detached pea leaves, disease development was not observed; probably as previously discussed due to dehydrated infected pod material being used as inoculum. The use of detached leaves is known method of bulking and

maintaining downy mildew of quinoa (*Peronospora farinosa* f. sp. *chenopodii*). Danielsen & Ames (2000) suggest preparing a spore suspension with fresh sporangia, pipetting the suspension onto water agar then placing healthy leaves over the agar; which is reflective of a biotrophs need for living host tissue for its survival and development. Similarly, Herath Mudiyanselage (2015) reported that inoculation of detached boysenberry leaves incubated on 1.5% water agar with *P. sparsa* sporangia, obtained by washing actively sporulating leaves sourced from infected potted boysenberry plants, resulted in infection and subsequent sporangial production. Maximum sporulation was also observed to occur when the inoculated leaves were incubated at 20˚C compared with 10˚C and 15˚C. Additionally, sporulation was higher on inoculated young leaves compared with older leaves. Further experiments using fresh sporangial inoculum and testing different temperature and relative humidity incubator conditions are required to determine whether this could be a successful method of maintaining *Pvp* in the absence of a host plant.

It was hypothesized that soaking or slicing the seed prior to sowing would increase the likelihood of achieving *Pvp* infection using ground-up, dehydrated, infected pods as a source of inoculum. After 72 h, only 6 out of 120 seeds had germinated; all 6 from the soaking treatment. Soaking seeds in water prior to sowing is considered an 'old gardeners' trick' which shortens the germination period by accelerating the seeds metabolic reactions (Silva et al. 2017). No seeds which had their seed coats sliced, or had no treatment applied had germinated after the 72 h period. After 4-weeks, no plants had developed any visible signs of disease and no asymptomatic *Pvp* infection was detected in the plants which underwent PCR. However, as *Pvp* infection was not achieved in the previously described experiments using dehydrated infected pea pods, no conclusions can be drawn on the efficacy of soaking or slicing seeds prior to inoculation. Further experiments using an alternative source of inoculum, such as fresh sporangia, would need to be conducted to truly assess the effects seed treatments have on the likelihood of achieving *Pvp* infection.

It has been speculated that *Peronospora* spp. isolates can become adapted to a certain cultivar, becoming more biotrophic and less damaging by reducing its demand on the host (Clark & Spencer-Phillips 2011). Site Two, from which the fresh sporangia for experiments G and H were collected, most recently hosted a crop of 'Utrillo' plants. 'Utrillo' seeds were one of the few cultivars included in the experiments with fresh sporangia, thus this pathogen-host interaction could have been reflected in the data, but the conclusion can only be speculated due to lack of repetition. The lack of disease expression from 'CS480-AF' and 'Rondo', whilst genetically being comparatively susceptible to *Pvp*, would not be explained by this phenomenon therefore it is unlikely that pathogen/host relationship was becoming more commensal in this instance. This concept has been previously been observed between downy mildew (*Peronospora hyoscyami*) and tobacco (*Nictotiana tabacum*), as a reduction in sporulation was detected subsequent to successive transfers of sporangia to new host plants

however it was not investigated further (Johnson 1988). In the current study, it is more likely that the lack of disease expression was more likely due to the incubator conditions not being conducive for sporulation.

3.5 Limitations and Further Research

Similarly to Chapter 2, the greatest limitations in this study was the inoculum collection and storage techniques. Initial sample collection occurred during height of the pea growing season, where *Pvp* was occurring sporadically in fields across sampled areas; undeniably the ideal time to be collecting samples. However, infected pods were collected as opposed to sporangia. Modes of infection are limited when using infected pods as an inoculum source as only oospores are produced in the pod (Stegmark 1994). Oospores are typically formed when environmental conditions are no longer favourable for disease development, or when a nutrient source becomes depleted (Frinking et al. 1985). At the time of collection pods were not heavily infected therefore the likelihood of oospore presence, and thus the likelihood of achieving infection from these samples was diminished.

When experiments using the dried inoculum were conclusively unsuccessful and the decision was made to locate fresh *Pvp* sporangia, the only pea plants still available were volunteer plants growing amongst the grass at Site Two. Of the small number of volunteer plants, only a few were observed to have sporulating downy mildew. Consequently, the number of experiments and replicates set up using fresh sporangia were limited. Any future studies should therefore aim to harvest pea plants with abundant sporangial production during the cropping season for use in inoculation studies.

Future studies could focus on replicating environmental conditions required for disease expression. A study in 1970 by Pegg & Mence investigated the environmental conditions, such as temperature, relative humidity (RH) and leaf wetness, required for *P. viciae* for various stages of its development and reported optimal conditions to be in the range of 12-20˚C, between 95-100% RH and a minimum of 1 h of leaf wetness. Despite the incubator being set up to best replicate these conditions in experiments across this study, sporulation was severely limited, thus the laboratory conditions required for sporulation in the current study were inconclusive. Unlike the 1980 study, leaf wetness was not measured in the current study therefore it is unknown to what effect this had on the observed results. Latent infection was achieved vis previously published inoculation methods, with the use of sporangial inoculum and an inoculum source within the soil. This suggests the methods tested in this Chapter and published in the wider literature can successfully cause downy mildew infection in pea plants and it is simply a matter of identifying the conditions that will trigger disease expression.

3.6 Conclusion

This study aimed to identify a successful and reliable method for screening pea plants against *Pvp* in a controlled setting. Latent infection was achieved by planting seeds in soil collected from a site with a known history of *Pvp* infection, placing inoculum alongside the seed at the time of planting, and exposing young plants to fresh *Pvp* sporangia. The two latter methods were tested using dehydrated inoculum, but infection was not detected. Due to variations between temperate and humidity conditions it is impossible to say definitively whether infection would have occurred for experiments A-E if fresh material was used instead of dried. The variation observed between visual inspection and molecular analysis of the plants from the soil experiments means we must be tentative with conclusions drawn from this study, however it is essential that we acknowledge that variations between visual inspection and PCR analysis exist for future research.

Chapter 4

General Discussion and Conclusion

This research aimed to characterise the genetic diversity of pea downy mildew in New Zealand's pea growing regions, develop a method to detect asymptomatic and symptomatic infection caused by the pathogen in *Pisum sativum*, and to develop a screening bioassay for pea cultivars inoculated with downy mildew. This work has indicated that the causal organism of pea downy mildew is *Peronospora viciae* f. sp. *pisi* with genetically different isolates being present in the populations infecting peas in New Zealand. Preliminary bioassays have been developed that have the potential to enable the future rigorous screenings of pea cultivars for susceptibility to New Zealand *Pvp* genotypes.

Peronospora viciae f. sp. *pisi* infected pods and miscellaneous leaves were collected from seven *Pisum sativum* growing sites throughout the North and South Islands of New Zealand in the 2018-2019 growing season. From each site, 12 plants were sampled and from each 5 pods were collected. At the time of collection, pods exhibiting 60% visible *Pvp* infection were collected from all South Island sites. North Island sites were sampled by another arable researcher, where pod *Pvp* infection was variable; many pods were not visibly infected with the pathogen whilst others exhibited moderate infection. Throughout this research, four different sample types were used (S1-4). S1 samples derived from the 382 dehydrated field pods, from which two samples were taken; one from a visibly infected section of the plant and another from an adjacent section which did not appear visibly infected with *Pvp*. All S1 samples contained dehydrated pod material and possibly other pathogens. Fifty-two pods which had the most visible *Pvp* mycelia were selected to be included as a subset of the dehydrated field pods; these were referred to as S2 samples. Mycelia only from *Pvp* was scraped from the pea pod sample. Fresh sporangia obtained from miscellaneous field leaves or symptomatic plants from the laboratory growth chamber were referred to as S3 samples. As the sporangia was lifted from the plant, all S3 samples were pure *Pvp*. S4 samples were derived from sections of the centre of the youngest leaf of asymptomatic plants, from the laboratory growth chamber. All S4 samples consisted largely of fresh plant material. The origin of each sample type influenced the efficacy of the molecular techniques used in this research.

To investigate the most effective method of amplifying *Pvp* with molecular means within different sample types (Chapter 2), the internal transcribed spacer (ITS) region and *cox* loci were amplified by polymerase chain reaction (PCR) using varying primers, DNA quantities and primer concentrations. Presumably, due to the inhibitory compounds and degraded DNA, *Pvp* was unable to be amplified in any reactions which used S1 samples (Abu Almakarem et al. 2012; Schrader et al. 2012). *Peronospora*

viciae f. sp. *pisi* was able to be amplified in samples S2, S3 and S4, each with different reactions. The partial ITS1, complete 5.8S and partial ITS2 (approximately 508 bp) was amplified in S2 samples. Products were then sequenced, compared to one another then BLASTed against *P. viciae* sequences in GenBank. A comparison of the sample sequences against each other indicated no genetic variation. Most notably, a BLAST of the sample sequences against a partial ITS sequence from a *P. viciae* isolate deriving from *Pi. sativum* (GenBank accession number: AY225471) indicated 99% identity with 2 bp differences, identifying *Pvp* as the casual organism of downy mildew in the samples from *Pi. sativum* pods in the current study. When compared to partial 18S, complete ITS1 and 5.8S region of a known *Pvp* isolate (GenBank accession number: DQ078696), no similarities were identified. Further confirmation is required of the entire ITS region and *Cox* loci to give a non-ambiguous identification. Upon examining *P. viciae* sample sequences from Liu et al. (2013) it was noted that their *Pvp* isolates were compared to sequences of *P. viciae* isolates deriving from common vetch (*Vicia austifolia*) (GenBank accession numbers: EF174953 and AY198230), highlighting the similarities between closely related *Peronospora* spp. and the necessity of using the *Cox* loci as a secondary barcode for species differentiation. Additionally, an RFLP analysis of the partial ITS region PCR product did not identify any genetic variation between the S2 samples, validating the results of sequence comparison.

Despite this, when a larger representation of the genomes of 24 representative S2 samples were examined for variations using RAPD PCR, varying polymorphic banding patterns were produced within and between sites, indicating genetic dissimilarities between isolates. Most variation was observed between geographically isolates sites; North Island sites were located across a broader geographic scale and had approximately 15 nucleotide substitutions, whilst South Island sites were all in close proximity and had approximately 5 nucleotide substitutions between them. As highlighted by García-Blázquez et al. (2007) and Liu et al. (2013), *P. viciae* on pea plants is very similar to *P. viciae* on common vetch, therefore the variation observed within the North Island sites may derive from *P. viciae* which has spread from nearby vetch plants. Vetch was not observed in any of the sampled South Islands sites, however due to differences between sample collectors between the North and South Islands it is unknown whether vetch was present within or growing nearby any of the North Island sites. The potential for a green bridge for *Pvp* and *P. viciae* between peas and other species could be investigated similarly to the method outlined by Sanders & Korsten (2002), whereby selected hosts are exposed to numerous known isolates at once and observed for signs of disease expression. If future research proves a green bridge between *Pi. sativum* and vetch plants, or closely related *Fabaceae* spp., for *Pvp,* then growers need to consider an integrated pest management strategy which includes the removal of both volunteer pea plants and Fabaceae plants in the off-

season to reduce the likelihood of *Pvp* inoculum being transferred to a new crop or cropping location.

Results obtained from the research undertaken in Chapter 2 has highlighted many opportunities for further research on the genetic composition of *Pvp* populations in New Zealand. Similarly, to the study of Milla et al. (2005), dominant alleles could be identified and utilized to develop correlating sequence characterised amplified region (SCAR) markers and primers that may aid in identifying resistance markers within the *Pvp* genome. Alternatively, the use of microsatellite markers could be used for analysing the population structure of *Pvp*. Whilst microsatellite loci have not been identified specifically for *Pvp*, microsatellite markers have been identified in other *Peronospora* spp. (Trigiano et al. 2011; Feng et al. 2018). Further, Perumal et al. (2008) identified and developed microsatellite primer sets from *Peronosclerospora sorghi* that have been successfully applied to other downy mildew species. Thus, these could be utilised in future research to streamline the process of selecting for resistance and alleviating growers of pressures associated with managing pea downy mildew.

A bioassay was developed to screen *Pi. sativum* cultivars against *Pvp* (Chapter 3). Visible symptoms were apparent with the use of fresh sporangia or fresh soil collected from a site with a known history of *Pvp* infected plants. Asymptomatic plants were observed using the previously described fresh inoculum and were confirmed to be infected with *Pvp* after a nested PCR was performed. *Peronospora viciae* f. sp. *pisi* was not detected in any of the sampled plants that were inoculated with dehydrated, infected field pods, thus it is concluded that the use of fresh sporangia or inoculum within the soil is imperative for any future screenings of *Pi. sativum* cultivars. Overall, only two plants expressed signs of disease therefore the most conducive incubator conditions required for disease expression cannot be ascertained. To investigate conducive growth chamber conditions for disease expression, one of the three successful inoculation methods identified in Chapter 3 could be utilised to cause asymptomatic infection; confirmed using the most suitable PCR protocol identified in Chapter 2. Plants confirmed to be asymptomatic could then be exposed to different growth chamber conditions to determine the most favourable conditions for expression within a controlled laboratory environment. Reflective of the results in this study, a conducive environment may not always be reproducible, thus phenotypic disease expression cannot always be directly related to genetic variation within *Pvp*. Without further research it will remain unknown whether no disease symptoms were expressed due to non-conducive conditions or natural tolerance within *Pi. sativum*. Similarly to Herath Mudiyanselage (2015), vaseline coated slides as spore traps could be set up within, and on the boundaries of cropping areas to determine the conditions under which spore production and release occurs. Data collected from the spore traps could also be used to track natural sporangia movement within and between sites throughout a season, potentially elucidating nearby sources of inoculum influencing the population structure of downy mildew on peas in a given area.

The rapid bioassay screenings have highlighted difficulties when screening pea cultivars for potential resistance to *Pvp*. To overcome this, the next step for *Pvp* research should focus on developing a quantitative real-time PCR (qPCR) protocol for examining *Pvp in planta* and to investigate any correlation between disease expression and plant genetics. Quantitative PCR can be used to quantify the concentration of *Pvp* in a given section of the plant. By taking subsections from different locations within the plant, the location where the highest concentration of *Pvp* is likely to occur can be detected, thus future studies using conventional or nested PCR can sample from the most suitable location. Even during the early stages of infection, qPCR may aid in observing differential growth supported by tolerant and susceptible cultivars (Shao & Tian 2018). Furthermore, during screening bioassays, qPCR could be utilized to discriminate between tolerant and susceptible pea germplasms, even in the absence of disease expression, by quantifying the pathogen in both asymptomatic and symptomatic infections. Additionally, a threshold for disease expression could be identified, allowing researchers to predict *Pvp* sporulation contingent with its concentration *in planta* as well as the impact *Pvp* may have on a crop based on the level of inoculum in the soil. Understanding such thresholds would allow researchers to determine the influence different crop and timing rotations have on the level of *Pvp* inoculum in the soil resulting in infection, within different soil types and under variable environmental conditions. Thus, allowing growers to test their soil to determine potential risks associated with sowing peas, with regard to *Pvp*, and aid in predicting the intensity of disease, given the right environmental conditions, before the crop is impacted economically.

In summary, bioassays inoculating pea plants with *Pvp* resulted in asymptomatic infection and conventional PCR failed to detect *Pvp* in the infected, dehydrated field pods (S1), thus a method to detect *Pvp* at low concentrations *in planta* was required. A nested PCR protocol determined the presence or absence of *Pvp* in asymptomatic, live plants, however, a discontinuous growth pattern was indicated, potentially impacting the designs of future bioassays and molecular techniques. Nested PCR was not able to conclusively elucidate *Pvp* within S1 samples, therefore a representative subset of pods were selected and pure mycelial samples were obtained. *Peronospora viciae* f. sp. *pisi* was able to be analysed when pure or fresh DNA samples were used. The results of the genetic diversity assessment is the first report to indicate that there are genotypically distinct isolates within New Zealand's *Pvp* population, however, more work is required to determine whether this is reflected in different pathotypes present in New Zealand.

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Appendix A

Supplementary Material for Chapter 2

A.1 PCR primers and their corresponding sequences

Primer	Sequence
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC
DC6	GAGGGACTTTTGGGTAATCA
DM3F	GCCGAGTGAGCCCTATCATGGTGAGTGTT
DM3R	TATGCTTAAGTTCAGCGGGTAATCTTGCCT
Oom*Cox*1-levup	TCAWCWMGATGGCTTTTTTCAAC
Oom*Cox*1-levlo	CYTCHGGRTGWCCRAAAAACCAAA
*Cox*2-F	GGCAAATGGGTTTTCAAGATCC
*Cox*2-RC4	TGATTWAYNCCACAAATTTCRCTACATTG

A.2 RAPD primers and their corresponding sequences

Primer	Sequence
DMp4	GAAGGGTCCC
DMp6	ACGAATGGAG
DMp50	GCGCTCTTAA
DMP51	ACGCCTACCC
DMp67	CTACCCGGCT
DMp73	AACCGCTCTC

A.3 Trimmed partial ITS1, complete 5.8S and partial ITS2 reverse complement sequence of 28 samples of *Pvp* used for genetic analyses

Trimmed partial IST1, complete 5.8S and partial ITS2 reverse complement sequence, reflective of *Pvp* samples; 1.3, 1.5, 1.7, 1.9, 1.10, 1.12, 2.7, 2.8, 2.12, 3.1, 3.10, 3.11, 4.1, 4.2, 4.4, 4.6, 4.7, 4.11, 5.3, 5.8, 5.10, 5.11, 6.2, 6.3, 6.5, 7.5, 7.8.

CAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATTGCAGG
ATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACAACCGGGGACCATCCCGGGAGTATGCCTGTATCAG
TGTCCGTACATCAAACTTGGTTTTCTTCTTTCCGTGTAGTCGGTGGAAGATATGCCAGATGTGAAGTGTCTTTCG
ACTGGTTTTCGAATCGGTTGTGAGTCCTTTGAAATGTATAGAACTGTACTTCTCTTTGCTCGAAAAGCGTGGCA
TTGTTGGTTGTGGAGGCTGTTCGTGTGACCAGTCGGCGATCGGTTTGTCTGCTGTAGCATTAATGGAGGAGTG
TTCGATTCGCGGTATGGTTGGCTTCGGCTAAACAGACGCTTATTGGGCTCTCTTTCTGCTGTGGCGGTATGAAC
TGGTGAACCGTAGTCATGTGTGACTTGGCTTTTGAATTGGCTTTGCTGGTGCGAA

A.4 Presence and absence matrix of polymorphic bands produced by RAPD primers DMp4 and DMp51 for 22 *Pvp* samples

Table A.4.1 Presence and absence matrix of polymorphic bands produced by RAPD primer DMp4 for 22 *Peronospora viciae* f. sp *pisi* samples collected during the 2018-2019 pea growing season from 7 different sites across New Zealand.

bp	1.5	1.7	1.8	1.11	2.2	2.5	2.7	2.9	3.1	3.10	3.11	4.3	4.4	4.7	5.3	5.7	5.10	6.1	6.2	6.3	6.5	7.8
2500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2000	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
1750	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	0	0	1
1650	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
850	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
700	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
650	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
550	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1
300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table A.4.2 Presence and absence matrix of polymorphic bands produced by RAPD primer DMp51 for 22 *Peronospora viciae* f. sp. *pisi* samples collected during the 2018-2019 pea growing season from 7 different sites across New Zealand.

bp	1.5	1.7	1.8	1.11	2.2	2.5	2.7	2.9	3.1	3.10	3.11	4.3	4.4	4.7	5.3	5.7	5.10	6.1	6.2	6.3	6.5	7.8
2500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1
2000	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1750	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
1650	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	1	1	1	0	0	0	1
1100	1	1	0	1	0	0	1	1	1	0	0	1	1	0	1	1	1	1	1	1	1	1
850	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1
700	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
650	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
550	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
300	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0

A.5 Representative gel images for PCR reactions trialled for the amplification of the ITS region of *Pvp* in different sample types using primers DC6 and ITS4

Figure A.5.1: Reaction #5: Products approximately 1500 bp of a partial internal transcribed spacer region amplified with primers DC6 and ITS4 from dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: Positive control. Lane 13: Negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

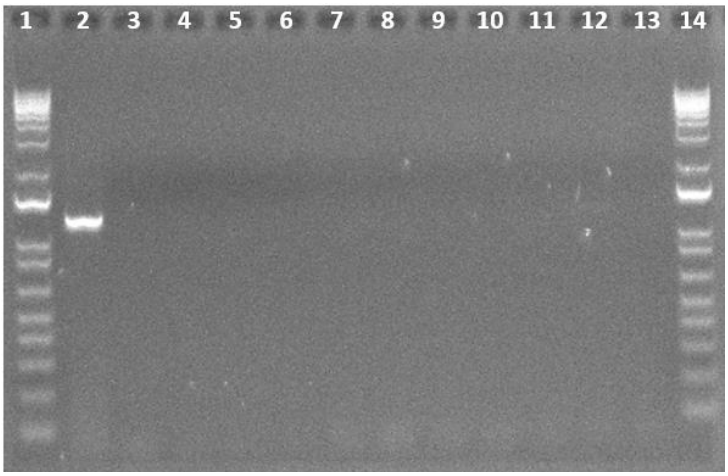

Figure A.5.2: Reaction #6: Products approximately 1500 bp of a partial internal transcribed spacer region amplified with primers DC6 and ITS4 from dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: Positive control. Lane 13: Negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

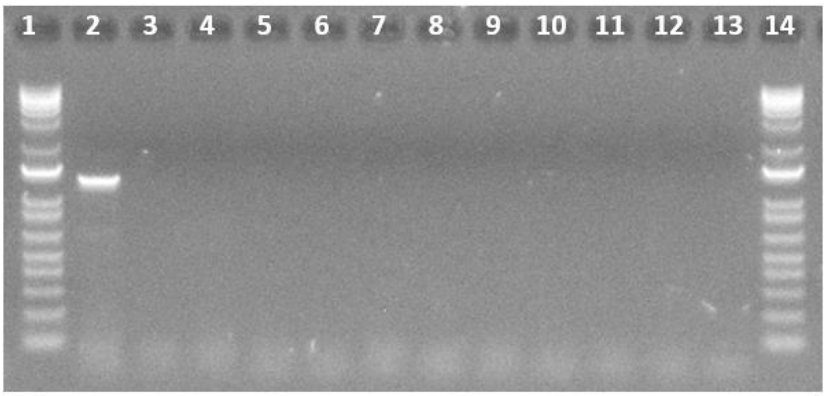

Figure A.5.3: Reaction #7: Products approximately 1500 bp of a partial internal transcribed spacer region amplified with primers DC6 and ITS4 from dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: Positive control. Lane 13: Negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

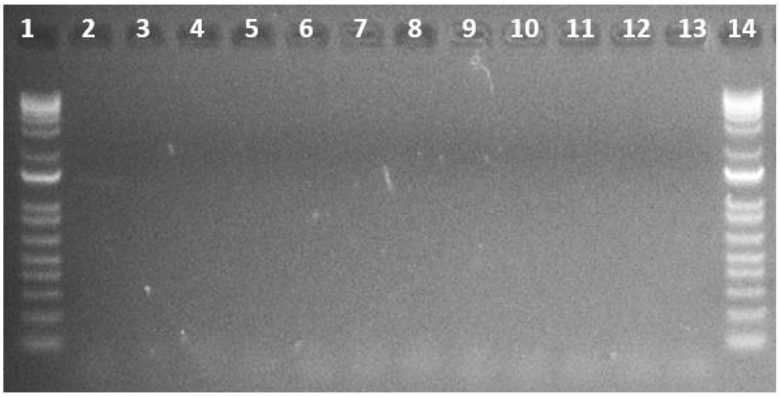

Figure A.5.4: Reaction #8: Products approximately 1500 bp of a partial internal transcribed spacer region amplified with primers DC6 and ITS4 from dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: Positive control. Lane 13: Negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

A.6 Representative gel images for PCR reactions trialled for the amplification of the partial ITS1, complete 5.8S and partial ITS2 region of *Pvp* in different sample types using primers DM3F and DM3R

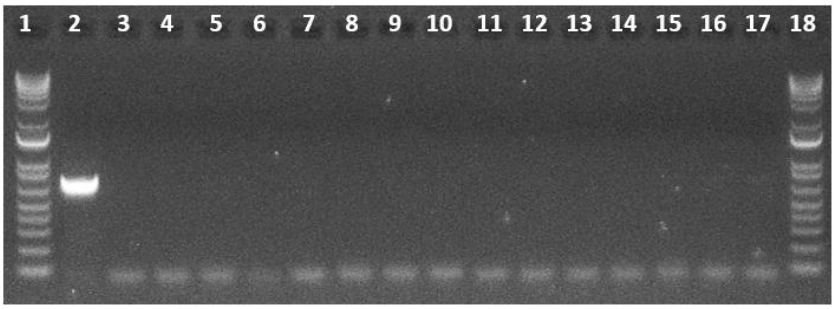

Figure A.6.1: Reaction #11: Products approximately 725 bp of a partial internal transcribed spacer region amplified with primers DM3F and DM3R from asymptomatic plants (S4 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 18: 1 kb+ ladder. Lane 2: Positive control. Lane 17: Negative control. Lanes 3-16: Asymptomatic 'Prelado' plants from Section 3.2.5.

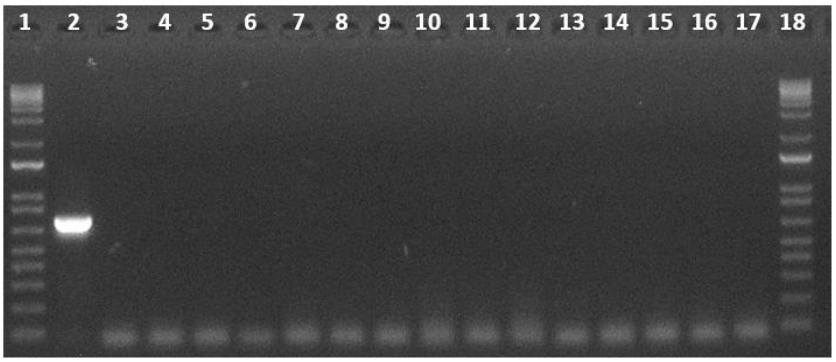

Figure A.6.2: Reaction #12: Products approximately 725 bp of a partial internal transcribed spacer region amplified with primers DM3F and DM3R from asymptomatic plants (S4 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 18: 1 kb+ ladder. Lane 2: Positive control. Lane 17: Negative control. Lanes 3-16: Asymptomatic 'Prelado' plants from Section 3.2.5.

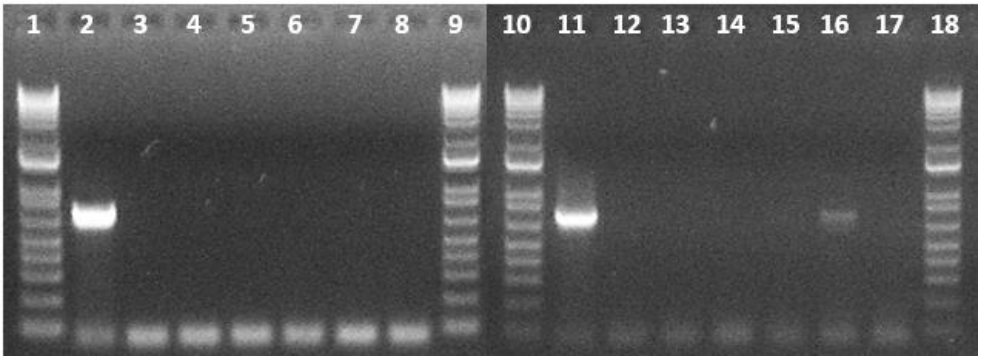

Figure A.6.3: Reaction #14: Products approximately 725 bp of a partial internal transcribed spacer region amplified with primers DM3F and DM3R from dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1, 9, 10 and 18: 1 kb+ ladder. Lanes 2 and 11: Positive control. Lanes 8 and 17: Negative control. Lanes 3-16: Dehydrated field pods from Site 1, Plant 1.

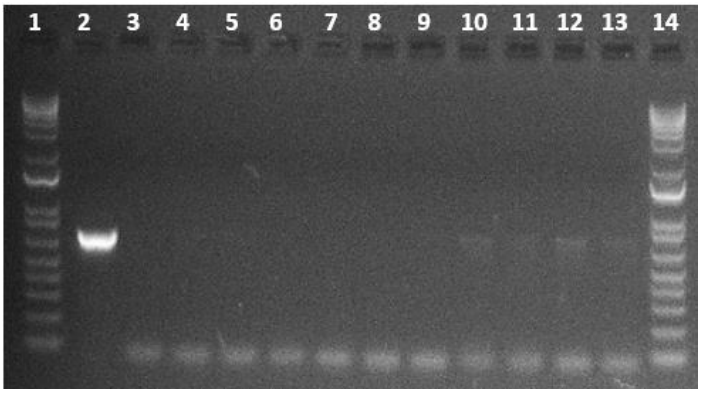

Figure A.6.4: Reaction #15: Products approximately 725 bp of a partial internal transcribed spacer region amplified with primers DM3F and DM3R from dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: Positive control. Lane 13: Negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

A.7 Representative gel images for two-step nested PCR reactions for the amplification of the partial ITS1, complete 5.8S and partial ITS2 region of *Pvp* in different sample types using primers DC6/ITS4 and DM3F/DM3R

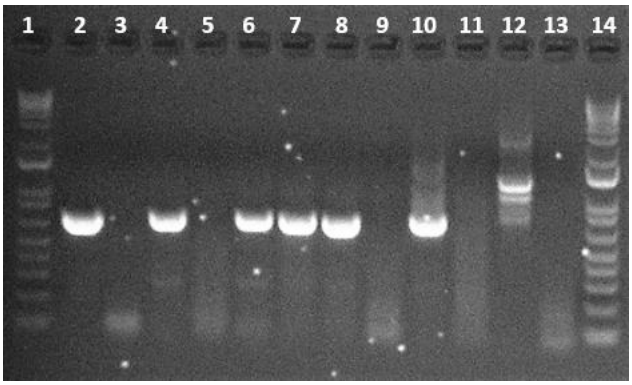

Figure A.7.1: Reaction #19: A product approximately 725 bp of a partial internal transcribed spacer region was amplified with a nested PCR using primers DM3F and DM3R, subsequent to an initial reaction using primers DC6 and ITS4, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: positive control. Lane 13: negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

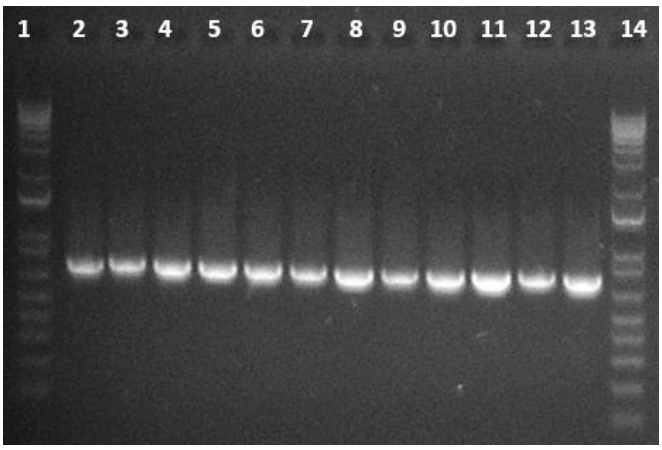

Figure A.7.2: Reaction #27: A product approximately 725 bp of a partial internal transcribed spacer region was amplified with a nested PCR using primers DM3F and DM3R, subsequent to an initial reaction using primers DC6 and ITS4, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: positive control. Lane 13: negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

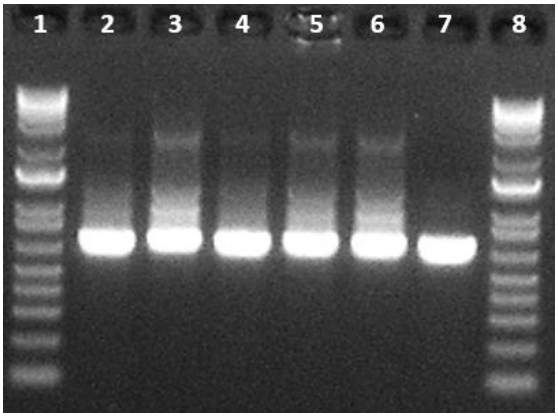

Figure A.7.3: Reaction #29: A product approximately 725 bp of a partial internal transcribed spacer region was amplified with a nested PCR using primers DM3F and DM3R, subsequent to an initial reaction using primers DC6 and ITS4, from selected samples of dehydrated field pods (S1 samples) which had the heaviest smearing in prior reactions. Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 8: 1 kb+ ladder. Lane 2: positive control. Lane 17: negative control. Lanes 3-6: Dehydrated field pods from Site 1, Plant 1.

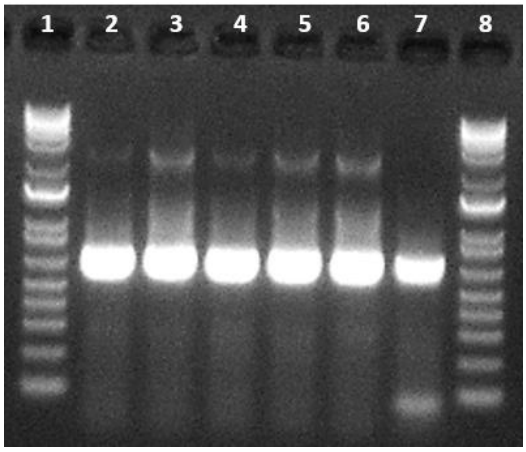

Figure A.7.4: Reaction #30: A product approximately 725 bp of a partial internal transcribed spacer region was amplified with a nested PCR using primers DM3F and DM3R, subsequent to an initial reaction using primers DC6 and ITS4, from selected samples of dehydrated field pods (S1 samples) which had the heaviest smearing in prior reactions. Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 8: 1 kb+ ladder. Lane 2: positive control. Lane 17: negative control. Lanes 3-6: Dehydrated field pods from Site 1, Plant 1.

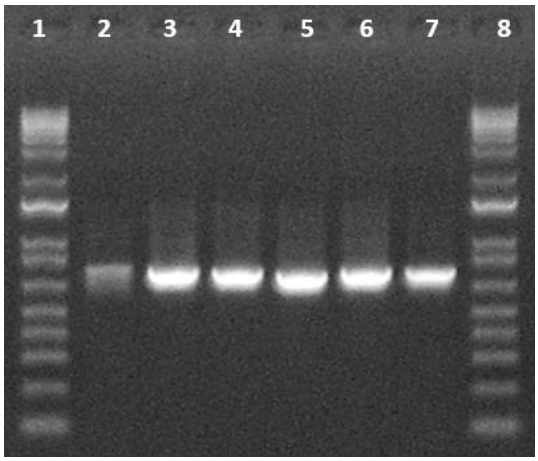

Figure A.7.5: Reaction #31: A product approximately 725 bp of a partial internal transcribed spacer region was amplified with a nested PCR using primers DM3F and DM3R, subsequent to an initial reaction using primers DC6 and ITS4, from selected samples of dehydrated field pods (S1 samples) which had the heaviest smearing in prior reactions. Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 8: 1 kb+ ladder. Lane 2: positive control. Lane 17: negative control. Lanes 3-6: Dehydrated field pods from Site 1, Plant 1.

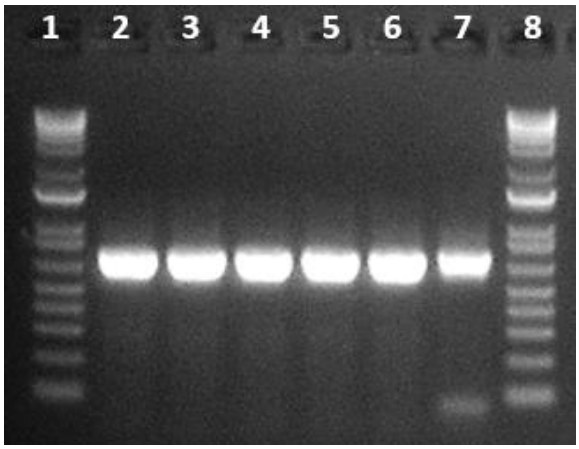

Figure A.7.6: Reaction #32: A product approximately 725 bp of a partial internal transcribed spacer region was amplified with a nested PCR using primers DM3F and DM3R, subsequent to an initial reaction using primers DC6 and ITS4, from selected samples of dehydrated field pods (S1 samples) which had the heaviest smearing in prior reactions. Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 8: 1 kb+ ladder. Lane 2: positive control. Lane 17: negative control. Lanes 3-6: Dehydrated field pods from Site 1, Plant 1.

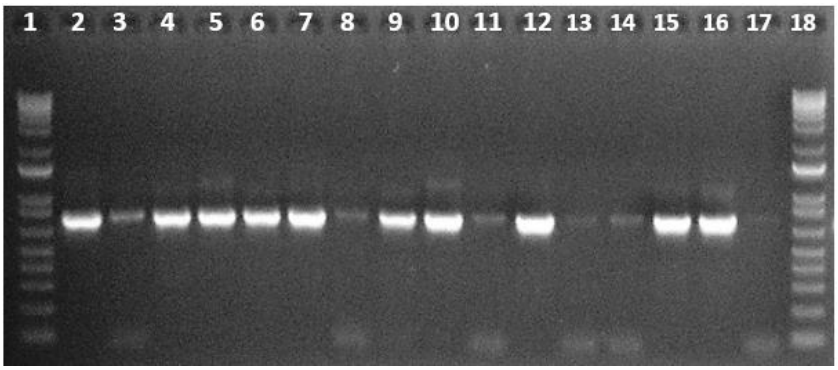

Figure A.7.7: Reaction #33: A product approximately 725 bp of a partial internal transcribed spacer region was amplified with a nested PCR using primers DM3F and DM3R, subsequent to an initial reaction using primers DC6 and ITS4, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 13: 1 kb+ ladder. Lane 2: positive control. Lane 12: negative control. Lanes 3-11: Dehydrated field pods from Site 1, Plant 1.

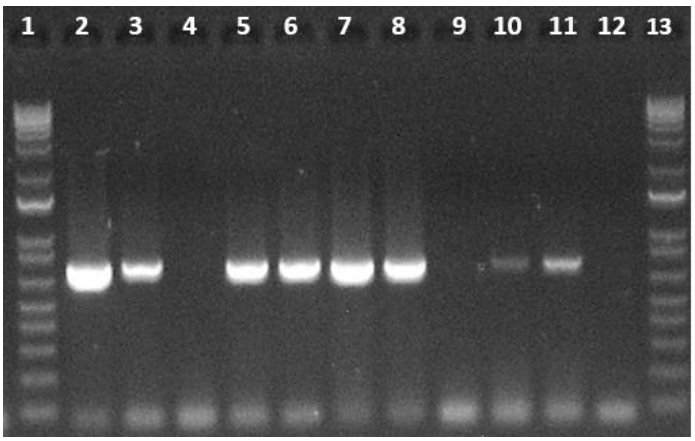

Figure A.7.8: Reaction #34: A product approximately 725 bp of a partial internal transcribed spacer region was amplified with a nested PCR using primers DM3F and DM3R, subsequent to an initial reaction using primers DC6 and ITS4, from asymptomatic plants (S4 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 13: 1 kb+ ladder. Lane 2: positive control. Lane 12: negative control. Lanes 3-11: Dehydrated field pods from Site 1, Plant 1.

A.8 Representative gel images for duplicate PCR reactions for the amplification of the ITS region of *Pvp* in different sample types using primers DC6 and ITS4

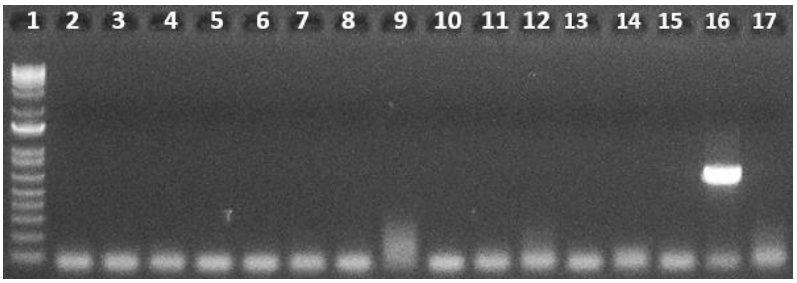

Figure A.8.1: Reaction #42: A product approximately 1500 bp of a partial internal transcribed spacer region was amplified with a duplicate PCR using primers DC6 and ITS4, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: positive control. Lane 13: negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1

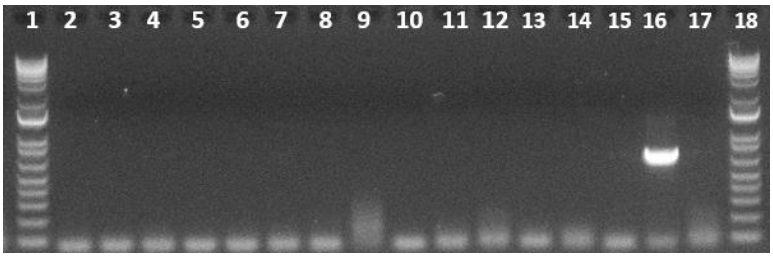

Figure A.8.2: Reaction #43: A product approximately 1500 bp of a partial internal transcribed spacer region was amplified with a duplicate PCR using primers DC6 and ITS4, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: positive control. Lane 13: negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

A.9 Representative gel images for duplicate PCR reactions trialled for the amplification of the partial ITS1, complete 5.8S and partial ITS2 region of *Pvp* in different sample types using primers DM3F and DM3R

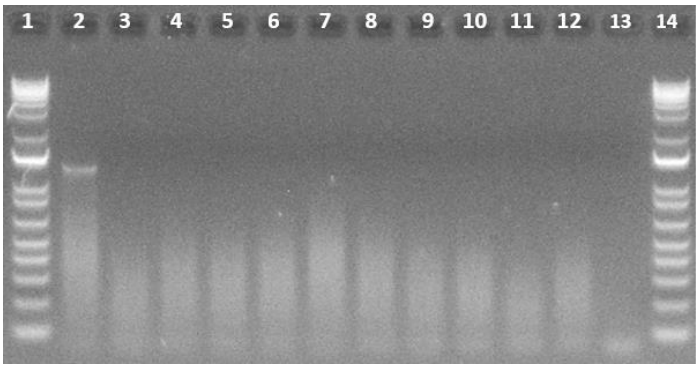

Figure A.9.1: Reaction #44: A product approximately 725 bp of a partial internal transcribed spacer region was amplified with a duplicate PCR using primers DM3F and DM3R, from samples of asymptomatic plants (S4 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 18: 1 kb+ ladder. Lane 2: positive control. Lane 17: negative control. Lanes 3-16: Asymptomatic plants (S4 samples).

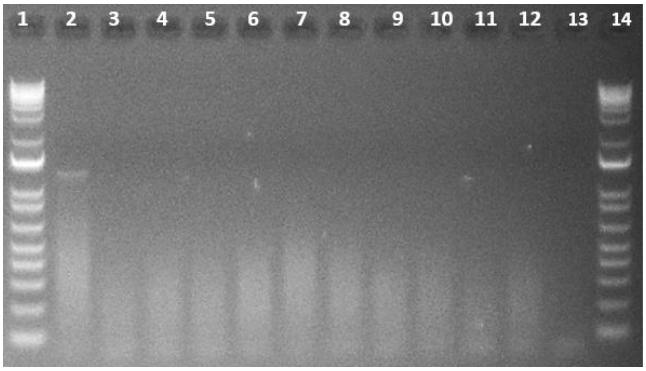

Figure A.9.2: Reaction #45: A product approximately 725 bp of a partial internal transcribed spacer region was amplified with a duplicate PCR using primers DM3F and DM3R, from samples of asymptomatic plants (S4 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lane 1: 1 kb+ ladder. Lane 2: positive control. Lane 17: negative control. Lanes 3-16: Asymptomatic plants (S4 samples).

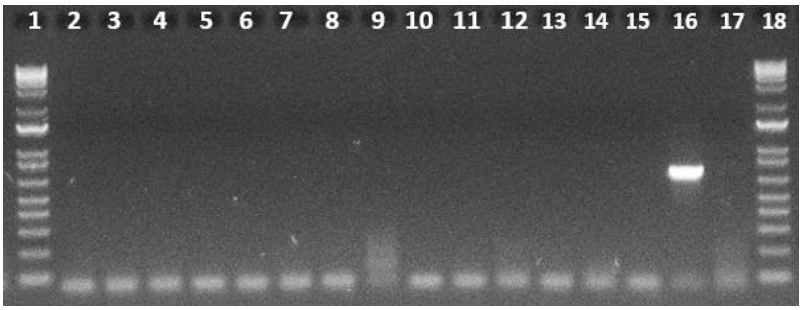

Figure A.9.3: Reaction #46: A product approximately 725 bp of a partial internal transcribed spacer region was amplified with a duplicate PCR using primers DM3F and DM3R, from samples of asymptomatic plants (S4 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 18: 1 kb+ ladder. Lane 2: positive control. Lane 17: negative control. Lanes 3-16: Asymptomatic plants (S4 samples).

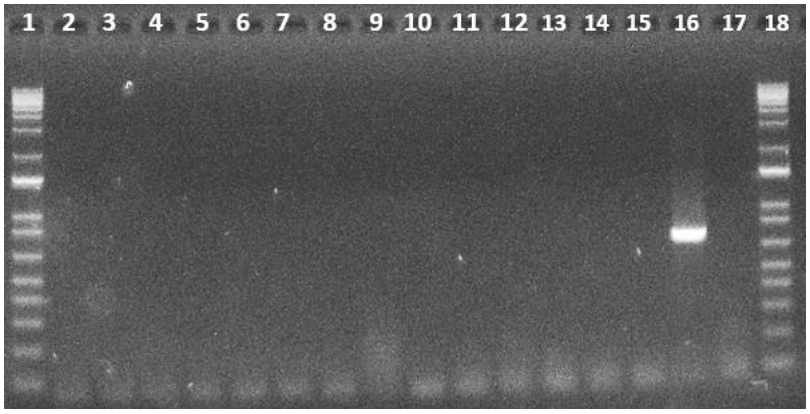

Figure A.9.4: Reaction #47: A product approximately 725 bp of a partial internal transcribed spacer region was amplified with a duplicate PCR using primers DM3F and DM3R, from samples of asymptomatic plants (S4 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 18: 1 kb+ ladder. Lane 2: positive control. Lane 17: negative control. Lanes 3-16: Asymptomatic plants (S4 samples).

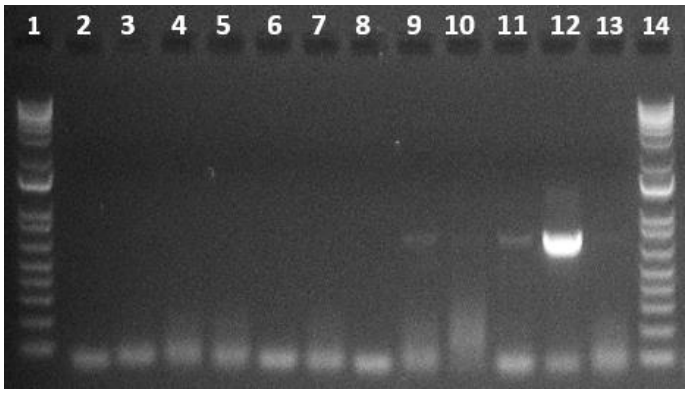

Figure A.9.5: Reaction #48: A product approximately 725 bp of a partial internal transcribed spacer region was amplified with a duplicate PCR using primers DM3F and DM3R, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: positive control. Lane 13: negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

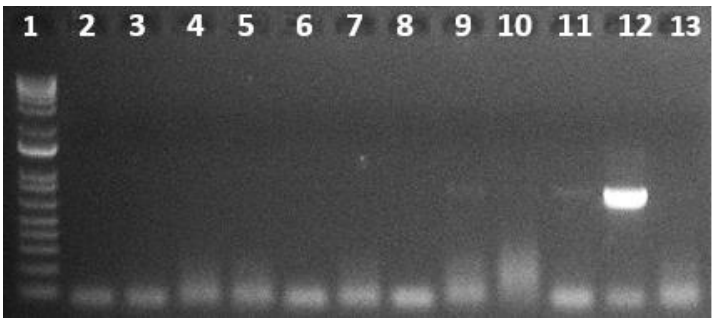

Figure A.9.6: Reaction #49: A product approximately 725 bp of a partial internal transcribed spacer region was amplified with a duplicate PCR using primers DM3F and DM3R, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lane 1: 1 kb+ ladder. Lane 2: positive control. Lane 13: negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

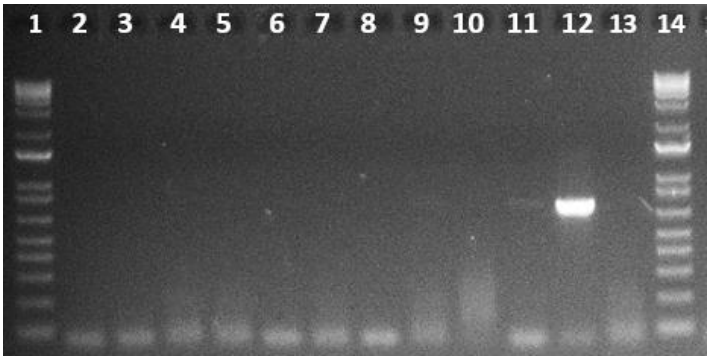

Figure A.9.7: Reaction #50: A product approximately 725 bp of a partial internal transcribed spacer region was amplified with a duplicate PCR using primers DM3F and DM3R, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: positive control. Lane 13: negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

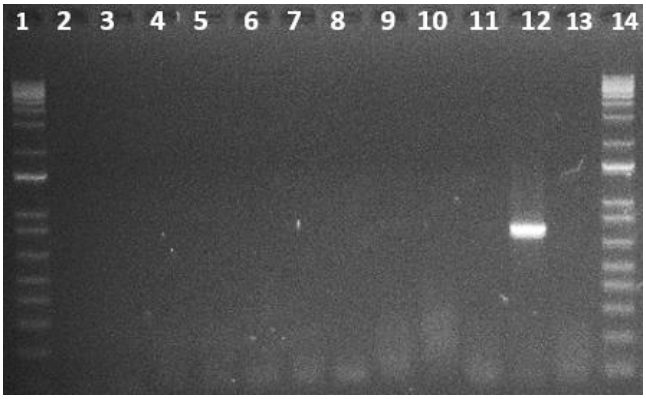

Figure A.9.8: Reaction #51: A product approximately 725 bp of a partial internal transcribed spacer region was amplified with a duplicate PCR using primers DM3F and DM3R, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: positive control. Lane 13: negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

A.10 Gel images from screening enzymes for their efficacy at digesting PCR products of *Pvp* initially amplified with primers DC6 and ITS4 in RFLP

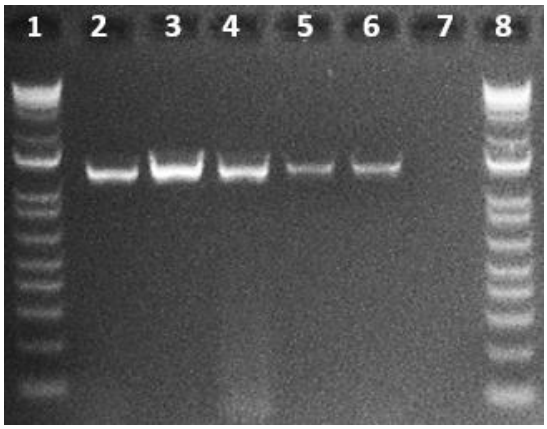

Figure A.10.1: Undigested PCR product of Reaction #1 with enzyme BamHI (G^GATCC). Lanes 1 and 8: 1 kb+ ladder. Lane 2: Positive control. Lane 7: Negative control. Lanes 3-6: Undigested PCR product of S3 sporangial samples from Reaction #1.

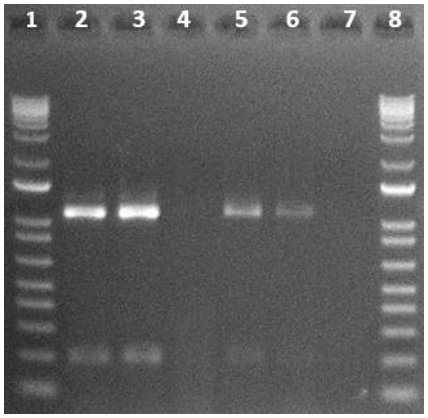

Figure A.10.2: Digested PCR product of Reaction #1. Product was digested into bands approximately 200 and 1300 bp, adding up to the original product of 1500 bp with enzyme EcoRI (G^AATTC). Lanes 1 and 8: 1 kb+ ladder. Lane 2: Positive control. Lane 7: Negative control. Lanes 3-6: Undigested PCR product of S3 sporangial samples from Reaction #1.

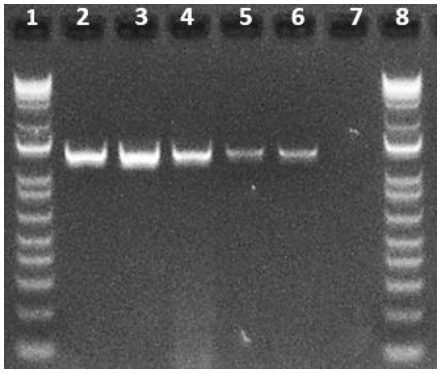

Figure A.10.3: Undigested PCR product of Reaction #1 with enzyme HindIII (A^AGCTT). Lanes 1 and 8: 1 kb+ ladder. Lane 2: Positive control. Lane 7: Negative control. Lanes 3-6: Undigested PCR product of S3 sporangial samples from Reaction #1.

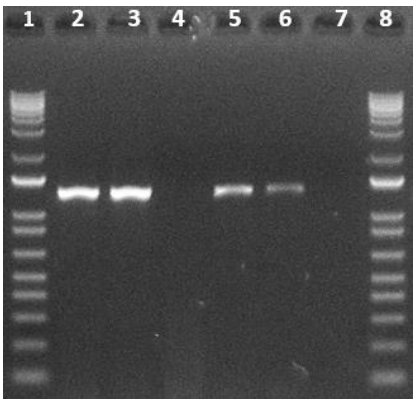

Figure A.10.4: Undigested PCR product of Reaction #1 with enzyme NotI (GC^GGCCGC). Lanes 1 and 8: 1 kb+ ladder. Lane 2: Positive control. Lane 7: Negative control. Lanes 3-6: Undigested PCR product of S3 sporangial samples from Reaction #1.

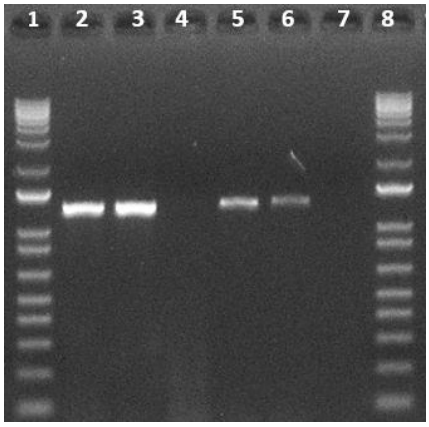

Figure A.10.5: Undigested PCR product of Reaction #1 with enzyme XbaI (T^CTAGA). Lanes 1 and 8: 1 kb+ ladder. Lane 2: Positive control. Lane 7: Negative control. Lanes 3-6: Undigested PCR product of S3 sporangial samples from Reaction #1.

A.11 Gel images for PCR reactions trialled for the amplification of the *Cox*2 region of *Pvp* in different sample types using primers *Cox*2-F and *Cox*2-RC4

Figure A.11.1: Reaction #52: A product approximately 650 bp of a *Cox*2 loci was amplified during PCR using primers, *Cox*2-F and *Cox*2-RC4, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: positive control. Lane 13: negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

Figure A.11.2: Reaction #53: A product approximately 650 bp of a *Cox*2 loci was amplified during PCR using primers, *Cox*2-F and *Cox*2-RC4, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: positive control. Lane 13: negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

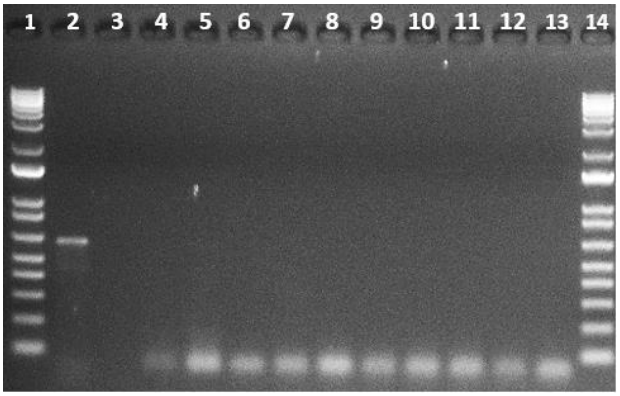

Figure A.11.3: Reaction #54: A product approximately 650 bp of a *Cox*2 loci was amplified during PCR using primers, *Cox*2-F and *Cox*2-RC4, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lane 1: 1 kb+ ladder. Lane 2: positive control. Lane 13: negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

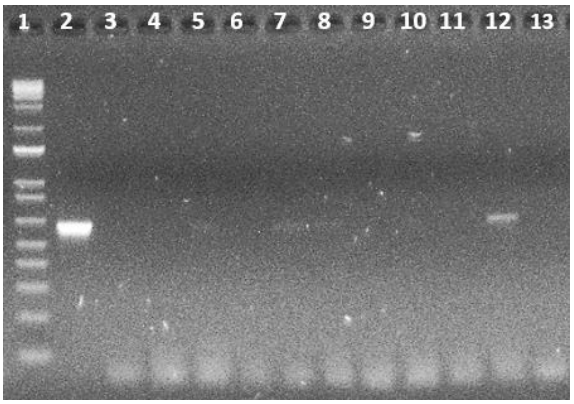

Figure A.11.4: Reaction #55: A product approximately 650 bp of a *Cox*2 loci was amplified during PCR using primers, *Cox*2-F and *Cox*2-RC4, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lane 1: 1 kb+ ladder. Lane 2: positive control. Lane 13: negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

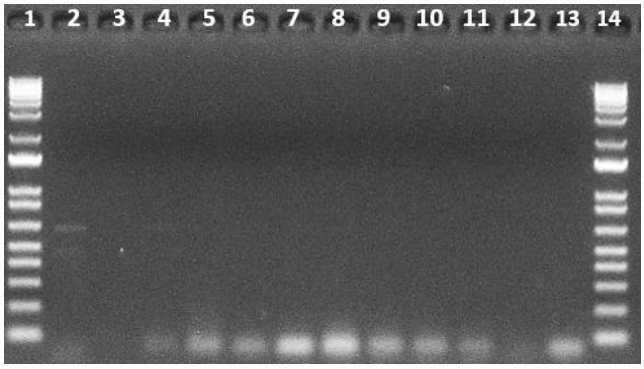

Figure A.11.5: Reaction #56: A product approximately 650 bp of a *Cox*2 loci was amplified during PCR using primers, *Cox*2-F and *Cox*2-RC4, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lane 1: 1 kb+ ladder. Lane 2: positive control. Lane 13: negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

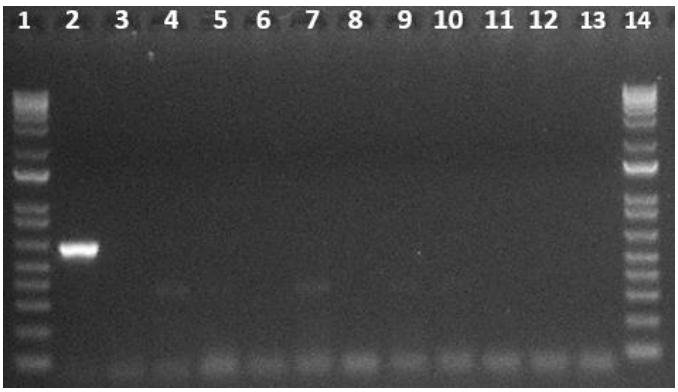

Figure A.11.6: Reaction #57: A product approximately 650 bp of a *Cox*2 loci was amplified during PCR using primers, *Cox*2-F and *Cox*2-RC4, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lane 1: 1 kb+ ladder. Lane 2: positive control. Lane 13: negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

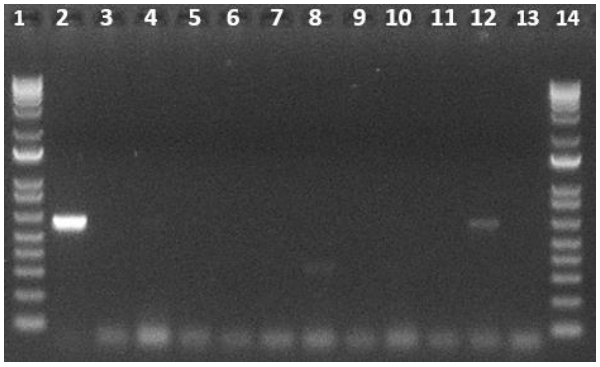

Figure A.11.7: Reaction #58: A product approximately 650 bp of a *Cox*2 loci was amplified during PCR using primers, *Cox*2-F and *Cox*2-RC4, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lane 1: 1 kb+ ladder. Lane 2: positive control. Lane 13: negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

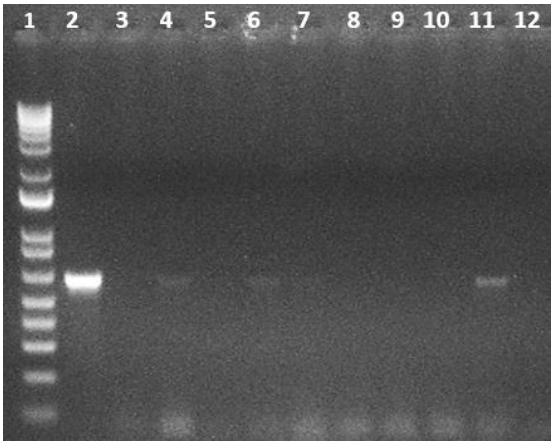

Figure A.11.8: Reaction #59: A product approximately 650 bp of a *Cox*2 loci was amplified during PCR using primers, *Cox*2-F and *Cox*2-RC4, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lane 1: 1 kb+ ladder. Lane 2: positive control. Lane 12: negative control. Lanes 3-11: Dehydrated field pods from Site 1, Plant 1.

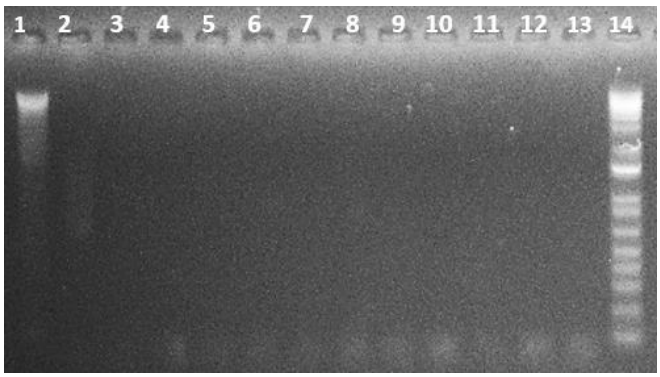

Figure A.11.9: Reaction #60: A product approximately 650 bp of a *Cox*2 loci was amplified during PCR using primers, *Cox*2-F and *Cox*2-RC4, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: Positive control. Lane 13: Negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

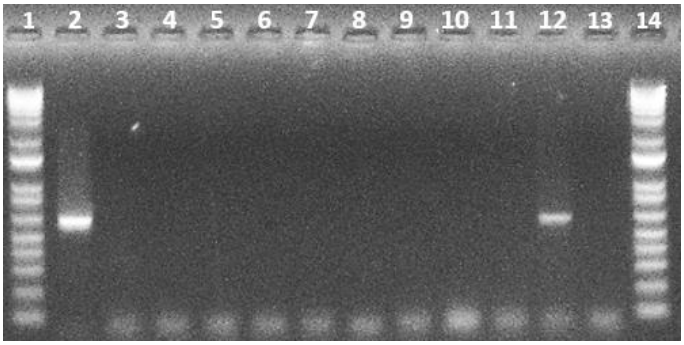

Figure A.11.10: Reaction #61: A product approximately 650 bp of a *Cox*2 loci was amplified during PCR using primers, *Cox*2-F and *Cox*2-RC4, from samples of dehydrated field pods (S1 samples). Bands indicate the presence of *Peronospora viciae* f. sp. *pisi*. Lanes 1 and 14: 1 kb+ ladder. Lane 2: Positive control. Lane 13: Negative control. Lanes 3-12: Dehydrated field pods from Site 1, Plant 1.

A.12 Gel images from screening RAPD primers for their efficacy at producing polymorphic bands from different *Pvp* isolates

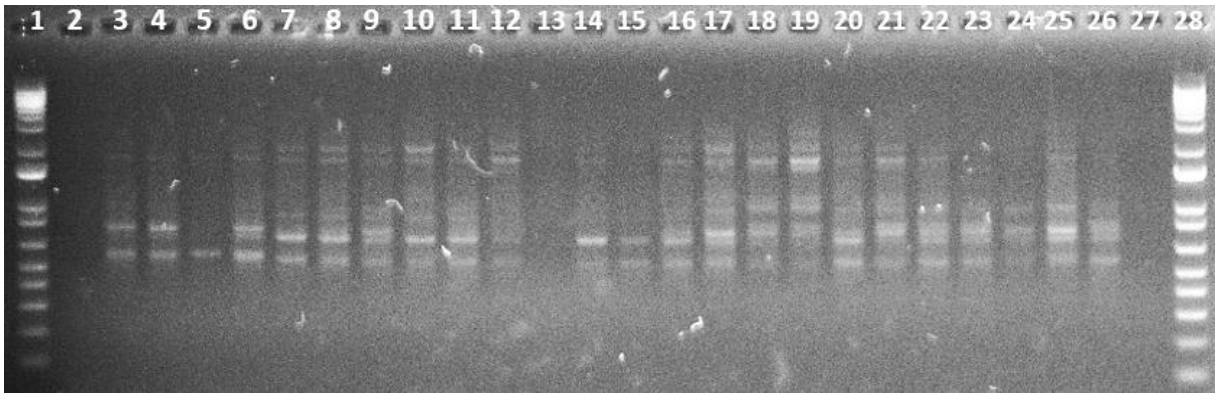

Figure A.12.1: Polymorphic bands produced with RAPD primer DMp4 for 24 representative S2 samples. Lanes 1 and 28: 1 kb+ ladder. Lanes 2 and 27: Negative control. Lane 3: Site 1, Plant 5. Lane 4: Site 1, Plant 7. Lane 5: Site 1, Plant 8. Lane 6: Site 1, Plant 11. Lane 7: Site 2, Plant 2. Lane 8: Site 2, Plant 5. Lane 9: Site 2, Plant 7. Lane 10: Site 2, Plant 9. Lane 11: Site 3, Plant 1. Lane 12: Site 3, Plant 10. Lane 13: Site 3, Plant 11. Lane 14: Site 4, Plant 3. Lane 15: Site 4, Plant 4. Lane 16: Site 4, Plant 7. Lane 17: Site 5, Plant 3. Lane 18: Site 5, Plant 7. Lane 19: Site 5, Plant 10. Lane 20: Site 6, Plant 1. Lane 21: Site 6, Plant 2. Lane 22: Site 6, Plant 3. Lane 23: Site 6, Plant 5. Lane 24: Site 7, Plant 5. Lane 25: Site 7, Plant 8. Lane 26: Site 7, Plant 9.

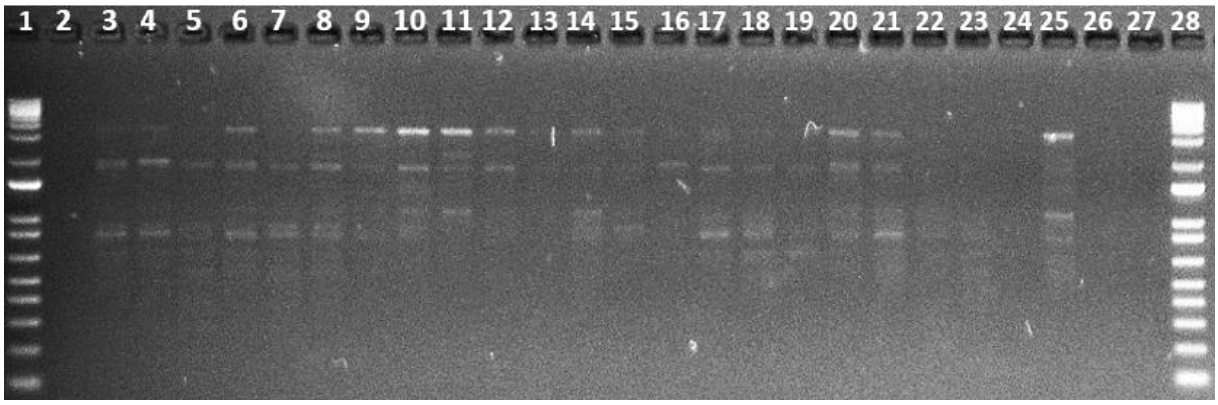

Figure A.12.2: Polymorphic bands produced with primer DMp6 for 24 representative S2 samples. Lanes 1 and 28: 1 kb+ ladder. Lanes 2 and 27: Negative control. Lane 3: Site 1, Plant 5. Lane 4: Site 1, Plant 7. Lane 5: Site 1, Plant 8. Lane 6: Site 1, Plant 11. Lane 7: Site 2, Plant 2. Lane 8: Site 2, Plant 5. Lane 9: Site 2, Plant 7. Lane 10: Site 2, Plant 9. Lane 11: Site 3, Plant 1. Lane 12: Site 3, Plant 10. Lane 13: Site 3, Plant 11. Lane 14: Site 4, Plant 3. Lane 15: Site 4, Plant 4. Lane 16: Site 4, Plant 7. Lane 17: Site 5, Plant 3. Lane 18: Site 5, Plant 7. Lane 19: Site 5, Plant 10. Lane 20: Site 6, Plant 1. Lane 21: Site 6, Plant 2. Lane 22: Site 6, Plant 3. Lane 23: Site 6, Plant 5. Lane 24: Site 7, Plant 5. Lane 25: Site 7, Plant 8. Lane 26: Site 7, Plant 9.

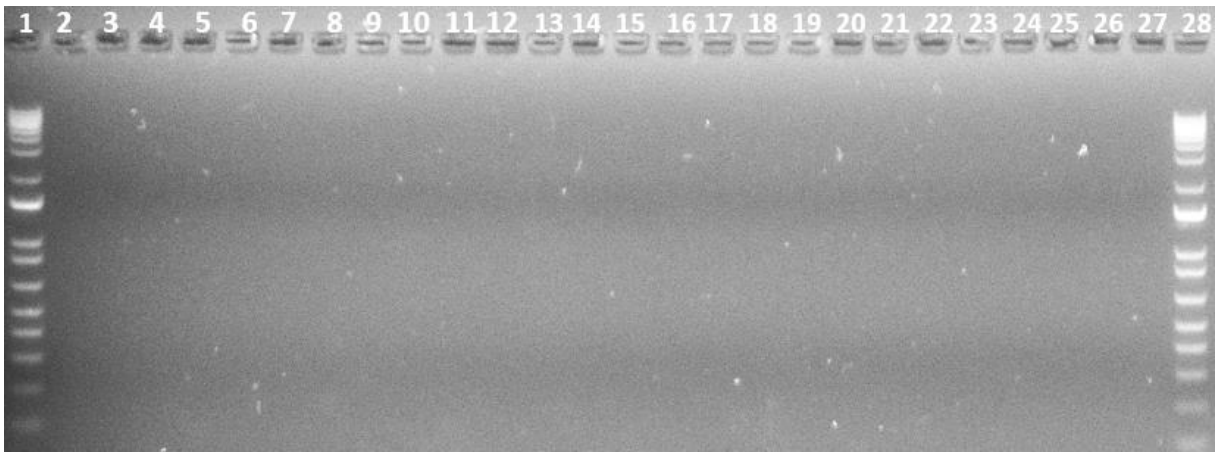

Figure A.12.3: Polymorphic bands produced with primer DMp50 for 24 representative S2 samples. Lanes 1 and 28: 1 kb+ ladder. Lanes 2 and 27: Negative control. Lane 3: Site 1, Plant 5. Lane 4: Site 1, Plant 7. Lane 5: Site 1, Plant 8. Lane 6: Site 1, Plant 11. Lane 7: Site 2, Plant 2. Lane 8: Site 2, Plant 5. Lane 9: Site 2, Plant 7. Lane 10: Site 2, Plant 9. Lane 11: Site 3, Plant 1. Lane 12: Site 3, Plant 10. Lane 13: Site 3, Plant 11. Lane 14: Site 4, Plant 3. Lane 15: Site 4, Plant 4. Lane 16: Site 4, Plant 7. Lane 17: Site 5, Plant 3. Lane 18: Site 5, Plant 7. Lane 19: Site 5, Plant 10. Lane 20: Site 6, Plant 1. Lane 21: Site 6, Plant 2. Lane 22: Site 6, Plant 3. Lane 23: Site 6, Plant 5. Lane 24: Site 7, Plant 5. Lane 25: Site 7, Plant 8. Lane 26: Site 7, Plant 9.

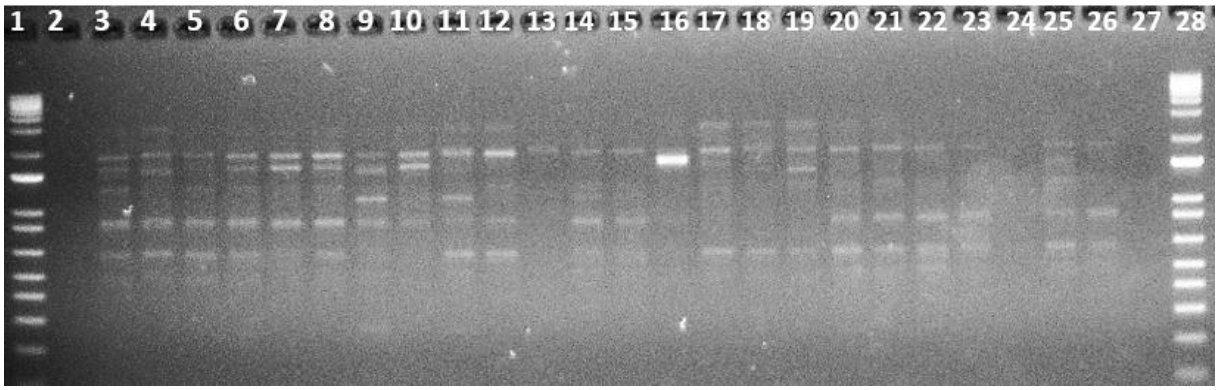

Figure A.12.4: Polymorphic bands produced with primer DMp51 for 24 representative S2 samples. Lanes 1 and 28: 1 kb+ ladder. Lanes 2 and 27: Negative control. Lane 3: Site 1, Plant 5. Lane 4: Site 1, Plant 7. Lane 5: Site 1, Plant 8. Lane 6: Site 1, Plant 11. Lane 7: Site 2, Plant 2. Lane 8: Site 2, Plant 5. Lane 9: Site 2, Plant 7. Lane 10: Site 2, Plant 9. Lane 11: Site 3, Plant 1. Lane 12: Site 3, Plant 10. Lane 13: Site 3, Plant 11. Lane 14: Site 4, Plant 3. Lane 15: Site 4, Plant 4. Lane 16: Site 4, Plant 7. Lane 17: Site 5, Plant 3. Lane 18: Site 5, Plant 7. Lane 19: Site 5, Plant 10. Lane 20: Site 6, Plant 1. Lane 21: Site 6, Plant 2. Lane 22: Site 6, Plant 3. Lane 23: Site 6, Plant 5. Lane 24: Site 7, Plant 5. Lane 25: Site 7, Plant 8. Lane 26: Site 7, Plant 9.

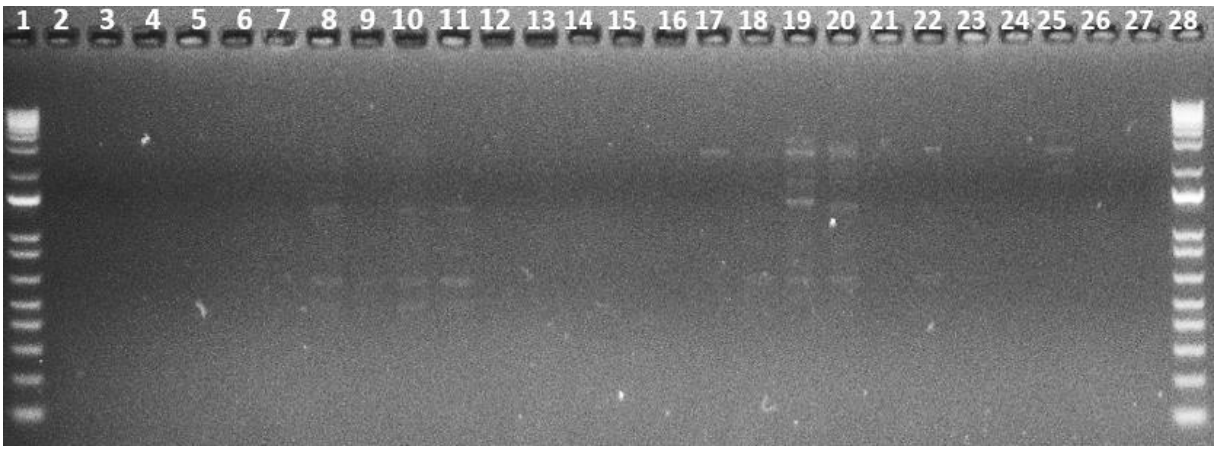

Figure A.12.5: Polymorphic bands produced with primer DMp67 for 24 representative S2 samples. Lanes 1 and 28: 1 kb+ ladder. Lanes 2 and 27: Negative control. Lane 3: Site 1, Plant 5. Lane 4: Site 1, Plant 7. Lane 5: Site 1, Plant 8. Lane 6: Site 1, Plant 11. Lane 7: Site 2, Plant 2. Lane 8: Site 2, Plant 5. Lane 9: Site 2, Plant 7. Lane 10: Site 2, Plant 9. Lane 11: Site 3, Plant 1. Lane 12: Site 3, Plant 10. Lane 13: Site 3, Plant 11. Lane 14: Site 4, Plant 3. Lane 15: Site 4, Plant 4. Lane 16: Site 4, Plant 7. Lane 17: Site 5, Plant 3. Lane 18: Site 5, Plant 7. Lane 19: Site 5, Plant 10. Lane 20: Site 6, Plant 1. Lane 21: Site 6, Plant 2. Lane 22: Site 6, Plant 3. Lane 23: Site 6, Plant 5. Lane 24: Site 7, Plant 5. Lane 25: Site 7, Plant 8. Lane 26: Site 7, Plant 9.

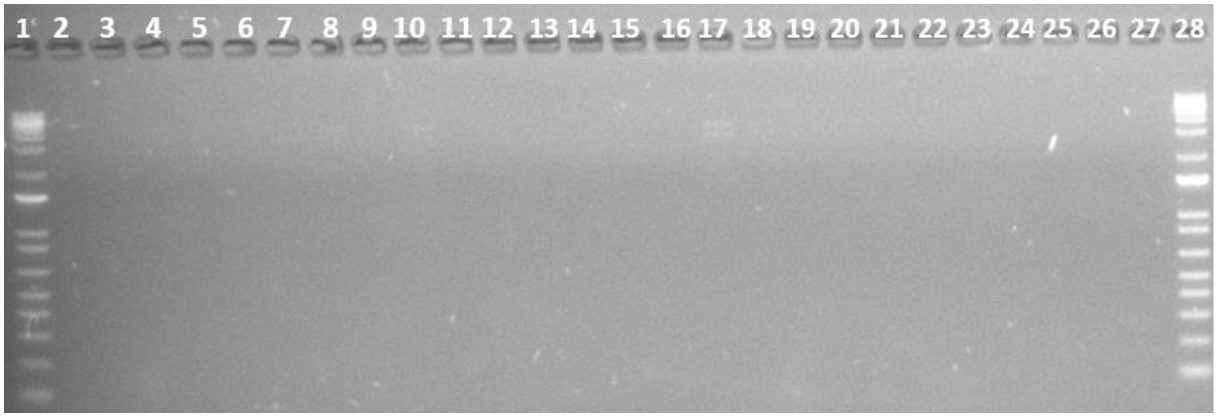

Figure A.12.6: Polymorphic bands produced with primer DMp73 for 24 representative S2 samples. Lanes 1 and 28: 1 kb+ ladder. Lanes 2 and 27: Negative control. Lane 3: Site 1, Plant 5. Lane 4: Site 1, Plant 7. Lane 5: Site 1, Plant 8. Lane 6: Site 1, Plant 11. Lane 7: Site 2, Plant 2. Lane 8: Site 2, Plant 5. Lane 9: Site 2, Plant 7. Lane 10: Site 2, Plant 9. Lane 11: Site 3, Plant 1. Lane 12: Site 3, Plant 10. Lane 13: Site 3, Plant 11. Lane 14: Site 4, Plant 3. Lane 15: Site 4, Plant 4. Lane 16: Site 4, Plant 7. Lane 17: Site 5, Plant 3. Lane 18: Site 5, Plant 7. Lane 19: Site 5, Plant 10. Lane 20: Site 6, Plant 1. Lane 21: Site 6, Plant 2. Lane 22: Site 6, Plant 3. Lane 23: Site 6, Plant 5. Lane 24: Site 7, Plant 5. Lane 25: Site 7, Plant 8. Lane 26: Site 7, Plant 9.

A.13 Gel images of polymorphic bands RAPD analyses with 24 *Pvp* samples using primers DMp4 and DMp51

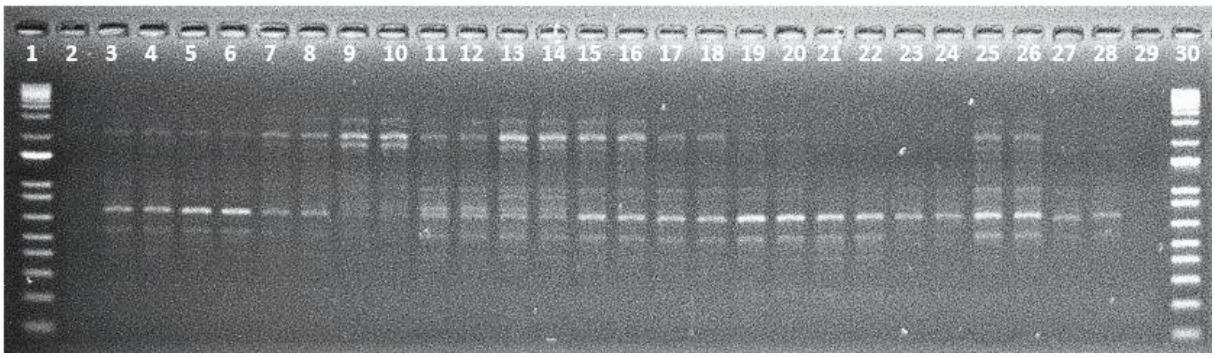

Figure A.13.1: Polymorphic bands produced with RAPD primer DMp4 for 13 S2 samples from the North Island (Sites 4, 5, 6 and 7). Lanes 1 and 30: 1 kb+ ladder. Lanes 2 and 29: Negative controls. Lanes 3 and 4: Site 4, Plant 3 duplicate 1 and 2. Lanes 5 and 6: Site 4, Plant 4 duplicate 1 and 2. Lanes 7 and 8: Site 4, Plant Seven duplicate 1 and 2. Lanes 9 and 10: Site 5, Plant 3 duplicate 1 and 2. Lanes 11 and 12: Site 5, Plant 7 duplicate 1 and 2. Lanes 13 and 14: Site 5, Plant 10 duplicate 1 and 2. Lanes 15 and 16: Site 6, Plant 1 duplicate 1 and 2. Lanes 17 and 18: Site 6, Plant 2 duplicate 1 and 2. Lanes 19 and 20: Site 6, Plant 3 duplicate 1 and 2. Lanes 21 and 22: Site 6, Plant 5 duplicate 1 and 2. Lanes 23 and 24: Site 7, Plant 5 duplicate 1 and 2. Lanes 25 and 26: Site 7, Plant 8 duplicate 1 and 2. Lanes 27 and 28: Site 7, Plant 9 duplicate 1 and 2.

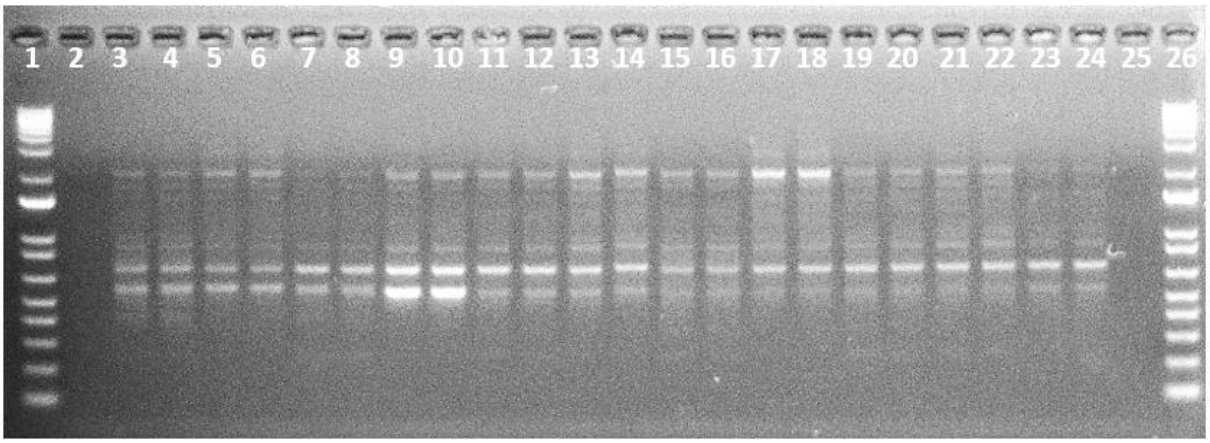

Figure A.13.2: Polymorphic bands produced with primer DMp4 for 11 S2 samples from the South Island (Sites 1, 2 and 3). Lanes 1 and 26: 1 kb+ ladder. Lanes 2 and 25: Negative controls. Lanes 3 and 4: Plant 1, Site 5 duplicate 1 and 2. Lanes 5 and 6: Site 1, Plant 7 duplicate 1 and 2. Lanes 7 and 8: Site 1, Plant 8 duplicate 1 and 2. Lanes 9 and 10: Site 1, Plant 11 duplicate 1 and 2. Lanes 11 and 12: Site 2, Plant 2 duplicate 1 and 2. Lanes 13 and 14: Site 2, Plant 5 duplicate 1 and 2. Lanes 15 and 16: Site 2, Plant 7 duplicate 1 and 2. Lanes 17 and 18: Site 2, Plant 9 duplicate 1 and 2. Lanes 19 and 20: Site 3, Plant 1 duplicate 1 and 2. Lanes 21 and 22: Site 3, Plant 10 duplicate 1 and 2. Lanes 23 and 24: Site 3, Plant 11 duplicate 1 and 2.

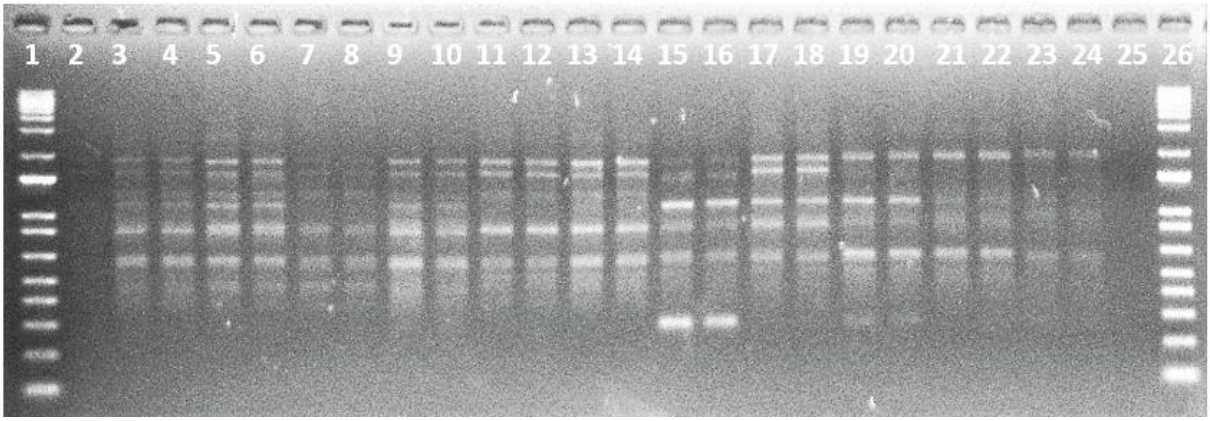

Figure A.13.3: Polymorphic bands produced with primer DMp51 for 11 S2 samples from the South Island (Sites 1, 2 and 3). Lanes 1 and 26: 1 kb+ ladder. Lanes 2 and 25: Negative controls. Lanes 3 and 4: Plant 1, Site 5 duplicate 1 and 2. Lanes 5 and 6: Site 1, Plant 7 duplicate 1 and 2. Lanes 7 and 8: Site 1, Plant 8 duplicate 1 and 2. Lanes 9 and 10: Site 1, Plant 11 duplicate 1 and 2. Lanes 11 and 12: Site 2, Plant 2 duplicate 1 and 2. Lanes 13 and 14: Site 2, Plant 5 duplicate 1 and 2. Lanes 15 and 16: Site 2, Plant 7 duplicate 1 and 2. Lanes 17 and 18: Site 2, Plant 9 duplicate 1 and 2. Lanes 19 and 20: Site 3, Plant 1 duplicate 1 and 2. Lanes 21 and 22: Site 3, Plant 10 duplicate 1 and 2. Lanes 23 and 24: Site 3, Plant 11 duplicate 1 and 2.

Appendix B

Supplementary Material for Chapter 3

B.1 Potting mix recipe

500 L of Potting mix contained 400L composted bark, 100L pumice, 1500g Osmocote extract (16-3.9-10 NPK), 500g horticultural lime and 500g hydraflo (wetting agent).

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