SPORE TRAPPING COUPLED WITH QUANTITATIVE PCR: POTENTIAL FOR APPLICATION TO BIOSECURITY SURVEILLANCE

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1 ABSTRACT

Plant disease surveillance is used in biosecurity to enable early detection of incursions of new pathogens, to monitor disease status during eradication programs, and to demonstrate that an area is free of a particular pathogen for trade purposes. Monitoring for plant pathogens currently relies on detection of symptoms by suitably skilled personnel, but the difficulties of distinguishing diseases based on symptoms, and of timing surveillance to coincide with symptom expression, can result in new pathogens not being detected until they are already widespread. The aims of this research were, firstly, to investigate the efficacy of spore trapping combined with quantitative PCR diagnostic assays for biosecurity surveillance and, secondly, to use the system to test predictions of spore release generated by epidemiological models.

Methodology for detection and quantification of airborne spores using spore traps and quantitative PCR assays was optimised and tested on three model pathogens endemic on pulse and oilseed crops in South Australia:

1. *Leptosphaeria maculans,* cause of blackleg (phoma stem canker) of canola (oilseed rape, *Brassica napus*)
2. *Didymella pinodes,* which causes ascochyta blight (blackspot) of field pea (*Pisum sativum*), and
3. *Ascochyta rabiei,* cause of ascochyta blight of chickpea (*Cicer arietinum*)

The PCR tests were shown to be both specific and sensitive when applied to spore trap samples, and results were borne out by close correlation with microscopic counts of ascospores on tapes of trapped spores. Poor reproducibility of results was largely addressed by replication of PCR assays and use of an exogenous control to allow variation to be taken into account. Implications of the imprecision remaining in the system are discussed in relation to epidemiological research leading to biosecurity surveillance. Storage of spore trap samples at -20°C was found to be a suitable system for ascospores of *L. maculans*, with no reduction in the yield of DNA after 6 months. The finding that DNA yield from conidia of *L. maculans* was reduced, however, indicated that further research may be needed before applying these findings to other species and/or types of spore. The potential for reduced yield of DNA from spore trap samples collected during periods of extremely hot weather, and the possibility that dust

may affect yields, were identified as factors to be considered in assessing qPCR results. Nevertheless, the system proved to be generally robust in weather conditions prevailing in southern Australia.

The data from 2 years of monitoring for model pathogens were used to calibrate epidemiological models to the field site and to refine the models, as required. The findings that ascospores of *D. pinodes* were released during rain events in the summer led to incorporation of an additional cycle of ascospore formation and release, commencing during the cropping season, into the G1 Blackspot Manager model (model pathogen 1). Furthermore, the detection of *D. pinodes* DNA in spore trap samples on rainless days led to the incorporation of a relative humidity factor into the model. A minor modification was made to the Blackleg Sporacle model to smooth the model prediction curve (model pathogen 2). These changes, and the calibration of the models to the field site, need to be validated in further seasons. The results of monitoring for *D. rabiei* indicated that few or no ascospores were released from chickpea stubble, suggesting that the second mating type required for sexual reproduction, Mat 1-1, either does not occur in South Australia or is present as only a very small proportion of the population (model pathogen 3).

In investigating questions arising from the above experiments, it was found that 7-month-old canola stubble exposed to prolonged dry conditions continued to release ascospores immediately upon wetting, for up to 5 months (i.e. up until early summer, 12 months after harvest), but did not continue beyond the (Australian) early summer into the mid- and late-summer and autumn of the following year. This suggested that infested canola stubble from the season before the last is unlikely to be a significant source of infection for the current year's canola crop emerging in autumn.

DNA of *D. pinodes* was readily detected in dust generated at harvest but application of the dust to field pea seedlings failed to cause blackspot. The implications of these findings in relation to disease spread and biosecurity surveillance are discussed.

The results obtained using the optimised methodology for spore trapping coupled with PCR diagnostics provided a good match with those obtained using trap plants, and with predictions of epidemiological models. Spore trapping coupled with qPCR proved to be a useful tool for epidemiological studies, which can be applied in biosecurity surveillance.

2 DECLARATION

This thesis contains no material which has been accepted for the award of another degree or diploma in any University and, to the best of my knowledge and belief, contains no other material previously published or written by another person, except where due reference is given. I give consent to this thesis being made available for photocopying and loan from the University Library.

___________________________________ ___/___/______

Bernadette Klazina Vogelzang

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4 ABBREVIATIONS

¼ PDA	One quarter strength potato dextrose agar
AWS	Automatic weather station
BOM	Australian Bureau of Meteorology
Ct	In quantitative PCR, the cycle threshold at which the increase in fluorescence exceeds the background for the fluorescence-labelled probe
DAFWA	Department of Agriculture and Food Western Australia
fg	Femtogram (10^{-15} gram)
g	Earth's gravitational acceleration (measure applied in centrifugation)
LCS	Lack of correlation weighted by the standard deviations
MSD	Mean squared deviation
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
pg	Picogram (10^{-12} gram)
qPCR	Quantitative PCR (real-time PCR)
RDTS	Root Disease Testing Service (SARDI)
RFLP	Restriction fragment length polymorphism
RH	Relative humidity
RMSD	Root mean squared deviation
RO water	Reverse osmosis water
RT-PCR	Reverse-transcriptase PCR
SARDI	South Australian Research and Development Institute
SB	Squared bias
SDSD	Squared difference between standard deviations
SE	Standard error of the mean
SNP	Single nucleotide polymorphism
Syn.	Synonym
UK	United Kingdom
USA	United States of America

1 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Australia is fortunate to be free of a large number of plant pathogens that cause significant disease elsewhere. Despite stringent quarantine requirements, new pathogens enter the country from time to time and these may become established and cause major crop loss to the industries concerned, or damage to natural ecosystems, if not eradicated (Anonymous 2004). Early detection of newly arrived plant pathogens is one of the most important factors determining the likely success of an eradication campaign. Plant disease surveillance is important for early detection of incursions, monitoring disease status during eradication programs and enabling the early development of targeted management strategies in situations where eradication is not feasible.

Plant disease surveillance is also needed to demonstrate that an area is free from a particular pathogen for trade purposes. While in the past the absence of a disease record has usually been accepted by trading partners as sufficient to indicate pest-free status, an evidence-based approach is now being adopted internationally ("evidence of absence" as opposed to "absence of evidence") (Donovan 2003). Surveillance for plant pathogens is therefore becoming increasingly important to maintain access to overseas or interstate markets, or to justify quarantine restrictions applied to trading partners.

Surveillance for plant pathogens currently relies on detection of symptoms by suitably skilled personnel. The difficulties of detecting disease at low incidence on the basis of symptoms, of finding diseases of limited distribution, and of timing surveillance to coincide with symptom expression, may result in new diseases not being detected until they have become widespread. More efficient surveillance techniques for plant pathogens are therefore needed to enhance Australia's early warning surveillance capability and its ability to demonstrate area freedom for pathogens of biosecurity concern.

One potential means of surveillance for plant pathogens is by strategic monitoring of airborne inoculum. Many economically important fungal plant pathogens produce airborne spores, which are dispersed over longer distances than other pathogen propagules, such as splash-dispersed or waterborne spores, and often serve as the

primary inoculum source for new infection foci and for epidemics (Salam, Fitt *et al.* 2007; Trapero-Casas, Navas-Cortes *et al.* 1996). Monitoring airborne inoculum could potentially enable earlier detection of the presence of a pathogen than monitoring for symptoms on plants, as spores of plant pathogens may be trapped before disease is present in local crops (Lacey 1988), (Jackson and Bayliss 2011).

Sampling of airborne inoculum has been undertaken in various ways since the 1860s (Lacey and West 2006). However its use has been limited until recently by the considerable time and expertise required to obtain accurate results using conventional methods for identification and quantification, based on microscopy and/or culturing on suitable growth media. With the advent of nucleic acid-based diagnostics, methods that are faster, cheaper, more accurate, sensitive, specific and reliable, and do not require specialist plant pathology expertise, have become available. A range of these methods has been investigated in recent years for use in conjunction with air sampling (Macneil, Kauri *et al.* 1995) (Peccia and Hernandez 2006); (West, Atkins *et al.* 2008).

This review aims to identify relevant research on techniques for the identification and quantification of airborne fungal plant pathogens from air samples and to determine what additional research may be needed to enable application of such techniques as a tool for biosecurity surveillance and epidemiological studies, in particular in Australian conditions.

1.2 Inoculum dispersal

Airborne spores can be dispersed over long distances. The distance travelled by spores results from the interplay of many factors including physical characteristics of the spores (size, shape, degree of surface roughness, density and electrostatic charges) and environmental factors including wind (speed, direction, turbulence, gradients near the ground and pattern of atmospheric circulation), rain and topography of the area (Lacey 1988). Ascospores originating in Britain have been trapped more than 500 km from the British coast, and turbulent weather is believed to have been associated with the movement of endospores of *Bacillus* species from near the Black Sea to Sweden, a distance of 1,800 km (Lacey and West 2006). Viability of spores may be reduced during dispersal but there is evidence that a small number of spores of some pathogens may survive dispersal over distances of thousands of kilometres. For example, meteorological data indicate that the introduction of sugarcane rust (*Puccinia melanocephala*) into America in 1978 was most likely the result of movement of

uredospores by cyclonic winds thousands of kilometres over the sea from West Africa (Brown and Hovmoller 2002). For ascomycetes, survival of ascospores during aerial dispersal is likely to be less than for spores of rust fungi which are comparatively robust against environmental damage (Brown and Hovmoller 2002). However, ascospores are known to move in the order of tens of kilometres and remain viable. For example, during an epidemic of Ascochyta blight of chickpeas in Northern Idaho in 1986, airborne ascospores of *Didymella rabiei* were implicated in the spread of disease to new infection foci 10 - 15 km from the nearest affected fields (Trapero-Casas and Kaiser 1992b). In a study of aerial dispersal of ascospores from infested pea stubble, significant numbers of blackspot lesions, assumed to be caused by *D. pinodes* ascospores, were detected on trap plants 4 km from the inoculum source, though highest numbers were detected immediately adjacent to infested stubble (Davidson, Kimber *et al.* 2006). It has been suggested that ascospores of *Leptosphaeria maculans* are blown several kilometres from infested canola stubble (Hall 1992).

1.3 Air sampling

A variety of air samplers has been developed, ranging from passive types such as microscope slides, Petri dishes and trap plants, to volumetric types such as suction traps, rotary impact samplers and ionic spore traps (Jackson and Bayliss 2011). Horizontal slides are particularly suitable for splash-dispersed spores, which are carried in droplets of a size that is not normally carried any great distance by wind (McCartney, Fitt *et al.* 1997). Vertical slides with an adhesive can be used to capture spores by impaction. This is a simple and cheap method, but does not allow for variation in particle deposition due to wind speed and direction (McCartney, Fitt *et al.* 1997). Trap plants give a more realistic picture of deposition of viable spores on crop plants than samplers, but there may be logistical challenges in assuring a timely supply of trap plants, and the amount of inoculum may be underestimated if conditions are suboptimal for disease development (Schoeny, Jumel *et al.* 2007). Rotary impact samplers are useful where spatial distribution of spores is of interest. Where absolute concentrations of airborne spores are required, volumetric samplers are usually essential (McCartney, Fitt *et al.* 1997). Suction traps, based on a design by Hirst (Hirst 1952), are among the most commonly used in agricultural settings. The Hirst-type volumetric spore trap (Neumeister-Kemp et al. 2004) featured in Figure 1-1 contains a rotating drum on which a strip of clear Melinex tape (Burkard, UK), coated with an adhesive, has been fixed. Air is drawn through a narrow aperture which is directed into the wind by means

of a wind vane, and airborne particles are impacted onto the drum. The drum can be set to rotate once a week or once a day, thus allowing precise linking with weather events if a weather station is located nearby. The tape can then be removed for microscopic examination and other types of laboratory processing.

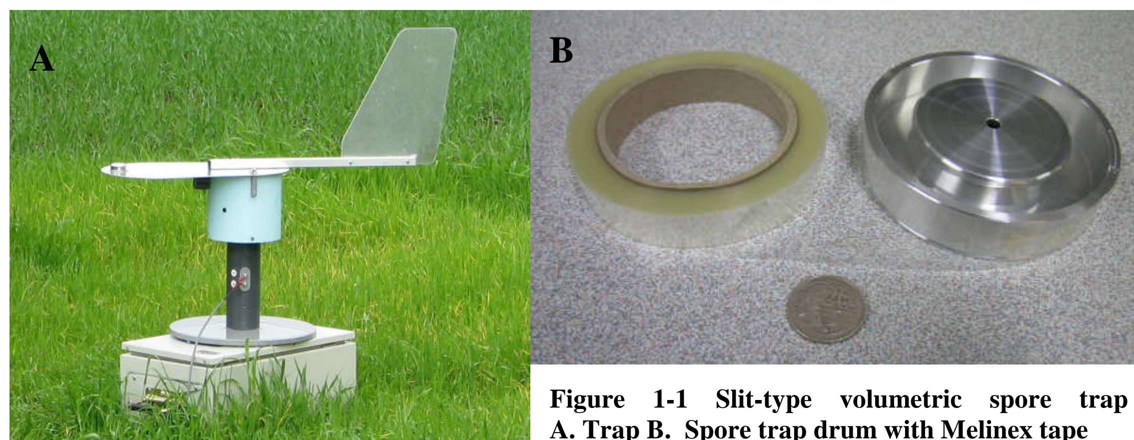

Figure 1-1 Slit-type volumetric spore trap A. Trap B. Spore trap drum with Melinex tape

Sampling of airborne inoculum of fungal plant pathogens has been used mostly for studying pathogen epidemiology, and traps are generally placed among or close to sources of inoculum (Freeman, Ward *et al.* 2002; Ma, Luo *et al.* 2003; Neumeister-Kemp, Maxwell *et al.* 2004). For example, Driessen (2005) used a Hirst-type volumetric spore trap to collect data on timing of basidiospore release of the boronia rust pathogen, *Puccinia boroniae,* which was correlated with weather factors, including minimum daily temperature, rainfall and relative humidity, as well as time of day. The trap was located 5 m from a row of boronia plants which acted as a source of inoculum, at a height of 1.5 m above ground. In a study of the development of fungicide resistance, Fraaije et al. (2005) placed spore traps in the centre of plots, of size 125 m x 120 m, of winter wheat infected with the Septoria leaf blotch pathogen, *Mycosphaerella graminicola*, to which different fungicide treatments were applied. Through monitoring ascospore movement, rapid shifts in the relative proportions of fungicide resistance alleles within the pathogen population were detected. Knowledge of the biology of the pathogen under study can inform the best positioning and timing of placement of the traps. For example, an understanding of the diurnal patterns of spore release for a targeted pathogen may enable the operator to select appropriate periods of the day for sampling which could exclude other potentially confounding propagules (McCartney, Fitt *et al.* 1997).

1.4 Methods for identification and quantification

To be useful in detection of spores from air samples, the methods used for identification of spores should be accurate, sensitive, fast and reliable. A number of methods have been used, including microscopic examination, culturing on a growth medium, serology and PCR-based tests.

1.4.1 Conventional methods

Conventional methods of identification and quantification of airborne fungal spores from spore traps rely on either direct microscopic examination or culturing, and consequently are time-consuming and laborious, and require specialist expertise. Because of morphological similarities among spores of many species / genera of fungi, visual identification can be difficult or, in some cases, impossible. When spores cannot be identified morphologically, culturing may be used. However, culturing is unsuitable for organisms that are slow-growing or not culturable *in vitro* and the choice of culture medium may influence which organisms can grow (Williams, Ward *et al.* 2001). These difficulties have limited the use of air sampling for the detection and quantification of plant pathogens.

1.4.2 Serological methods

Serological methods have been used to detect and quantify specific plant pathogens from air samples. The most promising methods are based on the enzyme-linked immunosorbent assay (ELISA), a technique which uses enzyme-mediated colour changes to detect enzyme-labelled antibodies when they bind with an antigen on a target plant pathogen (Ward, Foster *et al.* 2004). Kennedy et al. (2000) described a microtitre immunospore trapping device which captures fungal spores into microtitre wells, where ELISA is then applied directly to the spores. The device, which is portable, robust and inexpensive, trapped 1.7 times more ascospores of *Mycosphaerella brassicicola* than a Burkard suction spore trap, which is based on the Hirst design (Wakeham, Kennedy *et al.* 2004). ELISA-based techniques are quantitative, cost-effective to run and amenable to high-throughput, and therefore have good potential for the detection of airborne spores of plant pathogens, providing that suitable antibodies are available. However, specific antibodies need to be developed to avoid cross-reactivity for complex pathogens such as fungi, and therefore ELISA-based tests are time-consuming and costly to develop. They are also generally less sensitive than nucleic acid-based methods (Ward, Foster *et al.* 2004).

1.4.3 Polymerase Chain Reaction (PCR)

PCR is a technique for rapidly generating multiple copies of selected nucleic acid sequences. PCR-based detection tests are fast and accurate, and allow the detection of target organisms present in very small quantities in biological samples (Glick and Pasternak 2003). They also have the advantage that specialist diagnostic skills are not required.

In PCR the two strands of nucleic acid are separated through heating to 95°C, in the presence of thermostable DNA polymerase, deoxyribonucleoside triphosphates (dNTPs) and a primer pair. The primers are short sequences of nucleic acid complementary to those at either end of a sequence in the target nucleic acid specific to that target. The temperature of the reaction mix is then reduced to enable binding of the primers to the target nucleic acid through complementary base pairing, and then increased to enable replication of the selected nucleic acid sequence through extension from the primers. Copies of the target sequence are thus generated. With each sequential cycle of PCR the amount of target sequence doubles. This exponential increase results in a great many copies of the target sequence in a very short period of time. Once the selected sequence has been amplified it is then detected through a format such as electrophoresis on an agarose gel.

The degree of specificity of a PCR-based detection test is dependent on the primers used. Primers can be chosen to target taxa at higher levels (e.g. all fungi) or lower levels (particular species or genotypes). To generate PCR-based detection tools, ribosomal DNA (rDNA) has been widely targeted because it contains both highly conserved regions and more variable regions, the internal transcribed spacer (ITS) and intergenic spacer (IGS) regions, respectively. RDNA is also present in high copy number in all organisms, thus enabling the development of sensitive tests. The number of sequences in publicly-available databases is far greater for this part of the genome than for any other region (Ward, Foster *et al.* 2004), enabling screening to check the specificity of primers. When rDNA sequence variation or conservation is not suitable for a particular target organism, primers which target other conserved regions of the genome can be used (Ma and Michailides 2007). For example, beta-tubulin genes were used to detect fungi in situations where primers based on rRNA genes were unsuitable because of interference by DNA of other organisms (Hirsch, Mauchline *et al.* 2000). Beta-tubulin genes have also been targeted in studies of fungicide resistance (Ward,

Foster *et al.* 2004). Primers that target mating type genes may also be used (Wallace and Covert 2000).

Whatever taxonomic level a PCR test is designed for, it is important that the test have specificity for the organism(s) of interest and not detect amplicons of other organisms (Ma, Luo *et al.* 2003; Schaad, Frederick *et al.* 2003). With rapidly expanding sequence databases available in the public domain, such as GenBank and EMBL (Williams, Ward *et al.* 2001), there is an increasing body of information on which to base primer design.

There are two main approaches in PCR. End-point PCR involves amplifying the selected nucleic acid sequence and then detecting it through electrophoresis on an agarose gel or some other detection format. Real-time or quantitative PCR (qPCR) involves measurement of the amplified sequence within a real-time PCR machine through accumulation of a fluorescent signal.

1.4.3.1 Quantitative PCR (qPCR)

QPCR, as its name implies, enables the quantification of target nucleic acid. The principle behind it is that the more target nucleic acid present before the commencement of PCR, the fewer amplification cycles required before the amount is increased above a threshold level (Ward, Foster *et al.* 2004). QPCR involves the binding of either a fluorescent dye or a fluorescently-labelled probe specific to a region within the amplified DNA. The accumulation of PCR product is monitored at the end of each PCR cycle through an increase in fluorescence and is related to a cycle threshold (C_t), defined as the number of amplification cycles at which the fluorescence is statistically greater than the background level. Because of slight variations in the number of cycles required to reach the C_t from one PCR run to the other, standard sample(s) of known quantities of target nucleic acid are included with each set of test samples, A calibration curve, or "standard curve", is constructed which relates the C_t to these standards. The quantity of target nucleic acid in the test samples is then determined in relation to the calibration curve.

In addition to allowing quantification, qPCR has several other advantages over end-point PCR. It is faster and allows higher throughput (Ward, Foster *et al.* 2004); it has a greater dynamic range, i.e. it can detect a greater range of starting concentrations (six orders of magnitude rather than two) (McCartney, Foster *et al.* 2003); and it does not require post-reaction processing. Furthermore, if a specific probe is used, it can be more specific than end-point PCR, sufficiently so to be able to detect single nucleotide

polymorphisms (Ward, Foster *et al.* 2004). This could be an important consideration in biosecurity where it may be necessary to be able to distinguish between endemic and exotic strains of an organism. QPCR requires more specialised equipment and is more expensive than end-point PCR (Ward, Foster *et al.* 2004), but over time the technology has become cheaper and has been adopted more widely.

1.4.4 Reverse-transcriptase PCR (RT-PCR)

A disadvantage of PCR is that it does not indicate whether the material detected is viable or not. One way to overcome this problem is to use reverse-transcriptase PCR (RT-PCR) to detect RNA instead of DNA (Ward, Foster *et al.* 2004). In comparison with DNA, RNA is inherently short-lived and is therefore a good indicator of viability. However, RNA cannot serve as a template for PCR. For this reason, a preliminary step is required in the RT-PCR reaction, the reverse transcription of RNA into cDNA. This can be done using the enzyme RNA-transcriptase in a separate reaction, followed by the transfer of cDNA into a second reaction for the PCR, or as a combined one-tube reaction using a single heat-stable polymerase able to function both as an RNA- and DNA-dependent DNA polymerase (Bustin 2000). The first option may be more sensitive, and has the further advantage that it enables the cDNA to be stored for later reactions. The second option minimises handling time and the risk of contamination. RT-PCR is more prone to error than PCR because RNA is unstable and difficult to extract (West, Atkins *et al.* 2008). Furthermore, the variable efficiency of the reverse transcriptase, and the need for two sequential enzymatic steps, tend to compromise its reproducibility (Bustin 2000). RT-PCR can be carried out using conventional end-point PCR or real-time quantitative PCR (q-PCR). The latter significantly simplifies the process, potentially leading to improvements in reproducibility (Bustin 2000). Nevertheless successful application depends on a comprehensive appreciation of the technical requirements of the techniques, and careful design, application and validation of results (Freeman, Walker *et al.* 1999); Bustin 2000).

1.4.4.1 DNA extraction

Prior to any DNA-based diagnostic test, cells must be disrupted and DNA extracted. Various methods have been used, including homogenisation of plant samples using a mortar and pestle or similar implement. However more robust methods are often required to extract DNA from spores. These include agitation of samples together with glass beads or ball bearings in a specially designed homogenising apparatus. A potential difficulty with extraction of DNA from plant, soil or air samples is the

possibility of co-extraction of contaminants that may interfere with the PCR. To overcome inhibition caused by contaminants, DNA purification is required and this can be achieved by through various published protocols (McCartney, Foster *et al.* 2003), such as that described by Stonard et al. (Stonard, Marchant *et al.*). Commercial DNA extraction kits (Ma and Michailides 2007), such as the UltraClean plant DNA isolation kit (Mo Bio Laboratories Inc., California, USA) may also be used. Inhibition is sometimes overcome by dilution of the sample to reduce the concentration of inhibitors. For example Driessen (2005) diluted (1:10 and 1:100) samples of DNA extracted from air samples in a commercial boronia plantation, in order to detect *P. boroniae* prior to PCR amplification. She found that some samples required 1:100 dilution before a PCR product was obtained. Ma & Michailides (2007) pointed out that, while dilution of DNA has been used successfully by many researchers to overcome problems with inhibition, it involves a loss of sensitivity of the PCR assay. Other methods to reduce inhibition have been developed. A detailed analysis of inhibition of PCR reactions is beyond the scope of the present study but the topic is dealt with in detail by other authors, for example Ma & Michailides (Ma and Michailides 2007) and Paterson (Paterson 2007).

1.4.5 Use of PCR to detect fungal spores in air samples

Both end-point PCR and qPCR have been used in numerous studies to detect the presence of, and, in the case of qPCR, to quantify, fungal spores in air samples (Peccia and Hernandez 2006); (West, Atkins *et al.* 2008). While initial studies, such as those of Wakefield (1996) and (Haugland, Vesper *et al.* 1999), related to human health, the methodology has also been developed for the detection of plant pathogens. Williams et al. (2001) and Calderon et al. (2002b) initially used the fungus *Penicillium roqueforti*, which is a saprophyte and is the main fungus used to make blue cheese, as a model with which they developed DNA extraction and end-point PCR procedures. Similar procedures were then applied to the detection of spores of the plant pathogens *Pyrenopeziza brassicae* (causal agent of light leaf spot of brassicas), *Leptosphaeria maculans* and the omnivorous plant pathogen *Sclerotinia sclerotiorum,* from Burkard Hirst-type spore traps in canola crops (Calderon, Ward *et al.* 2002a); (Freeman, Ward *et al.* 2002).

Sensitivity of PCR assays can be very high but this may depend on the content of the air sample. In the study by Williams et al. (2001), it was possible to detect single spores of *P. roquefortii* in the absence of other particles. However, against a background of 4,500

spores of unidentified species, plus dust and pollen, 1,000 *P. roquefortii* spores were required to detect the fungus consistently. In contrast, Freeman et al. (2002) were able to detect 100, and in many replicates 10, spores of *S. sclerotiorum* in the presence of a 40-fold excess of DNA from the closely related fungus, *Botrytis cinerea*, and Calderon et al. (2002a) were able to detect as few as 10 spores each of the canola pathogens *P. brassicae* and *L. maculans* against a background of four other fungal species. The latter authors were also able to detect DNA from an estimated 1 to 30 spores of *L. maculans* in samples from a volumetric spore trap placed on open ground surrounded by infested canola stems. Similarly, Ma et al. (2003) were consistently able to detect as few as two conidia of *Monilinia fructicola*, the causal agent of brown rot of stone fruits, in spore trap samples from a prune orchard. This was achieved following dilution of the DNA extracts to overcome the effect of inhibitors, and by using nested PCR. Thus, with the use of appropriate spore disruption and DNA purification methods, a sensitivity of around 1 to 10 spores in the absence of other particles, and between 2 and 1,000 spores for air samples collected in the field, has been achieved using end-point PCR.

Specificity testing for PCR assays to be used on air samples has been addressed in various ways by different researchers. As mentioned previously, when developing PCR assays, sequences specific to the target organism(s) are chosen by examination of sequence information in public databases. Sequences are compared with those of related species from a range of regions. The assays may then be tested on environmental samples. For example, in the study by Williams et al. (2001), specificity was determined in the first instance by comparing sequence information of *P. roquefortii* with those of related and other species in Genbank or EMBL databases. Specificity was then tested using DNA extracted from 46 species (30 genera) of fungi from various culture collections. Finally, to test for inhibition, the assays were performed on DNA extracted from suspensions of air samples taken in greenhouses which contained a range of plants species, spiked with serial dilutions of *P. roquefortii* spores. These suspensions had many fungal spores and other particles from the greenhouses. No DNA of *P. roquefortii* was detected in any sample to which *P. roquefortii* spores had not been added, thus confirming the specificity of the assays. In the study by Fraaije et al. (2005), specificity of the PCR assay for *M. graminicola* was tested by applying the assay to DNA from a panel of fungi from leaves and aerosols not containing the pathogen. Other researchers testing specificity of PCR assays on environmental samples have limited the testing to DNA from a range of closely related

species (Carisse, Tremblay *et al.* 2009), or to other species associated with the same host plants as the test organism (Ma, Luo *et al.* 2003). As well, PCR results can be validated using another method such as microscopy. For example, in the study of Calderon et al. (2002a), duplicate spore trap tapes were created, one for microscopic examination, the other for PCR assay, by bisecting daily tape samples longitudinally. DNA of *L. maculans* was detected on duplicate tapes in 41 out of 54 field-collected samples found to contain *L. maculans* spores when examined microscopically (76%). No DNA of *L. maculans* was detected in the duplicate tapes of any of the remaining 52 tapes that had no *L. maculans*-like spores when observed under the microscope. These tapes did however have many different spore types and other particles, such as pollen. Although the primary purpose of this testing was to determine the sensitivity of the assays, the results also indicated their specificity. Such verification by microscopic examination is frequently problematic however. As previously mentioned (Section 1.4.1), enumeration of spores in air samples is time-consuming and often difficult due to the presence of other particles, and many fungi are not readily distinguishable on the basis of spore morphology. These problems are illustrated by the findings of Driessen (Driessen 2005), who conducted molecular analysis and microscopic examination, respectively, on two halves of 56 daily spore trap tape samples from a commercial boronia plantation. Of 12 tape segments that did not show basidiospores of *P. boroniae* when examined microscopically, ten duplicate tape segments gave negative results when tested using the molecular assay, as expected, but two tested positive, suggesting some cross-reactivity of the assay. Furthermore, of 44 samples on which *P. boroniae* basidiospores were observed microscopically, only 23 duplicate samples produced a nested PCR product which was positive for *P. boroniae* by restriction fragment length polymorphism (RFLP) analysis. These discrepancies were attributed to a number of factors, including inaccuracies in microscopic counting, potential differences in the numbers of spores on the two duplicate tape halves, and one or more steps in the nested PCR-RFLP analysis, *viz* DNA extraction, primer design and amplification parameters.

Quantitative PCR has been used to quantify spores of plant pathogenic fungi in air samples in numerous studies, including those of Fraaije et al. (2005), and Carisse et al. (2009). Rogers et al. (2009) used qPCR to quantify ascospores of *S. sclerotiorum* from Burkard Hirst-type spore traps and Fountaine et al. (2010) used spore traps together with qPCR to determine the relative importance of airborne inoculum of *Rhynchosporium secalis* in the initiation of epidemics of leaf blotch of winter barley.

The techniques have also been used in horticultural settings. For example, Luo et al. (2007) used Burkard Hirst-type spore traps and qPCR to monitor spore densities of *Monilinia fructicola* in stone fruit orchards.

Many researchers have validated the results obtained from qPCR assays through microscopic examination of spiked samples and/or of field-collected air samples, as discussed above in relation to end-point PCR. Some researchers have reported considerable variability in the relationship between the amount of DNA obtained using qPCR and spore counts conducted microscopically. For example Fraaije et al. (2005) found poor correlation ($R^2 = 0.12$) between the number of *M. graminicola* spores in air samples measured using qPCR and microscopy. In comparison, Luo e al. (2007) obtained a linear relationship between number of spores of *M. fructicola* counted microscopically on segments of Melinex tape, and the corresponding number of spores on duplicate tape segments determined with a qPCR, but the degree of fit to the regression line was poor ($R^2 = 0.60$). Others have reported a good correlation when comparing qPCR results with microscope counts of spores applied to samples in the laboratory. McDevitt et al. (2005) for instance reported a significant ($R^2 = 0.91$) linear relationship between qPCR and direct estimates of green fluorescent protein-expressing *Aspergillus fumigatus* conidia using fluorescence microscopy, in air samples collected onto filters. Carisse et al. (2009) reported a good correlation between yields of qPCR and microscope counts of *Botrytis squamosa* conidia in spiked samples prepared in the laboratory. However, correlations were not as close when the qPCR assay was used for the detection of airborne inoculum of *B. squamosa* in commercial onion fields. Sometimes the relationship has been poor even for spiked samples. Rogers et al. (2009) for instance, reported variability in the relationship between estimates of *S. sclerotiorum* ascospore numbers on artificially inoculated tape segments detected by qPCR and by microscopic counting ($R^2 = 0.76$). The discrepancies reported in these studies were attributed mostly to difficulties in microscopic counting of spores in air samples, although Luo et al. (2007) also noted that slight loss of spores in the DNA extraction process may have caused variation among samples. These reports demonstrate both the difficulties of quantifying spores in air samples by microscopy, and the potential of using qPCR and air sampling for studying the spatial and temporal distribution of airborne spores.

1.5 Use of spore trapping and PCR in biosecurity

A combination of air-sampling and PCR-based diagnostic methods is being used for various purposes, but there have been only a few reports in the literature of the use of these tools to monitor for plant pathogens of biosecurity concern.

A PCR assay was used in combination with spore trapping using a Burkard Hirst-type spore trap to track the spread of the sugarcane smut pathogen (*Ustilago scitaminea*) in Queensland, following its first detection in 2006 (Magarey, Bade *et al.* 2009a; Magarey, Braithwaite *et al.* 2008a). The researchers employed end-point PCR and the results were therefore qualitative rather than quantitative. The technique was found to be useful as an early warning tool, providing the first indication of the disease in several important sugarcane production areas. Spore trap results were later confirmed in a number of these areas by the subsequent detection of smut symptoms in sugarcane crops. The researchers used universal ITS primers in an endogenous test to check for inhibition of the molecular assay, and found that additional DNA purification steps were required for samples contaminated with ash or dust. However, samples from some sites could not be analysed because the amount of dust generated by haulage equipment in the vicinity of trap was too great; this was unfortunate as one of these sites was later found to have been in the midst of a smut infestation (Magarey, Bade *et al.* 2009a). Heavy rainfall during the wet season was also found to reduce atmospheric smut spore densities, necessitating further sampling in clear weather. The authors suggested that weather factors be taken into consideration in determining the timing of spore trapping. A further logistical difficulty was delay associated with the molecular assays. Consequently, smut symptoms were observed prior to the spore trap results at some sites. Nevertheless the spore trapping proved a valuable tool in assisting the industry to identify areas in which the disease was likely to be present. The technology provided early warning, which could be used by farmers to implement disease management plans, in particular transition to resistant varieties. The authors suggested that further use of the technology might include monitoring in heavily infested areas to determine the peak of the epidemic, and to gain more information about the potential for varieties of intermediate resistance to withstand inoculum. This would require development of qPCR assays to allow quantification of atmospheric spore densities.

In another biosecurity-related study, (Barnes, Szabo *et al.* 2009) applied a nested qPCR assay to quantify DNA from spores of the Asian soybean rust pathogen, *Phakopsora*

pachyrhizi, in rain samples. This technique was used to monitor long-distance dispersal (greater than 100 km) of the pathogen, which had recently been introduced into the USA, into soybean growing areas in which it does not overwinter. In laboratory simulations with artificially inoculated samples, the test was able reliably to detect two or more spores and, in 67% of cases, one spore. When applied to field samples the assay was sufficiently sensitive to detect the first deposition of *P. pachyrhizi* spores in a given area, as later confirmed by disease reports. To verify results, the researchers analysed all positive samples by gel electrophoresis. Contamination was a concern in the use of the nested qPCR assay, and this was addressed through implementation of hygiene measures in the laboratory, and monitoring through the use of negative controls. Use of the qPCR assay to test weekly rain collections from sites across the soybean growing area of central and eastern USA enabled temporal and spatial distribution of the pathogen to be tracked over a two-year period, and provided evidence to support models suggesting the pathogen was largely restricted to southern parts of the USA during winter and spring. A disadvantage of using a DNA assay was that it did not provide information about the viability of the spores. However the long distances between isolated fields in some parts of the study areas provided indirect evidence of long-distance dispersal of *P. pachyrhizi*.

A monitoring network, similarly based on detection of spores in rain samples, is also in place in the USA to monitor for cereal rusts (http://www.ars.usda.gov/Main/docs.htm?docid=14574), some strains and species of which, e.g. wheat stem rust (*Puccinia graminis* f. sp. *tritici* pathotype Ug99) pose an important biosecurity threat to many countries around the world (http://www.planthealthaustralia.com.au/pidd-docs/Ug99%20FS.pdf).

These studies have shown the value of spore trapping combined with PCR-based tests for biosecurity-related studies, and the potential to use these tools for epidemiological research to improve management of newly introduced pathogens. However, they have revealed some weaknesses in the system, such as the fact that the viability of inoculum detected using these tests is not known, and the need to factor weather considerations into timing of sampling. Jackson & Bayliss (2011) highlighted the need to incorporate data collection relevant to prevailing climatic conditions and optimal sampling times. Those authors also noted that information on how long spore samples can be stored is lacking, and that there is a need to develop protocols for sampling and for processing

and storage of spores collected during surveillance for pathogens of biosecurity significance.

1.6 Storage of spore trap samples

Storage of spore trap samples prior to extraction of DNA may be required for various reasons, such as to enable samples to be processed in bulk, or to enable sample selection at the end of a season when the likely duration and peak of spore production may be better understood. Little information was found in the literature regarding the impact of storage on yield of DNA from spores in air samples. Many researchers reporting application of PCR assays to air samples, for example Calderon et al. (2002a), Carisse et al. (2009) and Fountaine et al. (2010), appear to have extracted DNA from spore trap samples without prior storage. Luo et al. (2007) stored spore trap samples, collected in a stone fruit orchard to sample *Monilinia fructicola,* at 4°C until DNA extraction. Some researchers have reported freezing spores to store them before DNA extraction. For example, Rogers et al. (2009) froze ascospores of *Sclerotinia sclerotiorum* prior to inoculating them onto plastic tape segments and extracting DNA from them, and Kaczmarek et al. (2009a) reported storing field-collected spore trap samples at -20°C prior to extraction of DNA and performance of qPCR assays to detect *Leptosphaeria* spp. Others have frozen samples in liquid nitrogen. Falacy et al. (2007) extracted DNA from air samples that had been desiccated, flash frozen in liquid nitrogen and stored at -70°C (method as described by Stummer et al. (1999)), and used end-point PCR to detect DNA from the grape powdery mildew fungus, *Erysiphe necator*. However, in none of these studies was the duration of storage stated, and the researchers did not comment on whether storage of samples frozen affected the yield of DNA quantified by qPCR.

DNA content of spores may be linked with their viability, and considerable research has been conducted on viability of spores following freezing and storage. Most studies have related to lyophilisation (freeze-drying) or storage in liquid nitrogen, both of which are reported to allow fungal cultures to remain viable in long-term storage (40 years or more) (Ryan and Smith 2004). However, lyophilisation is a complex and time-consuming method, and both methods require expensive equipment. Furthermore, storage in liquid nitrogen usually requires the addition of cryoprotectants, such as glycerol or trehalose, which may be toxic to some fungi (Ryan and Smith 2004), and which could potentially affect subsequent DNA extraction and PCR. Stummer et al. (1999) reported that air-dried conidia of *Uncinula* (syn. *Erysiphe*) *necator*, retained

viability following cryopreservation in liquid nitrogen at -70°C, without the use of cryoprotectants, for up to one year, and survived five cycles of freezing and thawing. Furthermore, these cryopreservation procedures did not appear to cause any genomic rearrangements. This would potentially be a suitable method of storage of air samples. However, a simpler and more accessible method such as freezing at – 20°C, which is the approximate temperature of most household freezers, would be more convenient, and less expensive. Biddulph et al. (1999) reported that ascospore suspensions, from *L. maculans* pseudothecia which had been frozen for up to one month, infected oilseed rape leaves with an estimated infection efficiency of approximately one in four. It was not clear whether this represented any loss in viability of the spores as infection efficiency was not measured prior to storage. Toscano-Underwood et al. (2001) reported that suspensions of *L. maculans* ascospores which had been frozen at -5°C for 3 weeks were able to produce infections on all inoculated oilseed rape plants. There was no indication from these studies that spore viability was reduced by storage of the spores frozen. Research is required to ascertain whether storing spores frozen affects their DNA content.

1.7 Effect of heat and relative humidity on yield of DNA from air samples

Both temperature and relative humidity may affect recovery of spores from traps (McCartney, Fitt *et al.* 1997). This may occur via temperature effects on the adhesive used in the spore trap, or through direct effects of temperature and humidity on the spore viability or germination. The viability and recovery of spores will then impact on the amount of DNA detected in spore traps.

Exposure to heat leads to reduced spore viability and this may be associated with a decline in DNA, even though the impact of the heat may be on other aspects of cell biology, such as protein denaturation, destruction of hormones or other metabolic injury (Barkai-Golan and Phillips 1991). The degree to which heat reduces spore viability depends on various factors, including the moisture content of the spores, metabolic activity (particularly whether or not the spores have commenced germination), nutrient availability and the species of fungus (Barkai-Golan and Phillips 1991).

Temperature tolerance of fungal spores is strongly linked to the amount of moisture either before or during exposure to heat, with wet heat being more damaging to spores than dry heat. For example, Barkai-Golan & Phillips (1991) reported that moist conidia of *Penicillium digitatum* were more susceptible to exposure to 70°C than dry conidia.

After 30 minutes, 90% of the moist conidia, but only 10% of the dry conidia, had been killed. Similarly, Schein (1965) measured viability of uredospores of *Uromyces phaseoli* in humidity vessels exposed in the laboratory to all combinations of temperature in the range of 5 to 33.5^{o}C and relative humidity in the range of for 31 to 95%. Viability was inversely proportional to both temperature and relative humidity. High humidity might also result in spore germination, with a concomitant increase in DNA content.

Tolerance to heat varies between fungal species. For example, Karabulut (2002) found that spores of *Monilinia fructicola* were more sensitive than those of *Penicillium expansum* to temperatures between 50 and 60°C. Therefore measured effects on one species cannot necessarily be extrapolated to other species.

In conclusion, DNA yield from spores captured in air samples may be affected by the temperatures and relative humidities to which they are exposed in the field. However, there is little information available to determine under what conditions these effects are likely to be manifest, or to quantify these effects.

Other aspects of the interaction between fungi with airborne spores and the environment in which they are captured, such as the dynamics of spore release, are also important when considering surveillance and management of biosecurity pathogens.

1.8 Epidemiological models

The need for information relating to pathogen dynamics in biosecurity has been recognised by several authors. Jackson & Bayliss (2011) noted the need for research on data collection methods relevant to spore capture times and climatic conditions. Spore trapping has been suggested as a tool to understand the epidemiology of *Ustilago scitaminea*, newly introduced to Australia (Magarey, Bade *et al.* 2009a). Barnes (2009) used the data generated by PCR-based detection of soybean rust spores in rain samples to support models which suggested that the pathogen was largely restricted to warmer areas of the USA during winter and spring.

One means of describing disease epidemiology is by mathematical equations or models. Mathematical models may be used to describe in a formal way how a disease develops in space and time, in order to determine the relative contribution of various factors to disease development (Tivoli and Banniza 2007). For example, Zhang (2004) used epidemiological models to describe the spatial and temporal dynamics of

Mycosphaerella blight (syn. ascochyta blight or blackspot), caused by *D. pinodes*, in field pea in western Canada. The rate, direction and distance of spread were described by logistic models, with disease gradient explained by wind speed and direction. Epidemiological models may also be used to predict one or more events in the life cycle of the pathogen, usually one of significance in controlling the disease. This is illustrated by (Schoeny, Jumel *et al.* 2007) who developed a disease-onset model for ascochyta blight of field peas in France, based on weather-dependent variables. Daily infection values were calculated based on temperature and moisture requirements for initiation of disease, which had previously been determined experimentally. Cumulative daily infection values were then calculated from sowing date, and were used to predict the onset of disease. Predictive models may be based on hypotheses about the mechanism(s) causing disease development, or they may be empirical, with the development of mathematical equations that best fit the data (Tivoli and Banniza 2007). Salam et al. (2003) used a combination of these approaches when developing Blackleg Sporacle, a predictive model for release of ascospores of *Leptosphaeria maculans*. In that model some parameters, such as "*T_threshold*" (the upper limit of the mean daily temperature required for conditions to be favourable for pseudothecial maturation), were based on published data and refined by fitting a range of values to measurements from a field site over several seasons. Other parameters such as "AD-fraction" (the fraction of mature ascospores released during an ascospore discharge event) were set with no prior basis but gave the best mathematical fit of the model to calibration data.

The processes governing events in the pathogen lifecycle captured in epidemiological models are often complex and may be incompletely understood. Furthermore, the timing of such events may differ between seasons at a location and between locations within a season (Salam, Fitt *et al.* 2007). To compensate for inaccuracies in model predictions, and variations between sites and seasons, a process of fitting the model to field data (testing and calibration) is undertaken. For any one site, ideally data from at least three seasons with variable weather conditions is used for model calibration (Salam, M.U., Department of Agriculture and Food, Western Australia, personal communication, 2010).

Epidemiological models are often based on previously developed models, modified to suit the situation. For example, the model described by Schoeny et al. (2007) was based partly on initial work by other authors (Shane and Teng 1983; Wolf and Verreet 2005)

cited in (Schoeny, Jumel *et al.* 2007) on predictions of infection by *Cercospora beticola*. The Blackleg Sporacle model (Salam, Khangura *et al.* 2003) was the precursor of the models "Improved Blackleg Sporacle", "SporacleEzy" (Salam, Fitt *et al.* 2007), and G1 Blackspot Manager (Salam, Galloway *et al.* 2011).

Understanding the dynamics of plant pathogens of biosecurity concern is key to successful surveillance and management. Spore trapping is a means of generating data required for modelling pathogen dynamics. Furthermore, comparison of data generated from spore trapping with predictions of spore release from epidemiological models, offers a means of testing the spore trapping methodology. Both the spore trapping and the model predictions can be validated by a further monitoring tool such as the use of trap plants.

1.9 Model pathogens

In order to develop air sampling methodology appropriate for bioscecurity surveillance, three ascomycetous pathogens of pulse and oilseed crops (*Leptosphaeria maculans*, *Didymella pinodes* and *Didymella rabiei*) endemic to South Australia, were used as models in the research described in this thesis. These pathogens were chosen because they were likely to occur at varying concentrations of ascospores at the chosen trial site, specific PCR assays were available, and mathematical models to predict timing of release of airborne spores had been, or were being, developed for each of them.

Of these three pathogens, *D. pinodes* produces the highest concentrations of airborne ascospores in South Australia, followed by *L. maculans*, whereas *D. rabiei* ascospores have been recorded only once from chickpea stubble incubated in South Australia (Galloway, J., Department of Agriculture and Food, Western Australia, personal communication). *L. maculans* has large ascospores which are readily visually distinguishable on the Melinex tapes used in the spore trap, and the methodology of spore trapping combined with PCR-based diagnostics has been demonstrated to be suitable for this pathogen (Calderon, Ward *et al.* 2002a).

1.9.1 Leptosphaeria maculans

Leptosphaeria maculans (Desm.) Ces. Et de Not. (anamorph *Phoma lingam* (Tode: Fr.) Desm.) is part of a disease complex which causes blackleg (syn. phoma stem canker) of canola (oilseed rape, *Brassica napus*). The disease was initially thought to be caused by two strains of *L. maculans*, a virulent and a weakly virulent strain (*L. maculans* A-group and B-group, respectively), but these are now classified as two species, *L. maculans* and

L. biglobosa (Williams and Fitt 1999); (Shoemaker and Brun 2001). *L. maculans* is usually the more damaging of the two pathogens, being associated with basal (crown) stem cankers, while *L. biglobosa* is more often associated with upper stem lesions (West, Balesdent *et al.* 2002); (Fitt, Huang *et al.* 2006).

Blackleg is an important disease of canola. While it normally results in yield losses of less than 10% (West, Kharbanda *et al.* 2001), it has also been been reported to cause severe, widespread epidemics in Canada, Europe and Australia (Gugel and Petrie 1992). It is considered the most important disease of canola in Australia (Marcroft, Sprague *et al.* 2004), where canola cultivation has increased significantly over the past few decades. *L. maculans* is present in most major canola producing areas (Australia, Europe and North America), but not in China (Fitt, Hu *et al.* 2008).

L. maculans infects cotyledons, leaves, stems, roots, pods and seeds of canola. When seedlings are infected before or after emergence, they may be killed and the symptoms resemble damping-off (Gugel and Petrie 1992). Infection arising from infected seed is initially seen as distinct round lesions with numerous pynidia on the cotyledons (West, Kharbanda *et al.* 2001). Leaf lesions, arising from infection by ascospores, first appear as pale green spots, which enlarge to 1-2 cm diameter and become pale brown, or greyish to dirty-white (Gugel and Petrie 1992); West et al, 2001). Eventually the centre of the lesion may break or fall out. Pycnidia, appearing as pinhead-sized black dots, form in the dead tissue in the centre of the lesion. The fungus spreads asymptomatically from cotyledon or leaf lesions down the petiole and into the stem where it forms stem lesions. These interrupt the flow of nutrients and water, causing the plant to mature early, and may result in smaller and shrivelled seeds (Gugel and Petrie 1992). If infected at a young stage, the stem lesion may girdle the stem and cause the plant to lodge. Stem lesions typically have a purple or dark brown margin and are associated with leaf scars. They may expand and coalesce during pod set and crack open to form dry rots or stem cankers (West, Kharbanda *et al.* 2001). Stem lesions and cankers (Figure 1-2) are usually the most damaging symptom of the disease.

The life cycle of *L. maculans* is shown in Figure 1-3. Ascospores, which are the most common primary inoculum, are produced by the heterothallic fungus in pseudothecia on canola stubble. They are released after wetting by rain, heavy dews or high humidity (McGee 1977), and infect cotyledons and leaves of young plants. Pycnidia are formed on lesions on the cotyledons, leaves and stems, and these produce conidia of *Phoma*

lingam, which act as a secondary inoculum source, infecting leaves, stems and seed pods (and thereby seeds). After harvest the pathogen survives as a saprophyte on stubble. Pycnidia and pseudothecia are formed on the stubble and release spores which infect the next crop. Ascospores are wind-dispersed and may be carried several kilometres, whereas conidia are splash-dispersed over short distances, up to several metres in windy conditions.

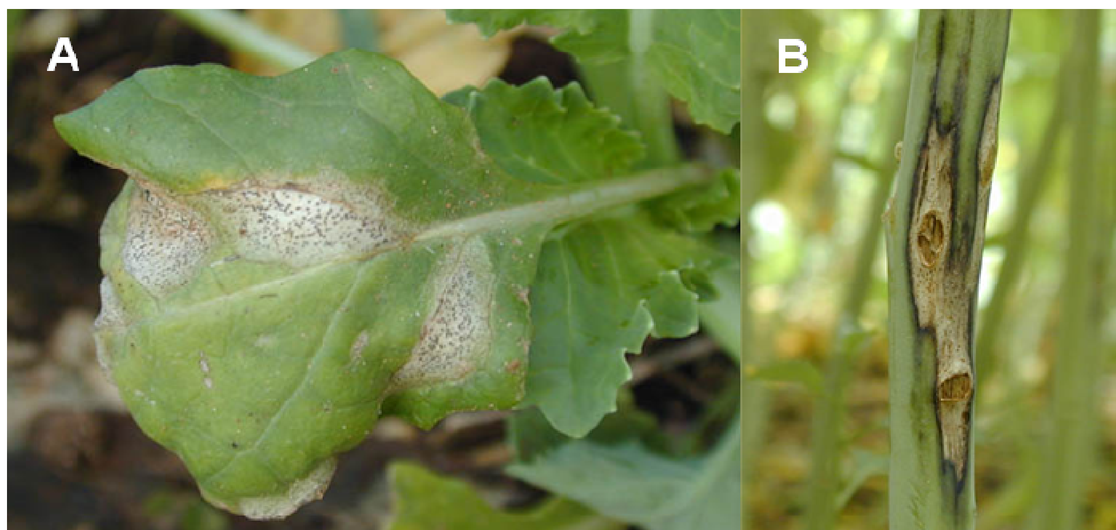

Figure 1-2 Symptoms of *L. maculans* infection on canola A. Leaf lesions B. stem canker (Sosnowski Mark 2002)

Ascospores are 50 (35-70) x 6 (5-8) µm in size, cylindrical to ellipsoidal, with ends mostly rounded, yellow brown, guttulate, and may be slightly constricted at the central septum (Punithalingam and Holiday 1972b).

In addition to canola, *L. maculans* has been reported from a range of other, mostly cruciferous, hosts, which may form an alternative source of inoculum. The pathogen does not survive more than a few months in soil (Hall 1992).

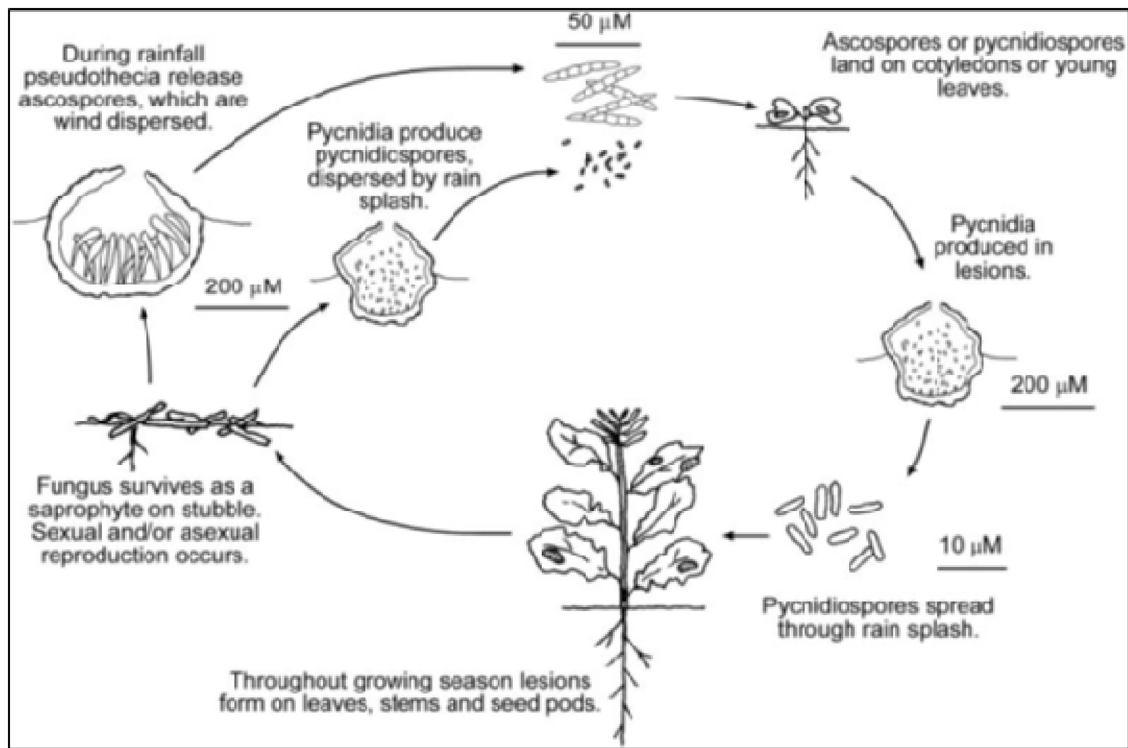

Figure 1-3 Life cycle of *Leptosphaeria maculans* on *Brassica napus* (Howlett, Idnurm *et al.* 2001).

Measures to control blackleg include the use of resistant cultivars, crop rotations, stubble management, use of disease-free seed and fungicides (West, Kharbanda *et al.* 2001). Breeding for resistance is a key component to management of the disease. However, durability of resistance has been problematic, due to rapid changes in the pathogen population, and major-gene resistance has been overcome in France and Australia (Fitt, Brun *et al.* 2006); (Sprague, Balesdent *et al.* 2006). *L. maculans* has a high propensity for rapid shifts in the population gene pool because it has an annual cycle of sexual recombination resulting in ascospores which are widely dispersed, and also produces large numbers of conidia each season. For this reason, it is important to incorporate more durable quantitative resistance into new cultivars (Fitt, Brun *et al.* 2006), and work is underway to achieve this (Brun, Chèvre *et al.* 2010); Jestin et al., 2011). Cultural methods, as noted above, are also important, in order to reduce inoculum potential. Destruction of stubble has traditionally been achieved by cultivation, but with increasing adoption of minimum tillage systems, other methods have been used, including burning (in Australia), chopping, slashing and/or harrowing to break up the stubble (in Europe, where burning is prohibited) (Gladders et al., 2006). Crop rotations of up to 4 years have previously been recommended (West et al., 2001). However, a one-year crop break, combined with isolation distance of 500 m from 6-

month-old canola stubble has been shown to be sufficient to reduce inoculum to manageable levels (Gossende et al., 2003, cited in Gladders et al., 2006; Marcroft et al., 2004), and *L. maculans* inoculum in soil has been shown to decline to negligible levels 3 years after cropping with oilseed rape in South Australia (Sosnowski et al., 2006). Early planting to avoid the most damaging symptoms (Hammond & Lewis, 1986) is also recommended (Gladders et al., 2006).

Fungicide usage to control blackleg varies depending on whether the canola crop is high yielding (Western Europe) or low yielding (Canada, Australia) (West et al., 2001). In Australia, fungicide applied either as a seed dressing or as a coating on fertiliser, which is placed below the seed at sowing, is used for most canola crops (Marcroft & Potter, 2008). Foliar fungicides may be economical in high yielding areas when inoculum levels are high and the canola cultivar is susceptible but, because fungicides with eradicant ability have not been available, several applications are usually needed (West et al., 2001). Timing of fungicide use could be improved through the use of forecasting schemes to predict risk (West et al., 1999).

A number of epidemiological models have been developed which describe and forecast aspects of the life cycle of *L. maculans* and relate these to weather conditions. Bernard et al. (1999) described two preliminary models developed for use in France, incorporating the effect of temperature, rainfall and humidity on pseudothecial maturation. The Blackleg Sporacle model (Salam et al., 2003) was developed for Australian conditions and uses daily mean temperature and daily total rainfall to predict timing of pseudothecial maturation and ascospore release. Improved Blackleg Sporacle and Sporacle Ezy (Salam et al., 2007) were developed for international use with both *L. maculans* and *L. biglobosa*. Huang (2007) developed a model which uses temperature and rainfall data for August and September to predict the first major ascospore release in the UK. The purpose of all these models is to assist in formulating strategies for management of blackleg disease.

1.9.2 Didymella pinodes

Didymella pinodes Berk. & Blox. [Petrak] (syn. *Mycosphaerella pinodes* [Berk. & Blox.] Vestergen; anamorph: *Ascochyta pinodes* L.K. Jones) is one of several pathogens in a fungal complex which causes ascochyta blight (blackspot) of field pea (*Pisum sativum*), the others being *Phoma medicaginis* Malbr. & Roum. var. *pinodella* (Jones) Boerema (syn. *Ascochyta pinodella* L.K. Jones; *Phoma pinodella* [L.K. Jones] Morgan-

Jones & KB. Burch), *Ascochyta pisi* Lib., and *Phoma koolunga* (Davidson et al., 2009a; Salam et al., 2011a).

Ascochyta blight is a common disease of peas worldwide, and has been reported to cause yield losses of between 10 and 75% (McDonald & Peck, 2009). *D. pinodes* is considered the most aggressive pathogen in the complex and causes the most economic loss (Kraft John et al., 1998). Symptoms consist of small purple lesions on leaves, stems and pods, which expand under moist conditions and become brown-black with a zonate appearance. As plants mature, affected leaves dry out, the whole lower stem may be girdled by lesions, and the lower plant generally takes on a blackened appearance (Bretag et al., 2006). *D. pinodes* is the only fungus in the ascochyta blight complex which produces ascospores (Kraft John et al., 1998; Salam et al., 2011a). The ascospores are 15 (12-18) x 6 (4-8) µm in size, hyaline, ellipsoid, guttulate, constricted at the septum and rounded at the ends (Punithalingam & Holliday, 1972c).

The lifecycle of *D. pinodes* (Figure 1-4) is similar to that of *L. maculans* (Section 1.9.1). Aerial inoculum from infested pea residues is the main source of infection (McDonald & Peck, 2009), although seed infection and soil-borne inoculum (dormant mycelium, sclerotia or chlamydospores) are also important (Bretag et al., 2006). Ascospore release from pea residues occurs mainly with autumn rain before or early in the growing season. As the disease progresses, pycnidia are produced on fresh and senescent pea tissue, leading to the release of conidia, which are splash-dispersed. Unlike *L. maculans*, both pycnidia and pseudothecia form on senescent tissue resulting in the release of conidia and ascospores during the growing season (Hare & Walker, 1944; Carter & Moller, 1961; Roger & Tivoli, 1996). Most ascospores are released in response to rainfall events, although some are also released with dew (Carter, 1963; Bretag, 1991; Zhang et al., 2005). A diurnal rhythm to ascospore release has been reported, with peak release in the afternoon (Carter, 1963; Bretag, 1991).

Didymella pinodes has been shown to attack a range of legumes other than pea in host range studies in the glasshouse, but the importance of other hosts in the field is not clear (Bretag et al., 2006). The most commonly infected alternative hosts are *Lathyrus* and *Vicia* (Lawyer, 1984).

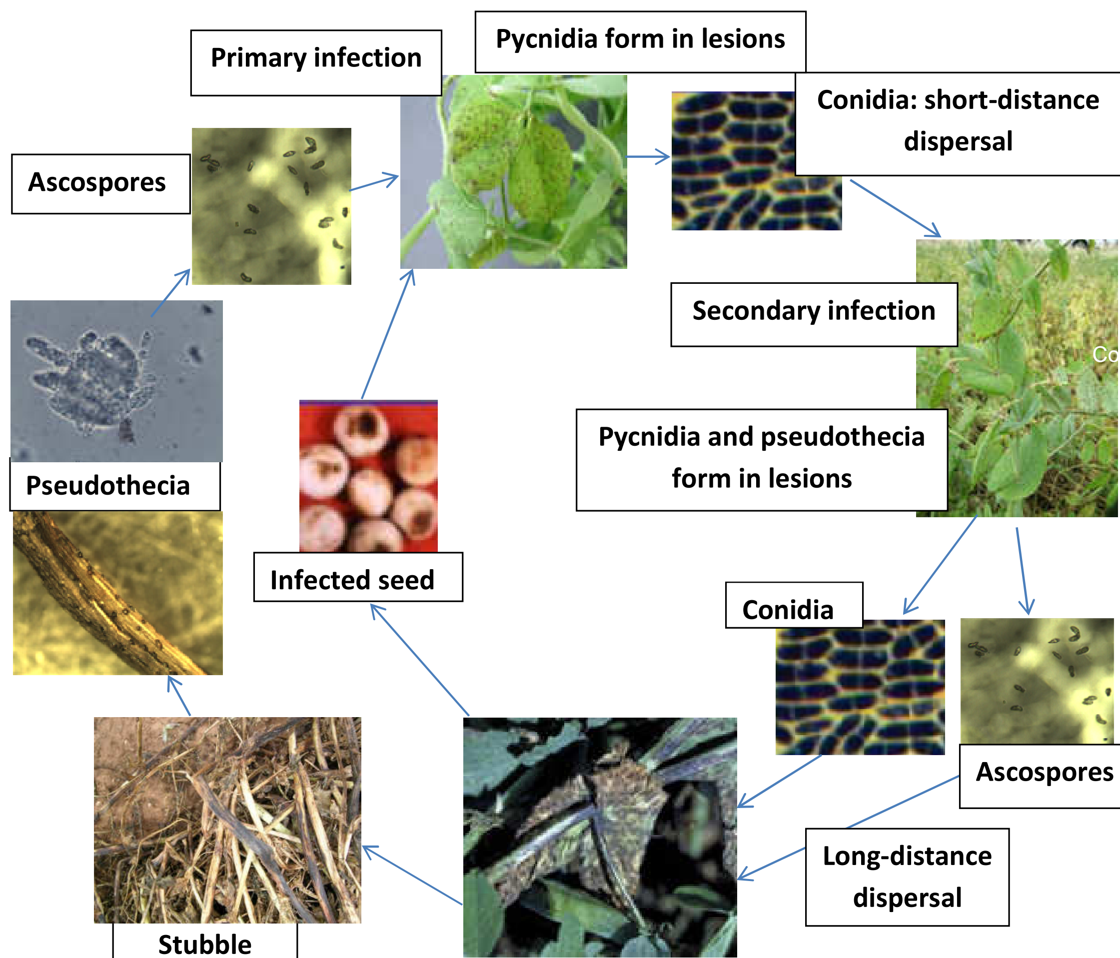

Figure 1-4 Disease cycle of ascochyta blight (*Didymella pinodes*) on pea (adapted from Tivoli & Banniza, (2007), including images courtesy Davidson, J.A., SARDI)

A number of different methods of disease control are practised. Seed treatment, particularly with systemic fungicides such as those in the benzimidazole group, is effective at controlling ascochyta seed infections and is widely used in most pea-growing regions (Bretag et al., 2006). Foliar fungicides are used in regions where the crop is sufficiently valuable, such as in France, but, as several applications are required to control the disease effectively (Bretag, 1985), their use is not economic in areas where yields are relatively low, such as Australia (Bretag et al., 2006). Resistance breeding for all pathogens in the ascochyta blight complex is difficult and few good sources of resistance are available to date (Bretag et al., 2006; McDonald & Peck, 2009), although there is resistance to *A. pisi*. Disease management can be achieved through various cultural methods including the use of disease-free seed, crop rotation and avoiding planting adjacent to previous pea crops. Another method is the strategic timing of crop sowing to minimise exposure to inoculum originating on pea residues from the previous season. In most cases, early-sown crops are more likely to be

exposed to high numbers of ascospores than later-sown crops. Delayed sowing can be used to avoid peak spore release and minimize disease. However there may be a yield penalty associated with the shorter growing season (McDonald & Peck, 2009). In some cases late sowing may be associated with increased disease (Hare & Walker, 1944), particularly if the crop is exposed to ascospores produced during the growing season in nearby early-sown pea crops (Bretag et al., 2006). The main control method practised in Australia is to delay sowing to 4-6 weeks after the first rains (Davidson & Ramsey, 2000; Bretag et al., 2000). In order to better time sowing date to minimise losses, a computer simulation model 'G1 Blackspot Manager' has been developed which uses daily mean temperature and daily total rainfall to predict the timing of pseudothecial maturation and ascospore release (Salam et al., 2011a). This model is based on ascospore release data from stubble collected on a weekly basis from various sites, wetted for 5 minutes and placed in a wind tunnel. A second generation (G2) of the Blackspot Manager model predicts disease severity and yield loss based on ascospore release predictions generated using the G1 module (Salam et al., 2011b). Release of *D. pinodes* ascospores has not previously been measured directly in the field and spore trapping combined with PCR-based diagnostics provides an opportunity to do so.

1.9.3 Didymella rabiei

Ascochyta rabiei (Pass.) Labrousse (teleomorph *Didymella rabiei* (Kovachevski) Arx), causes ascochyta blight of chickpeas (*Cicer arietinum* L.), a major disease around the world. The disease has been reported to cause losses ranging from 5 to 100% (Haware et al., 1998). In Australia, it severely curtailed the development of the chickpea industry after first detection of the disease in commercial crops in 1999, and is particularly difficult to manage in winter-dominant rainfall areas of southern Australia (Bretag et al., 2008).

Ascochyta blight affects all aerial parts of the chickpea plant. Initial symptoms on leaves are small water-soaked spots which expand rapidly to form round, grey lesions with brown margins and concentric rings of black pycnidia in the necrotic tissue at the centre (Pande et al., 2005). Lesions coalesce under favourable conditions causing a blighting of the foliage. Stem lesions are elongated, and may girdle the stem, causing it to break. Pod lesions are usually round, up to 0.5 cm in diameter and with concentric rings of pycnidia (Galloway, 2000). Diseased pods may lead to seed infection, but more typically cause seed abortion, resulting in direct yield reductions (Galloway, 2000).

The pathogen survives either on seed or on plant debris. Infected seed is one of the main sources of infection and is an important means of long-distance dispersal and establishment of compatible mating types (Kaiser, 1997; Pande et al., 2005). Pycnidia, either on stem lesions resulting from seed infection, or on plant debris, release conidia which are splash-dispersed to infect the newly emerging crop (Galloway, 2000; Shtienberg et al., 2005). Conidia from lesions formed in the crop act as secondary inoculum. Pseudothecia have not been reported on newly infected plants (Pande et al., 2005), but form on plant debris under cool, moist conditions (Trapero-Casas & Kaiser, 1992a). Ascospores are discharged during winter through to spring in Spain and Israel (Trapero-Casas et al., 1996; Shtienberg et al., 2005), with timing depending on moisture and pseudothecial maturity, and cause outbreaks of the disease in crops sown in autumn and winter. In Spain some inoculum remains to infect early-sown spring crops, and later sowing is recommended to avoid this inoculum source (Trapero-Casas et al., 1996). Ascospores are wind-dispersed over long distances and are thought to be a major source of initial inoculum in Spain (Trapero-Casas et al., 1996), Israel and northwest United States (Shtienberg et al., 2005). Release of ascospores from crop debris remaining between cropping seasons has been reported from many countries (Gan et al., 2006). *Didymella rabiei* is heterothallic, and requires two mating types to form viable pseudothecia (Trapero-Casas & Kaiser, 1992). Only one of the mating types has been conclusively shown to occur in Australia (Galloway & Macleod, 2003). This mating type was initially identified as MAT 1-1 but, is now known as MAT 1-2 (syn. MAT 2) due to nomenclatural changes (Phan et al., 2003). However, ascospores of the pathogen were detected in Western Australia in 2002 and have been detected once, in very low numbers, on stubble from South Australia (Payne, P., Department of Agriculture and Food, Western Australia, personal communication). This suggests that either the mating type MAT 1-1 is present in Australia, albeit at very low numbers, or occasional homothallic production of ascospores occurs.

Control of ascochyta blight of chickpea is difficult and requires integration of a number of disease control strategies (Pande et al., 2005). These include cultural methods such as use of disease-free seed, crop rotation, isolation from previous season's stubble, destruction of infected stubble, seed treatments and application of foliar fungicides, and the use of resistant cultivars (Shtienberg et al., 2006). Sources of resistance have been identified (Pande et al., 2005) and durable resistance to ascochyta blight is a major focus of chickpea breeding programs in many parts of the world (Shtienberg et al.,

2006). Recently new cultivars which are less susceptible to the disease have been released in Australia (Bretag et al., 2008). However, resistance is only partial, and there is a risk of it breaking down, particularly if sexual recombination were to occur (Gan et al., 2006; Bretag et al., 2008). Foliar fungicides have been evaluated in various studies around the world (Gan et al., 2006; Shtienberg et al., 2006; Bretag et al., 2008). However, the disease is difficult to control chemically because of rapid spread, particularly in epidemic situations, and even multiple applications have been found ineffective in conditions conducive to the disease (Shtienberg et al., 2006). An empirical model has been developed, which uses temperature and leaf wetness parameters to predict the timing of ascospore release (Shtienberg et al., 2005). This model is used to advise growers in Israel when to time fungicide sprays for optimal disease control. A study is underway to identify key environmental factors influencing the short distance (rain-splashed) and long-distance (wind-borne) distribution of spores of newly introduced foliar pathogens of annual field crops, using Ascochyta blight as a model (Coventry, 2011a).

1.9.4 Use of model pathogens to test the spore trapping system

The three model pathogens chosen for the studies described in this each infect aerial parts of the plant through release of airborne ascospores and/or splash-dispersed conidia, and affect field crops in the cropping systems practised in southern Australia. Because the numbers of airborne ascospores released in the southern Australian environment are likely to differ markedly between the three species, they offer an opportunity to test the sensitivity of the spore trapping system to different amounts of aerial inoculum.

1.10 Conclusions

Detection of plant pathogens of biosecurity concern could be improved by the development of more efficient methods, such as the use of conventional air samplers in combination with nucleic acid-based detection and quantification techniques. Air sampling in combination with PCR-based tests has been successfully applied to detect airborne spores of fungal plant pathogens in various contexts, but its application to biosecurity surveillance is in its infancy. There is a need to define the parameters within which these techniques will be useful for the early detection of plant pathogens of biosecurity concern. In particular, research is needed to determine sensitivity of PCR-based tests in the context of air sampling, how sensitivity is affected by the

presence of non-target particles, and how climatic factors such as temperature and humidity affect the detection of target pathogens. As well, baseline protocols detailing how to process and store samples are required. Definition of these parameters will help determine the strengths and limitations of these methods for the early detection of plant pathogens of biosecurity concern.

1.11 Research Aims

The primary aim of the research reported in this thesis was to investigate the efficacy of spore trapping combined with PCR-based diagnostic assays in relation to biosecurity surveillance. A secondary aim was to test predictions generated by epidemiological models for endemic plant pathogens chosen as models in this study.

1.12 Research Objectives

The objectives of the research were;

1. To develop a robust methodology for detection of plant pathogens using spore traps and PCR-based diagnostic assays
2. To test specificity of the PCR-based diagnostic assays in the context of air sampling
3. To investigate the effects of high temperature, a range of relative humidities and other factors, such as presence of dust, on detection of model pathogens
4. To test the spore trap/PCR-based diagnostic system and compare it with a trap plant system for monitoring airborne inoculum
5. To use the spore trap/PCR-based diagnostic system to study aspects of the epidemiology of three endemic pathogens, and in particular to test predictions of spore release generated by epidemiological models.

2 GENERAL MATERIALS AND METHODS

2.1 Trial site

Field trials were conducted at the South Australian Research and Development Institute (SARDI) Kingsford Research Station, approximately 40 km north of Adelaide, in South Australia (34°36'S, 138°45′E). This site is located in a rural area in which broad-acre cropping is practised, including crop rotation with field peas and canola as components of the rotation. An automatic weather station (AWS, Measurement Engineering Australia Pty Ltd, Magill, South Australia) was on site and measured rainfall, maximum and minimum temperature, wind speed and direction and relative humidity at 30-minute intervals. The automatic weather station consisted of a portable tripod mast supporting an enclosure with star-logger [model 6004-21] and 12V battery, solar panel and multi-channel input for sensors.

Spore traps (see Section 2.3.2) were placed at the site, at a distance of approximately 500 m north-west of the AWS, to monitor air-borne spores of the three model pathogens. Trap plants (see Section 2.3.1) were placed alongside the spore traps as a means of checking results.

2.2 Model pathogens

Three endemic ascomycetous pathogens of pulse and oilseed crops (*Ascochyta rabiei*, *Didymella pinodes* and *Leptosphaeria maculans*, as described in Chapter 1) were used as model organisms to develop the air sampling methodology. These pathogens were chosen because it was expected that they would occur at different concentrations of airborne ascospores at the trial site, they are morphologically distinguishable, and real-time PCR detection tests were available for each of them and in routine use at SARDI.

2.2.1 Fungal isolates

Isolates of the model pathogens were selected at random from a collection maintained by the Pulse and Oilseed Pathology group at SARDI. Isolates were maintained by periodically sub-culturing on agar and storing colonised agar plugs in sterile RO water at 4°C.

L. maculans isolate 66/97 was obtained from canola grown near Millicent in the south-east of South Australia (37°35′S, 140°21′E) in August 1997 and maintained on quarter strength potato dextrose agar (¼ PDA – Appendix 1) and stored as agar plugs in sterile distilled water at 3-4°C (Sosnowski et al., 2001). It was subcultured onto fresh agar medium and placed into storage twice, on 11 July 2008 and 18 June 2009.

D. pinodes isolate 123/02 was isolated from peas grown at Warrachie on Eyre Peninsula, South Australia (33° 38' 60 S, 135° 43' 0 E) in August 2002 and maintained on PDA. It was subcultured onto fresh agar medium and placed into storage once, on 17 January 2008.

D. rabiei isolate 144/00 was isolated from chickpeas grown near Elliston on Eyre Peninsula, South Australia (33° 47' 60 S, 135° 50' 60 E) in September 2000, and maintained on PDA. It was subcultured onto fresh agar medium and placed into storage in February 2008.

2.2.2 Infested stubble

2.2.2.1 Leptosphaeria maculans

Canola stubble, consisting of stem and root pieces of length 40–80 cm, with blackleg cankers, was supplied by Mr Trent Potter, SARDI, Struan, in the south-east of South Australia (37° 7' S, 140° 47' E), from fields which had severe disease in the previous season.

i. 2006 stubble was collected from SARDI Struan Research Station in mid-April 2007, from a blackleg nursery containing commercial cultivars and breeding lines with blackleg resistance rating ranging mostly from 5 to 7 (Potter et al., 2007), which was not harvested but had matured by mid-December 2006.

ii. 2007 stubble was collected on two occasions; firstly in late February 2008 from a nearby commercial property windrowed in the last week of November 2007, and secondly in mid-October 2008 from the blackleg nursery at Struan Research Station which had not been harvested but had matured by mid-December 2007.

iii. 2008 stubble was collected in late February 2009 from a commercial property at Mayhall near Struan harvested in the last week of November 2008.

2.2.2.2 *Didymella pinodes*

Pea stubble infested with *D. pinodes* was collected from the SARDI Research Station at Kingsford shortly after harvest in December 2006, November 2007 and November 2009.

i. 2006 stubble was from a pea crop sown in early June which had a disease severity rating of up to 17% for blackspot (i.e. up to 17% of internodes infected per plant).

ii. 2007 stubble was from disease trial plots sown in May 2007 and harvested in November 2007, which had up to 57% blackspot disease severity.

iii. 2008 stubble was from disease trial plots sown in May and harvested in November 2008, which had up to 53% blackspot disease severity.

iv. 2009 stubble was from disease trial plots sown in May and harvested mid- to late-November 2009, which had up to 62% blackspot disease severity.

2.2.2.3 *Didymella rabiei*

Chickpea stubble infested with *D. rabiei* was collected from disease epidemiology trial plots sown on 5 June 2008 at SARDI Turretfield Research Station, at Rosedale, South Australia (34 ° 33’ S, 138° 50’ E). The plots were harvested in late November and stubble collected on 14 December 2008. The stubble was obtained from cultivars Howzat (susceptible) and Almaz (moderately resistant; (McMurray *et al.* 2006) at a ratio of 2:1.

2.2.3 *Collection of ascospores from infested stubble*

2.2.3.1 *Leptosphaeria maculans*

Canola stubble collected in February 2008 and February 2009 was induced to produce mature pseudothecia by keeping it in a shallow tray lined with slightly moist sand (approximately 600 ml water applied to 3 kg sand) in a controlled environment at 15°C and 12-hour light using the method of Naseri (2008). Pseudothecia were deemed to be mature when they released spores upon wetting. This procedure was not necessary for stubble collected in October 2008, since abundant mature pseudothecia had formed in the field. The infested stubble pieces with mature pseudothecia were trimmed to 3-6 cm long, washed under tap water as needed to remove sand or dirt, bisected longitudinally and attached to the underside of a Petri dish lid using petroleum jelly. They were then wetted to run-off with RO water using a hand-held sprayer, and drained. The lid was blotted dry using tissue paper and replaced over the base. After one hour the base of the

Petri dish was examined under a dissecting microscope for the presence of ascospores. If necessary, the stems were re-wetted and left a further hour before checking again.

2.2.3.2 *Didymella pinodes*

Field pea stems infested with *D. pinodes* were attached to the underside of Petri dish lids and wetted as described for *L. maculans*, except the pea stems were not bisected since they were much thinner and could be affixed to the lid whole. In some cases (December 2007 and January 2008 for stubble collected November 2007) the stubble on the Petri dish lids required wetting twice a day with a hand-held sprayer and incubation at room temperature (approximately 22°C) for several days before ascospore release occurred. Subsequently (February and March 2008), new batches of stubble from the same source (Kingsford 2007) released spores after the first wetting.

2.2.4 *Assessment of fruiting bodies on stubble*

The number and maturity stage of *L. maculans* fruiting bodies on stubble were determined as follows:

The required number (three or five) of randomly-selected sections of canola stubble of length 10–15 cm, each with many mature *L. maculans* pseudothecia, was examined under 40 x magnification for the presence of fruiting bodies. On each stem a section 0.5 x 1 cm was randomly selected and the number of fruiting bodies on that section counted. Five pseudothecia were then removed from the selected section, or, if insufficient pseudothecia were present on that section, from an adjacent section of the stem, placed in a drop of water on a microscope slide and covered with a cover slip. Gentle pressure was applied to force asci and ascospores from the pseudothecia. The pseudothecia were examined under 100 x magnification (Olympus Light Microscope BH-2 with 12V 100W halogen lamp) and grouped into one of six maturity classes (Table 2-1). The maturity classes were adapted from (Naseri et al., 2008), with the following changes;

1. Stage P (pseudothecia absent) was not included, as most canola stems had sections with no pseudothecia, which could potentially be selected at random, regardless of the stage of maturity of pseudothecia on other parts of the stem. Instead an adjacent section of stem was selected as described above.
2. Stage D was divided into two sub-classes, D1 and D2. In both stages pseudothecia, asci and ascospores were mature, but in Stage D1 asci and many ascospores were released upon application of gentle pressure to the

pseudothecium, whereas in Stage D2 intact asci, containing mature ascospores, were ejected from the pseudothecium but few or no ascospores were released.

Table 2-1. Pseudothecial maturation stages of *Leptosphaeria maculans* (modified from the scale developed by Naseri et al. (2008)

Stage [1]	Pseudothecia	Asci	Ascospore
A	Not mature	Not mature	Absent
B	Not mature	< 8 spores	< 5 septae
C	Not mature	8 spores	5 septae
D1	Mature. Discharges many ascospores upon application of presure	Mature	Mature [2]
D2 [3]	Mature. Discharges few or no ascospores upon application of pressure	Mature	Mature
E	Empty	Empty	Discharged

[1] A pseudothecium was considered to have reached each stage of maturation when even one observed ascus and ascospore in the pseudothecium had reached the relevant stage of development
[2] Ascospore is 5-septate, constricted at first septum and yellow (Shoemaker & Brun, 2001)
[3] Newly introduced class. Discharges asci upon gentle pressure but asci release few or no ascospores

2.2.5 Assessment of ascospore release from stubble

A method modified from that used by Salam, M.U. (Department of Agriculture and Food, Western Australia, personal communication) was used to assess ascospore release from stubble. A rotorod (whirling arm) air sampler (Lacey & West, 2006; Figure 2-1), with double-sided adhesive tape (Scotch tape 3M 94253/4 x 72 respositionable D/C) on the leading edge of each arm, trimmed to the dimensions of the rotorod arm (60 x 1.5 mm), was placed at one end of a purpose-built wind tunnel (Coventry, 2011a). The wind tunnel (5.5 m long, 0.5 m wide and 0.5 m high) was of a design modified from that described by Fitt et al. (1986). This design allowed filtered air to be directed along the tunnel in a laminar flow at speeds of between 1 and 4.7 m second^{-1}. The required number (nine or five) of randomly-selected sections of canola stubble of length 10 – 15 cm, each with many mature *L. maculans* pseudothecia, was placed in a nylon mesh bag, wetted by immersion in water for 5 minutes, and placed in the wind tunnel at 45–60 cm upwind of, and level with the arms of, the rotorod sampler. The rotorod sampler was switched on and the wind tunnel operated for 1 hour at the maximum wind speed (4.7 m second^{-1}). The double-sided tapes were removed from one arm of the rotorod sampler, placed on a microscope slide and cut into four sections, of length approximately 15 mm,

which were then stuck to a microscope slide, taking care to position them parallel with the long side of the slide. The area between the tape sections was flooded with lactoglycerol (1:1:2 lactic acid: glycerol: water) and a large cover slip placed quickly over the tapes. The cover-slip was tapped gently to remove any air- bubbles trapped under the tapes. Slides were left overnight and ascospores on tape segments were counted under 200x magnification.

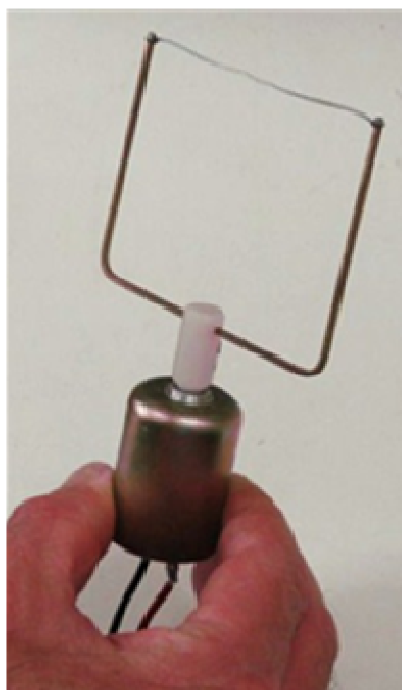

Figure 2-1 Rotorod sampler

2.3 Air sampling

2.3.1 Trap plants

To determine the relationship between aerial concentration of ascospores and infection of host plants, seedlings of field pea, canola and chickpea were placed alongside the spore trap to monitor for the model pathogens. Methods in routine use at SARDI were employed (Davidson et al., 2006) whereby trap plants were replaced weekly at the field site throughout the growing season (May to November) and returned to the glasshouse for incubation.

Trap plants were produced by filling nine pots of volume 900 ml with potting soil and sowing three pots each with seed of field pea (cultivar Parafield, susceptible to blackspot), canola (cultivar ATR-stubby; susceptible to blackleg) and chickpea (cultivar

Desavic; susceptible to ascochyta blight). The seed had been sourced from disease-free seedlots. Seedlings were thinned to four per pot (12 seedlings of each host plant), grown in a glasshouse at temperatures 18-29°C and watered every 2 or 3 days as required. Three weeks after sowing, the pots of seedlings were placed alongside the spore trap at the field site at Kingsford. They were placed in a tray containing water to a depth of several centimetres to minimise desiccation, and the tray was placed under a wire cage to prevent animals from feeding on the seedlings (Figure 2-2A). After exposure to air-borne inoculum for 1 week they were returned to the glasshouse, sprayed with pyrethrum for insect control, incubated in a humidity chamber (Figure 2-2B) for 5 days, then removed to a glasshouse bench and regularly watered. Plants were assessed for disease symptoms 10–30 days after returning from the field, by counting the number of leaf lesions caused by the three model pathogens. If there was uncertainty whether symptoms were caused by the model pathogen in question, leaves with symptoms were removed and placed in small humidity chambers (Figure 2-2C), and incubated at room temperature for several days to induce sporulation. Identification was based on morphology of pycnidia and conidia.

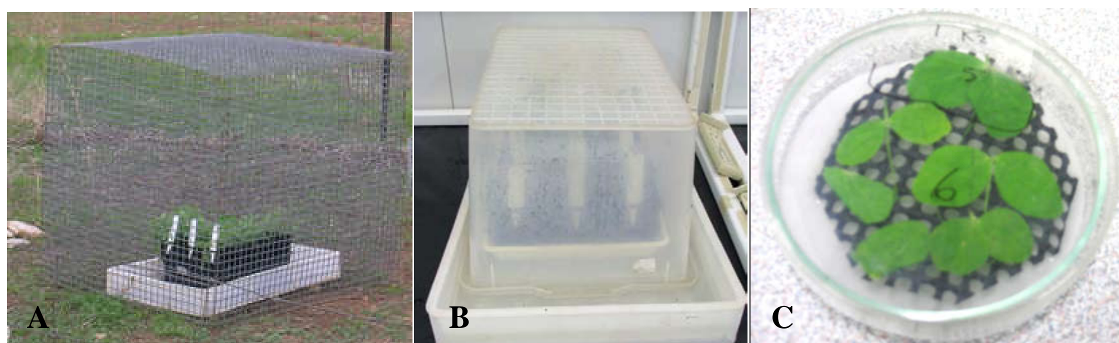

Figure 2-2 A. Trap plants (field pea cultivar Parafield, canola cultivar ATR-stubby and chickpea cultivar Desavic) at the Kingsford field site B. The same trap plants in a humidity chamber C. Leaves from trap plants of field pea cultivar Parafield with symptoms similar to blackspot in a small humidity chamber (filter paper at base of Petri dish was moistened with RO water)

2.3.2 Spore traps

Slit-type volumetric spore traps (Hirst-type) manufactured by Murdoch University, Western Australia (Neumeister-Kemp et al., 2004) were used in this study (Figure 1-1). For each sampling period, a length of Melinex tape (Melinex clear tape, 200 gauge, Burkard Manufacturing, Hertfordshire, UK), thinly coated with adhesive (see Section 2.3.3), was fixed to the rotating drum. The intake air flow on the spore trap was set to

10 l/min. At the end of the sampling period the spore trap drum was removed into an airtight container manufactured for this purpose and brought back to the laboratory for processing.

2.3.3 Application of adhesives to spore tape

Adhesives commonly used to coat Melinex tapes in Hirst-type spore traps include mixtures containing petroleum jelly and paraffin wax (Lacey & West, 2006) but petroleum jelly has been reported to be unsuitable for use in hot conditions, due to its low melting point (Gálan & Dominguez-Vilches, 1997). Therefore Tanglefoot® adhesive (product number E95113, Australian Entomology Supplies, NSW, Australia), which has been reported to be suitable for the hot, dry conditions of southern Australia (Driessen, 2005), was chosen for use in the experiments reported in this thesis. Initially the Tanglefoot® was applied undiluted to the Melinex tape in a thin layer using a Pasteur pipette held horizontally with the narrow part flat against the tape, as described by Driessen (2005). As this was time-consuming, experiments were conducted to assess the suitability of hexane as a solvent, so that the Tanglefoot® could be applied to the tape while it was on the spore trap drum mounted on a Burkard roller frame (Figure 2-3), using a paint brush as described in Lacey & West (2006), and to determine the best ratio of Tanglefoot® to hexane for this purpose.

Tanglefoot® was added to 10 ml of hexane, starting with 5 g Tanglefoot® and increasing in approximately 1 g aliquots. After each stepwise addition, the solution was thoroughly mixed using a magnetic stirrer, and then painted onto Melinex tape using a number 20 S&S Flat Taklon paint brush. The tape had been fixed onto the spore trap drum, which was mounted on the roller frame. All experimentation was conducted in a fume hood. The tape was allowed to dry in the fume hood for 20 minutes before it was removed and examined under a dissecting microscope to determine if the tape was evenly covered. A dilution of 12.2 g Tanglefoot® in 10 ml hexane gave the best consistency to produce even coverage of the Melinex tape. The final method is summarised in Appendix 2.

2.3.4 Effect of dissolving adhesive in hexane on spore capture

An experiment was conducted to determine if dissolving the Tanglefoot® in hexane affected its ability to trap spores compared with the undiluted Tanglefoot®.

Two slit-type volumetric spore traps were operated side-by-side at the University of Adelaide Waite Campus, Urrbrae, South Australia, on a 7-day cycle (i.e. rotating at a

rate of 48 mm per day) for 3 days in early February 2008, in order to capture spores and other particles from various sources present in the air. In one trap the Melinex tape was covered with Tanglefoot® dissolved in hexane, in the other the tape was covered with undiluted Tanglefoot®. The tapes were removed from the trap to the laboratory, and dissected vertically into 48 x 19 mm segments, each segment representing 24 hours of sampling. Each segment was bisected horizontally, making the segments 48 x 9.5 mm. For each sampling day the tape segments from the two traps were aligned in parallel, ensuring that the start and finish of the two tapes lined up, and fixed onto microscope slides with a thin film of water under each segment as described in Lacey & West (2006). The tape segments were stained using 0.1% aniline blue in lactoglycerol (Appendix 3) and mounted together under one cover slip (22 x 50 mm). The edges of the cover slip were sealed with nail polish. Tapes were examined using 200 x magnification and the total number of spores on four transects evenly spaced across the two segments was counted.

The mean numbers of spores on tapes with Tanglefoot® dissolved in hexane and undiluted were 48 and 32, respectively. There was no significant difference between the two treatments (P=0.205).

Dissolving Tanglefoot® in hexane did not reduce the number of spores caught in the spore trap. Painting the dissolved Tanglefoot® onto the tape using a paint brush and the Burkard roller frame was therefore deemed to be a suitable method to prepare the spore tapes.

2.3.5 Spore tape processing

After each sampling period, the tape was removed from the drum onto a perspex cutting block (Figure 2-3), held in place by a length of double-sided tape, and bisected horizontally using a scalpel. The cutting block was manufactured by Eglinton Engineering, Adelaide, South Australia, and was similar to the perspex cutting block supplied with volumetric spore traps by Burkard Manufacturing Company Limited, Hertfordshire, UK (Figure 2-3). However, instead of the vertical grooves which enable tapes to be cut into daily sections (48 mm long), on the Burkard cutting block (Lacey & West, 2006), the Eglinton cutting block had a horizontal groove to enable accurate longitudinal dissection of the tape into two halves – one for microscopic examination and the other for molecular diagnostics. Tapes were stored (Section 2.3.6) and, when required, were bisected vertically into seven equal-sized daily segments using the

Burkard cutting block, before being examined microscopically or processed for PCR analysis.

2.3.6 Storage of spore tapes

After horizontal dissection, each tape half was attached with double-sided sticky tape to the base of a purpose-built container, consisting of a length of flat electrical conduit (25 x 16 x 360 mm), purchased from a local hardware store (Figure 2-3), which was then sealed using the top of the length of conduit as a lid (Figure 2-3), and duct tape at the ends (storage system devised by staff of Bureau of Sugar Experimental Stations, Braithwaite, K., personal communication, 2007). To prevent dessication, the conduit was placed in a sealed plastic bag. Tapes for PCR assays were stored at -20°C to preserve DNA; tapes for microscopy at 4°C to preserve spore morphology.

2.3.7 Microscopic examination of the spore tapes

Several methods to stain and mount spore tapes were compared. Full descriptions of the methods are provided in Appendix 3, and a summary of their relative advantages and disadvantages is given in Appendix 4. The combination of stain and mountant eventually selected for use was 0.2% trypan blue in Mowiol with phenol. This was selected on the basis that spores were reasonably well stained and the Mowiol mountant made it easier to achieve good coverage of the tape than other methods.

The mounted tape segments were examined under the light microscope at 100 x or 200 x magnification. Spores were identified on the basis of morphology and counted at 200 x magnification using an eyepiece squared graticule with 100 squares each measuring 50 µm x 50 µm at 200 x magnification (Olympus Australia, Mt Waverley, Victoria). Unless otherwise stated, every fourth vertical transect was counted, i.e. one-quarter of the tape. Images were taken using a Moticam 2000 (Motic® MC camera) and Motic Images Plus 2.0 ML software.

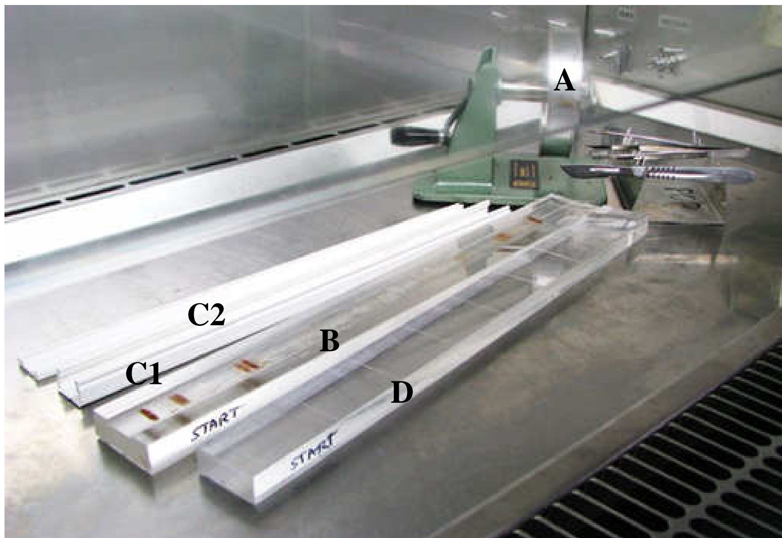

Figure 2-3 Equipment used to process and store spore trap tapes following their removal from the field. A. Spore trap drum on Burkard roller frame B. Eglinton cutting block for bisecting tape longitudinally into lengths of width 9.5 mm C1. Base of container for storage of spore trap tape after longitudinal bisection, consisting of a length of flat electrical conduit (25 x 16 x 360 mm), onto which the tape was fixed using a short length of double-sided tape near each end C2. Lid of the same container D. Burkard cutting block for dissecting tapes into daily segments of length 48 mm

2.4 Estimation of spore numbers on tapes

2.4.1 Spore concentration in suspension

In experiments to determine the sensitivity and specificity of PCR assays, suspensions of conidia or ascospores were applied to Melinex tape segments. Spore concentration was estimated using a Bright-Line® Improved Neubauer 0.1 mm deep haemocytometer (Reichert, Buffalo, NY, USA). The chamber and cover slip were first cleaned using lens paper with a little 70% ethanol to remove any grease. Suspensions were thoroughly mixed by inverting the vial at least four times. The suspension was immediately sucked into a glass Pasteur pipette, then, holding the pipette at a 45° angle, the pipette bulb was squeezed gently till a small droplet protruded which was quickly touched to the chamber inlet groove. If the chamber did not fill almost instantaneously it was cleaned and the procedure repeated. The amount of suspension added was such that little or no excess ran off into the drainage channels. Counting was repeated at least once (i.e. two counts). For dilute suspensions (less than 10^5 spores/ml), additional replicate counts were conducted so that at least 100 spores in total had been counted.

2.4.1.1 Preparation of conidial suspensions and application of conidia to tape

L. maculans

Isolate 66/97 was cultured on ¼ PDA and grown under 12 hour light (Philips TLD 6W/840 and NEC FL40SBL black light) at room temperature for approximately 6 days. The culture was flooded with 4-5 ml sterile RO water and passed through a sterile sieve (pore size 15 µm) to remove hyphal fragments and other structures, including fragments of pycnidial wall and swollen cells with thickened walls that had formed within aerial hyphae. Concentration of the spore suspension was estimated as described in Section 2.4, and the suspension was diluted to the required concentration.

If required, 50 or 100 µl of the suspension was pipetted onto Melinex tape segments with or without adhesive, in droplets distributed evenly across the whole segment (20 - 30 droplets), using an Eppendorf 10-100 µl pipette. Tape segments were left to dry in a laminar flow cabinet for 90 minutes.

D. pinodes

Isolate 123/02 was cultured on Coon's agar (Appendix 1) to promote sporulation, and grown under 12 hour light as described for *L. maculans*, at room temperature for approximately 6 days. The culture was then flooded with 4-5 ml RO water and sieved through a sterile 50 µm sieve to remove hyphal fragments, fragments of pycnidial wall and chlamydospores. The concentration was adjusted and, if required, conidia were applied to tape segments as described for *L. maculans* above.

D. rabiei

Isolate 144/00 was cultured on Coon's agar to promote sporulation and grown under 12-hour light as described for *L. maculans*, at room temperature for approximately 9 days. The culture was then flooded with 4-5 ml RO water and, where necessary, passed through a 105 µm or a 50 µm sieve to remove fragments of hyphae and pycnidial wall. The concentration was adjusted and, if required, conidia were applied to tape segments as described for *L. maculans*.

2.4.1.2 Application of ascospores to tape

In order to determine the number of ascospores on tape segments before DNA extraction and analysis, two methods of applying ascospores were used.

Method 1. Ascospores were collected from infested stubble as described in Section 2.2.3. Once sufficient spores had been released, they were suspended in 1-2 ml of

chilled (4°C) sterile RO water with 0.05% Tween 80, using a paint brush (number 3 Premier 355 Synthetic) to dislodge the spores from the Petri dish base. The suspension was pipetted into a 10-ml sterile screw-capped centrifuge tube (Sarstedt number 62.9924.284) and held at 4°C while the next Petri dish was processed in the same way. Each sequential suspension was added to the centrifuge tube. Spore concentration was estimated using a haemocytometer as described in Section 2.4.

Method 2. Ascospores were collected from infested stubble as described in Section 2.2.3. Segments of Melinex tape, with or without adhesive, were placed on microscope slides under the infested stems.

2.4.2 Spore counts on tapes

The number of ascospores deposited on a tape segment (Section 2.4.1.2 method 2) was counted using 100 x magnification (without using a cover slip).

2.5 Quantitative PCR assays

The amount of DNA in spore trap samples was estimated using qPCR assays with primers specific for each of the three model pathogens (RDTS, SARDI, http://www.sardi.sa.gov.au/products__and__services/entomology/diagnostic_service/predicta_b). All three tests were based on the ITS regions of ribosomal DNA and employ TaqMan® MGB™ assays (Applied Biosystems, Foster City, CA, USA). Details of the primers and probes for *L. maculans*, *D. rabiei* and *D. pinodes* are provided in Table 2-2. The primer set for *L. maculans* (as for the other target pathogens) was species-specific and enabled discrimination between *L. maculans* and the closely related *L. biglobosa*. QPCR reactions were performed in 10 µl volumes with an ABI PRISM® 7900HT Sequence Detection System using Qiagen QuantiTect® Probe PCR Master Mix (1 x Qiagen QuantiTect PCR Master Mix, 400 nM forward primer, 400 nM reverse primer, 200 nM probe) incorporating the specific primers and probes for the target (6 µl) and template DNA (4 µl) extracted from spores, and run over 40 cycles. Thermal cycling conditions were: an initial temperature of 95°C for 15 minutes to activate *Taq* polymerase, followed by melting step of 95°C for 15 seconds and annealing/extension step (combined) of 60°C for 1 minute for 40 cycles. Quantification was carried out for each organism by comparison with a reference standard (10-fold dilution series 0.2 – 200,000 fg/µl of the test organism in TE buffer (EDTA 0.1mM [pH 8.0]; Tris 10mM), which was used to create a standard curve. An exogenous organism, dry yeast (Defiance, NSW, Australia) was added to the first extraction buffer at 0.05 mg/ml (final

concentration) and was co-extracted. Yeast was quantified using a specific TaqMan assay as part of the Root Disease Testing Service standard protocol.

Table 2-2 Details of primers and probes used in qPCR to detect *D. rabiei, L. maculans* and *D. pinodes*

Primer/ Probe	*D. rabiei* [1]	*L. maculans* [1]
Forward	5'-CCTAGAGTTTGTGGGCTTTGTCC- 3'	5'-GGCGGCAGTCTACTTTGATTCT-3'
Reverse	5'-ATTGCAACTGCAAAGGGTGTG-3'	5'-GTTTTAGGGGATCCAATTGGTG-3'
Probe	6FAM-ACCCGCCGAGGAAACG-MGBNFQ	6FAM-TTTTTGCGTACTATTTGTTTCC-MGBNFQ
	D. pinodes [2]	
Forward	5'- AGAGACCGATAGCGCACAAG - 3'	
Reverse	5'- AGTCCAGGCTGGTTGCAGGA -3'	
Probe	6FAM-CATGTACCTCTCTTCGGG-MGB	

[1] (McKay, A., SARDI; Ophel-Keller, K., SARDI; Hartley, D., CSIRO Entomology; unpublished data)

[2] (Davidson et al., 2011)

2.6 Statistical analysis

Data were analysed using Microsoft Excel® 2003 edition for analysis of variance (ANOVA) or regression analysis unless otherwise stated. Advice and assistance with statistical analysis was provided by Chris Dyson, Biometrician, SARDI and Jenny Davidson, Senior Plant Pathologist, SARDI.

3 DEVELOPMENT OF DNA METHODOLOGY

3.1 Introduction

QPCR has been used by various researchers to quantify spores from air samples, as discussed in Chapter 1, and is sufficiently sensitive to detect very small numbers of spores. While some researchers, particularly those reporting on early studies, for example Williams et al. (2001), found that sensitivity was reduced in the presence of DNA from other species and PCR-inhibiting substances in air samples, some researchers, for example Rogers et al. (2009) and Ma et al. (2003), have reported development of DNA extraction and qPCR methodologies which have been sufficiently sensitive to detect as few as one to two spores in field-collected air samples. In relation to the model pathogens used in the studies reported here, ascospores of *L. maculans* have been detected in Burkard Hirst-type spore trap samples using end-point PCR (Calderon et al., 2002a), and more recently, qPCR (Kaczmarek et al., 2009). Spores of *D. pinodes* and *D. rabiei* have not been previously reported from spore trap samples using qPCR.

The aims of the experiments described in this chapter were to develop DNA extraction and purification techniques which enable accurate and sensitive detection of *D. pinodes*, *D. rabiei* and *L. maculans* spores from Hirst-type volumetric spore trap samples and to determine a relationship between spore numbers and the calculated amount of DNA from qPCR assays. DNA was extracted from spores on Melinex tape segments, as used in the spore traps, coated with Tanglefoot® adhesive.

A further aim was to determine whether applying Tanglefoot® dissolved in hexane to tapes, as discussed in (Sections 2.3.3 and 2.3.4), affected the detection of DNA from spore trap samples. Although Tanglefoot® applied directly has been used as an adhesive on spore trap tapes, it was not known whether dissolving it in hexane would change its composition and thereby release chemicals which might interfere with the DNA extraction or PCR reaction.

In optimising the DNA extraction and purification techniques several methodological problems were encountered. Adequate disruption of spores, removal of spores from

tape and variability between one experiment and another were all addressed. Conidia were used in the initial experiments as they could be more readily produced in culture than could ascospores. Spore disruption methods then had to be refined when applied to ascospores.

In the course of developing the methodology for DNA analysis of spore trap samples, a number of experiments were conducted which did not lead to any improvement in the amount of DNA or consistency between samples. A list of these experiments, together with a brief summary of outcomes, is provided in Appendix 5.

3.2 Materials and methods

In these experiments, unless otherwise stated, a conidial suspension of *D. pinodes* was prepared as outlined in Section 2.4.1.1, and diluted to the required concentration. The suspension (100 µl) was either applied directly to 2 ml "bead tubes" (2 ml tubes suitable for use in a homogeniser, as described in Appendix 6 Method 1, and kept at 4°C till ready to commence DNA extraction, or pipetted onto segments of Melinex tape (48 x 9.5 mm) and allowed to dry, as described in Section 2.4.1.1. Each of the tape segments with spores was then immediately inserted into a bead tube and DNA extracted.

DNA was extracted using the standard protocol for either the MoBio UltraClean® Plant DNA Isolation Kit (Catalogue No. 13000, MoBio Laboratories, Inc., CA, USA) or the MoBio PowerPlant® DNA Isolation Kit (Catalog No. 13200-S) as detailed in Appendix 6. The latter was used either with or without modification as described for individual experiments. Unless otherwise stated, 50 µl elution buffer (PB6) was used in the PowerPlant® protocol.

The amount of DNA in spore trap samples was estimated using qPCR assays as described in Section 2.5.

Microscopy was undertaken using either a Leica MZ6 dissecting microscope with Leica CLS150D cold light source at 6.3 to 40 x magnification, or an Olympus Light Microscope BH-2 with 12V 100W halogen lamp at 100, 200 or 400 x magnification.

Data were analysed by analysis of variance (ANOVA) or regression analysis, using Microsoft Excel® 2003 edition, unless otherwise stated (see Section 2.6).

3.2.1 Initial experiment using UltraClean® kit

3.2.1.1 Experiment 1

A conidial suspension of *D. pinodes* (2.35 x 10^5 conidia/ml) was prepared fresh and pipetted either onto tape segments with Tanglefoot® adhesive or directly into 2 ml UltraClean® bead tubes. DNA was extracted using the standard UltraClean® Plant DNA Isolation Kit protocol. QPCR was performed as described in Section 2.5 and microscopy was undertaken as described in Section 3.2. The experimental design was a randomised complete block with two replicates. The replication was of the extraction of DNA and performance of the qPCR on samples of conidia either on tape segments or in solution.

3.2.2 Comparison of methods for disrupting conidia

3.2.2.1 Experiment 2

The aim of this experiment was to compare several methods for disrupting spores on spore trap tapes. A conidial suspension of *D. pinodes* (5.4 x 10^5 conidia/ml) was prepared and 100 µl pipetted onto Melinex tape segments with Tanglefoot® adhesive. Once dry, the tapes were placed into 2 ml bead tubes with various combinations of bead solution and beads (outlined below), and subjected to "bead beating" using a FastPrep® homogeniser (FP120 Savant Instruments, Holbrook, NY, USA). Tapes were then examined microscopically at 40 x magnification to determine how much adhesive remained. Tapes and samples of fluid were examined microscopically at 200 x magnification to check for the presence of conidia. DNA was not quantified. The experimental design was a randomised complete block with two replicates. Treatments were;

1. UltraClean® kit bead tubes containing 550 µl UltraClean® bead solution plus UltraClean® beads plus 60 µl UltraClean® solution P1
2. PowerPlant® kit bead tubes containing 500 µl PowerPlant® bead solution plus four stainless steel ball bearings (2 mm diameter) plus 60 µl PowerPlant® solution PB1
3. Bead tubes with 0.1% Nonidet P40 (Roche Applied Science, Mannheim, Germany Cat. No. 1 1 754 599 001) non-ionic detergent with 0.2 g Ballotini glass beads (0.85 mm diameter).

3.2.2.2 Experiment 3

Several methods were compared to determine which was the most suitable for extracting DNA from spore trap samples.

A conidial suspension of *D. pinodes* (3.0 x 10^5 conidia/ml) was prepared and 100 µl pipetted either onto tape segments with Tanglefoot® adhesive or directly into 2 ml bead tubes then subjected to various DNA extraction methods as listed in Table 3-1. Extracted DNA was quantified using qPCR assays as described in Section 2.5. Microscopic examination of conidia was not carried out. The experimental design was a factorial with five replicates. Two-factor ANOVA was conducted using Statistix 8.0.

Table 3-1 DNA extraction methods applied to conidia either on tape segments with Tanglefoot® adhesive or in suspension

Treatment number	DNA extraction method
1	PowerPlant® kit[1]
2	PowerPlant® kit with 0.1% Nonidet P40 in nanopure water (220 µl) substituted for the Powerplant® bead solution, and 0.2 g Ballotini glass beads (0.85 mm diameter) substituted for the Powerplant® ball bearings
3	PowerPlant® kit but with 0.1% Nonidet P40 in B1 buffer (0.2 M sodium phosphate, pH 8.0; 220 µl) substituted for the PowerPlant® bead solution, and 0.2 g Ballotini glass beads (0.85 mm diameter) substituted for the PowerPlant® ball bearings
4	PowerPlant® kit but with 0.1% Nonidet P40 in B1 buffer (220 µl) substituted for the PowerPlant® bead solution

[1] The PowerPlant® bead tubes contained four stainless steel ball bearings

3.2.3 Efficacy of DNA Extraction Method 1 on different pathogens

Two experiments were conducted to determine if the PowerPlant® kit modified by using 0.1% NonidetP40 in B1 buffer instead of PowerPlant® bead solution, and glass beads instead of ball bearings (as per treatment 3, Table 3-1, hereafter referred to as “Method 1”), was suitable for conidia of the other two model pathogens (*D. rabiei* and *L. maculans*). This extraction method had previously been tested using *D. pinodes* conidia (Section 3.2.2.2).

3.2.3.1 Experiment 4

A conidial suspension of *D. rabiei* (1.4×10^5 conidia/ml) was prepared and pipetted onto tape segments or directly into bead tubes (100 µl per sample). DNA was extracted using Method 1 (Appendix 6). Extracted DNA was quantified using qPCR assays as described in Section 2.5. The experimental design was a randomised complete block with five replicates. The data were analysed by ANOVA.

3.2.3.2 Experiment 5

The methodology was the same as for Experiment 4, except that *L. maculans* conidia were used instead of *D. rabiei*, and the concentration of the suspension was 1.65×10^5 conidia/ml.

3.2.4 Removal of conidia from tape segments

3.2.4.1 Experiment 6

In previous experiments, the yield of DNA from conidia of model pathogens on spore trap tapes was consistently lower than from conidia in suspension. An experiment was conducted to determine whether this difference was due to one or a combination of the following factors; (i) the presence of the tape, (ii) the adhesion of the spores to the tape or (iii) the presence of the adhesive.

A conidial suspension of *D. rabiei* (8.2×10^4 conidia/ml) was prepared fresh. The suspension (100 µl) was applied either to segments of Melinex tape, with or without Tanglefoot® adhesive, or directly to 2 ml bead tubes; tape segments, with or without conidia, were either inserted into bead tubes or not, in the following combinations;

1. Tape with Tanglefoot®, conidia on tape
2. Tape without Tanglefoot®, conidia on tape
3. No tape, conidia in suspension
4. Tape with Tanglefoot®, conidia in suspension

DNA was extracted using Method 1 (Appendix 6) and quantified using qPCR assays as described in Section 2.5. The experimental design was a randomised complete block with two replicates. The replication was only of the DNA extraction from conidia on tapes or in suspension, not the preparation of the conidial suspension. ANOVA was performed using Statistix 8.0.

3.2.5 Sensitivity of DNA extraction/qPCR system on conidia

Two experiments were conducted to determine the limit of detection of conidia of the model pathogens *D. rabiei* and *L. maculans* on spore trap tapes, i.e. the minimum number of conidia that could be detected by qPCR following extraction of DNA from spore trap samples. These experiments were performed on conidia rather than ascospores, as conidia could be produced in culture and were therefore easier to obtain.

3.2.5.1 Experiment 7

A 10-fold dilution series of a conidial suspension of *D. rabiei* (4.5×10^5, 4.5×10^4, 4.5×10^3, 4.5×10^2 and 4.5×10 conidia/ml) was prepared. The suspensions, or RO water for the controls (100 µl per sample), were applied to segments of Melinex tape with Tanglefoot® adhesive, and allowed to dry. DNA was extracted using Method 1 (Appendix 6). Extracted DNA was quantified using qPCR assays as described in Section 2.5. There were four replicates for each of the five dilutions and the controls. Regression analysis was performed to compare concentration of conidia and quantity of DNA.

3.2.5.2 Experiment 8

A 10-fold dilution series of a conidial suspension of *L. maculans* (4.2×10^5, 4.2×10^4, 4.2×10^3, 4.2×10^2 and 4.2×10 conidia/ml) was prepared. The suspensions, or RO water for the controls (100 µl per sample), were applied to segments of Melinex tape with Tanglefoot® adhesive, and allowed to dry. DNA was extracted using Method 1 (Appendix 6). Extracted DNA was quantified using qPCR assays as described in Section 2.5. There were two replicates for each of the five dilutions and the controls. Regression analysis was performed to compare concentration of conidia and quantity of DNA.

3.2.6 Variability in calculated DNA values from conidia between qPCR assays

In previous experiments the calculated DNA value from conidia of model pathogens varied on a per-conidium basis from one experiment to another, with the largest variation observed for *D. rabiei*. An experiment was designed to test whether this variation was attributable to variability between qPCR assays.

3.2.6.1 Experiment 9

D. rabiei DNA from five samples each from three previous experiments was selected for repeat qPCR assays. All selected DNA samples had been extracted from conidia on

Melinex tape segments and the DNA had been stored at –20°C immediately after extraction. Extracted DNA was quantified using qPCR assays as described in Section 2.5.

3.2.7 Spore disruption and DNA extraction from ascospores

The experiments described above were conducted using conidia, but ascospores are more likely to be detected in the spore trap as they are wind-dispersed over long distances, whereas conidia are primarily splash-dispersed over short distances. Therefore, two experiments were conducted to determine if the DNA extraction method worked well on ascospores, and to compare DNA yield of ascospores and conidia.

3.2.7.1 Experiment 10

A 10-fold dilution series of a conidial suspension of *D. pinodes* (2.2 x 10^2, 2.2 x 10^3, 2.2 x 10^4 and 2.2 x 10^5 conidia/ml) was prepared. The suspensions, or RO water for the controls (100 µl per sample), were applied to segments of Melinex tape without Tanglefoot® adhesive, and allowed to dry. Ascospores were collected from infested stubble directly onto segments of Melinex tape without adhesive and counted, as described in Section 2.4.1.2. On some of the tapes ascospores had begun to germinate. Any tapes with more than 1% of spores germinating were rejected for use in the experiment.

Tape segments (with either conidia or ascospores) were placed into 2 ml bead tubes and DNA was extracted using Method 1 (Appendix 6). Extracted DNA was quantified using qPCR assays as described in Section 2.5. There was one replicate for each of the conidial dilutions and seven replicates of ascospores (with ascospore numbers varying from 17 to 3,780 ascospores per sample). Regression analyses were conducted between concentration of conidial suspension and measured DNA using Genstat Version 11.1.

3.2.7.2 Experiment 11

The method was as described in Section 3.2.7.1 except that the spores were of *L. maculans* and only one concentration of conidial suspension (4.64 x 10^2 conidia/ml) was used. There were five replicates of the conidial treatment and five replicates of the ascospore treatment (with ascospore numbers varying from 37 to 59 ascospores per sample) and two controls without spores.

3.2.8 Investigation of ascospore disruption

3.2.8.1 Experiment 12

In initial experiments to extract DNA from ascospores, results were variable and in some cases little or no DNA was obtained. It appeared that ascospores were not consistently disrupted by the DNA extraction procedures used. The aim of this experiment was to determine if various pre-treatments and variations in the homogenisation step aided in breaking open ascospores of *L. maculans*.

A suspension of *L. maculans* ascospores in RO water with 0.05% Tween 80 was prepared as described in Section 2.4.1.2 Method 1. The suspension (600 µl) was pipetted into 2 ml bead tubes together with various types and amounts of beads as listed below, and either homogenised or not, using a FastPrep® machine at 6,000 rpm for two periods of 40 seconds with 2 minutes cooling on ice in between. Lysate was pipetted onto microscope slides and allowed to dry, then stained with 0.1% aniline blue in lactoglycerol (Appendix 3) and viewed under 100 x magnification. The number of ascospores was counted for Treatments 0, 1, 2 and 5. For Treatments 3 and 4 ascospore were not counted as a quick perusal was sufficient to indicate that few ascospores had been disrupted.

Treatment 0: Control (no homogenisation)

Treatment 1: 0.2 g Ballotini glass beads, 0.85 mm diameter (designated "fewer beads")

Treatment 2: 0.6 g Ballotini glass beads, 0.85 mm diameter ("more beads")

Treatment 3: Ceramic beads (1.4 mm ceramic beads, 03961 CK14, Bertin Technologies, France) ["Ceramic BT"]

Treatment 4: Ceramic beads (1.4 mm, catalogue number 13113.50 MoBio) ["Ceramic MoBio"]

Treatment 5: Spores frozen at -80°C (liquid nitrogen for 2 minutes), then as for Treatment 1 ("-80°C, fewer beads")

A completely randomised layout was used. ANOVA was performed using Statistix 8.0.

3.2.9 Sensitivity of DNA extraction/qPCR system on ascospores

3.2.9.1 Experiment 13

The aims of this experiment were firstly, to determine if variability of DNA yield from ascospores of *L. maculans* was reduced when 0.6 g of beads were used rather than 0.2 g during the homogenisation step, and secondly, to determine the sensitivity of detection of *L. maculans* from ascospores on spore trap tapes.

Ascospores of *L. maculans* were collected directly onto Melinex tape segments with Tanglefoot® adhesive, as described in Section 2.4.1.2 Method 2. Tape segments with ascospores were frozen at –20°C immediately after the ascospores had been counted (within 3 days of ascospore release onto tapes). Tape segments were removed from the freezer after 16 hours and placed into bead tubes with either 0.2 g (Treatment 1) or 0.6 g (Treatment 2) of Ballotini glass beads (0.85 mm diameter). DNA was extracted using Method 1 (Appendix 6). There were 12 replicates for each treatment, with ascospore counts varying from 0 to 510 ascospores per sample. Extracted DNA was quantified using qPCR assays as described in Section 2.5, in duplicate. Calculated DNA values for *L. maculans* were standardised to the median calculated yeast DNA value. Regression analysis of the square root-transformed data for both ascospore numbers (x) and calculated DNA values (y), was performed using Genstat version 11.1 and the slopes of the regression lines for each of the two treatments were compared statistically using the Student's t-test. The square-root transformation was performed to take into account clumping of points near the origin with low "residuals" (differences between the sample value and its value estimated from the regression equation) and consequent bias on the definition of the slope by the relatively few large numbers with higher "residuals".

3.2.10 Effect on DNA yield of applying Tanglefoot® dissolved in hexane to spore trap tapes

3.2.10.1 Experiment 14

The aim of this experiment was to determine if applying the Tanglefoot® adhesive dissolved in hexane, as described in Section 2.3.4, affected the yield of DNA from spores caught on the tape segments.

A suspension of 4.8 x 10^{5} *D. rabiei* conidia/ml was prepared, and 100 µl pipetted onto each of several segments of Melinex tape with Tanglefoot® which had either been applied dissolved in hexane or applied directly. DNA was extracted using Method 1

(Appendix 6) and DNA quantified using qPCR assays as described in Section 2.5. Results were analysed using ANOVA.

3.3 Results

3.3.1 Initial experiments using UltraClean® kit

3.3.1.1 Experiment 1

The mean DNA yields per spore from conidia of *D. pinodes* in suspension and on tape segments were 0.24 fg and 0.10 fg, respectively.

3.3.2 Comparison of methods for disrupting conidia

3.3.2.1 Experiment 2

Some conidia of *D. pinodes* remained on the tapes in the UltraClean® treatment, while few or none were seen on the tapes subjected to either of the other two treatments (Table 3-2). In the case of the PowerPlant® treatment, some conidia may have remained on the tapes but been obscured by Tanglefoot®, some of which remained in blobs on the tapes. The PowerPlant® and Nonidet P40 treatments both disrupted conidia, although a few whole conidia remained in suspension following the Nonidet P40 treatment. All tubes had fragments of unknown origin in the fluid, presumably derived from the beads and/or the tape.

On the basis of these results it was decided to include the PowerPlant® kit separately and in combination with Nonidet P40, in an experiment to optimise extraction of DNA from conidia on Melinex tape segments.

3.3.2.2 Experiment 3

DNA yield from conidia of *D. pinodes* subjected to Treatment 3 (PowerPlant® kit modified with Nonidet P40 in B1 buffer, with glass beads) was significantly greater than from all the other treatments ($P=0.05$) (Table 3-3). The DNA yield was significantly less ($P < 0.05$) from conidia on tapes than in suspension. There was no significant interaction between DNA extraction protocols and whether conidia were on tapes or in suspension.

Table 3-2 Description of tapes and fluid from samples after being subjected to homogenisation in a FastPrep® machine, with various combinations of beads and bead solutions, viewed under 10 – 200 x magnification

Treatment number	Treatment name	Tape segment	Fluid
1	UltraClean® kit	Some conidia on tape. Also quite a lot of Tanglefoot®, still spread across tapes	No conidia. Many tiny (<15 μm^2) clear or brown angular fragments
2	PowerPlant® kit	No conidia but some Tanglefoot®, in blobs on tapes. Thin fragments of length approximately 15 µm	No conidia. Thin fragments of length approximately 15 µm
3	Nonidet P40	Tapes mostly free of Tanglefoot® and with detergent bubbles. One conidium on one tape.	Some whole conidia. Angular fragments of size approximately 10-15 μm^2

3.3.3 Efficacy of DNA Extraction Method 1 on different pathogens

3.3.3.1 Experiment 4

The mean yields of *D. rabiei* DNA from conidia on tapes and in suspension were 737 and 909 pg (52.6 and 64.9 fg/conidium), respectively. The difference in DNA yield from conidia on tapes and in suspension was significant ($P < 0.05$).

3.3.3.2 Experiment 5

The mean yields of *L. maculans* DNA from conidia on tapes and in suspension were 31.3 and 71.8 pg (1.90 and 4.35 fg/conidia), respectively. The difference in DNA yield from conidia on tapes and in suspension was significant ($P < 0.05$).

Table 3-3 Mean yield of DNA (pg) extracted from *D. pinodes* conidia (3.0×10^4 conidia per sample; n = 5), either on Melinex tape segments or in suspension, subjected to various DNA extraction protocols[1, 2]

Treatment	1	2	3	4
Tape	157^A	94^A	365^B	160^A
Suspension	246^C	159^D	714^C	384^C

[1] Treatments:
1. PowerPlant® kit;
2. PowerPlant® kit with 0.1% Nonidet P40 in nanopure water (220 µl) substituted for the Powerplant® bead solution, and 0.2 g Ballotini glass beads (0.85 mm diameter) substituted for the Powerplant® ball bearings;
3. PowerPlant® kit but with 0.1% Nonidet P40 in B1 buffer (0.2 M sodium phosphate, pH 8.0; 220 µl) substituted for the PowerPlant® bead solution, and 0.2 g Ballotini glass beads (0.85 mm diameter) substituted for the PowerPlant® beads;
4. PowerPlant® kit but with 0.1% Nonidet P40 in B1 buffer (220 µl) substituted for the PowerPlant® bead solution

[2] Means with the same superscript letter are not significantly different from one another ($P < 0.05$)

3.3.4 Removal of conidia from tape segments

3.3.4.1 Experiment 6

DNA yields from spores in suspension, with or without tape with adhesive (Treatments 3 and 4), were significantly higher ($P < 0.05$) than from spores that had been applied to tape with or without adhesive (Treatments 1 and 2) [Table 3-4].

Table 3-4 Mean yield of DNA from *D. rabiei* conidia either applied to Melinex tapes with (Treatment 1) or without (Treatment 2) Tanglefoot® adhesive, or in suspension with (Treatment 4) or without (Treatment 3) presence of tapes with Tanglefoot® adhesive. DNA extracted using Method 1[1]

Treatment	1	2	3	4
Mean DNA (pg) [2]	74.55^{A}	72.02^{A}	161.31^{B}	166.41^{B}
Mean DNA (fg/spore)	9.09^{A}	8.78^{A}	19.67^{B}	19.74^{B}

[1] Method 1: PowerPlant® kit but with 0.1% Nonidet P40 in B1 buffer (0.2 M sodium phosphate, pH 8.0; 220 µl) substituted for the PowerPlant® bead solution, and 0.2 g Ballotini glass beads (0.85 mm diameter) substituted for the PowerPlant® ball bearings

[2] Means with the same superscript letter are not significantly different from one another ($P < 0.05$)

3.3.5 Sensitivity of DNA extraction/qPCR system on conidia

3.3.5.1 Experiment 7

There was a significant ($P < 0.01$) linear relationship between $\log_{10}$ number of conidia and $\log_{10}$ DNA in the range 45 – 450,000 conidia/ml (Figure 3-1). Controls without conidia and the lowest concentration of conidia in the dilution series were not included in the regression analysis because at low conidial concentrations the relationship was distorted by contamination of samples, as indicated by the detection of *D. rabiei* DNA in control samples without conidia (Table 3-5). To determine what yield of DNA should be attributed to false positive results, the regression equation ($y = 0.9242\ x + 1.2487$ (Figure 3-1), was solved for number of conidia = 0, and this gave a value of 307 fg (0.3 pg). To determine the limit of detection, the regression equation was solved for number of conidia = 1, which gave a value of 320 fg, and the value of false positives (307 fg) was subtracted from that, giving a value of 13 fg. As this was greater than the minimum amount of DNA detectable of 5 fg (at threshold cycle 40; data not shown), the limit of detection was one conidium, equating to 13 fg (0.013 pg) of DNA.

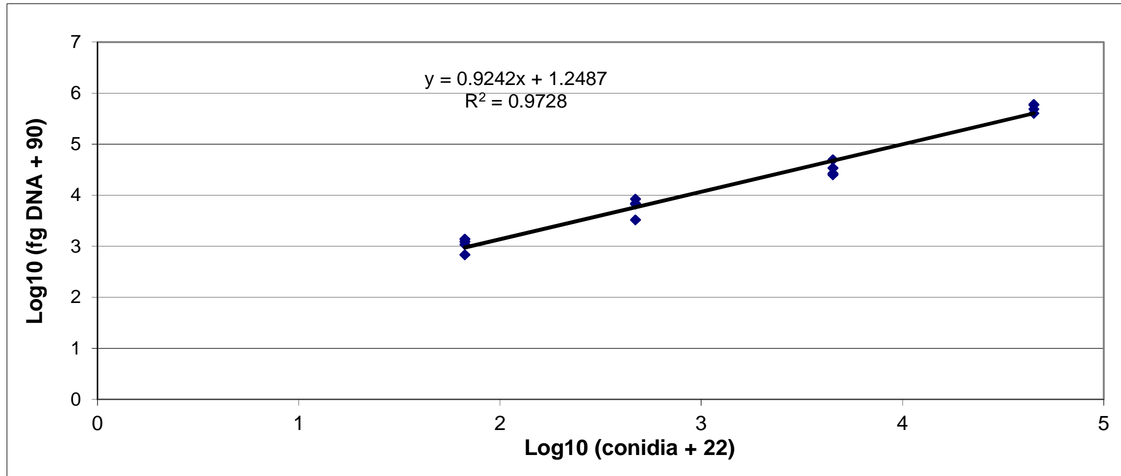

Figure 3-1 DNA yield from *D. rabiei* conidia applied to Melinex tape segments with Tanglefoot® adhesive at concentrations of 4.5 x 10^1 – 4.5 x 10^5 conidia/ml (log_{10} – transformed data)

3.3.5.2 Experiment 8

There was a significant (P < 0.001) linear relationship between log_{10} number of conidia and log_{10} DNA (Figure 3-2), with no contamination of samples, as indicated by the controls (Table 3-6). The limit of detection was 10 conidia, equating to 5.2 fg of DNA (detectable at threshold cycle 40).

Table 3-5 DNA yields (mean and standard error, SE) from *D. rabiei* conidia applied to spore trap tape segments at various concentrations

Conidia/ml	**0**	**45**	**450**	**4,500**	**45,000**	**450,000**
Conidia/sample	**0**	**4.5**	**45**	**450**	**4,500**	**45,000**
Mean DNA (pg)	3.9	0.4	1.0	6.2	33.5	513.5
SE	3.2	0.0	0.1	0.9	4.8	39.8

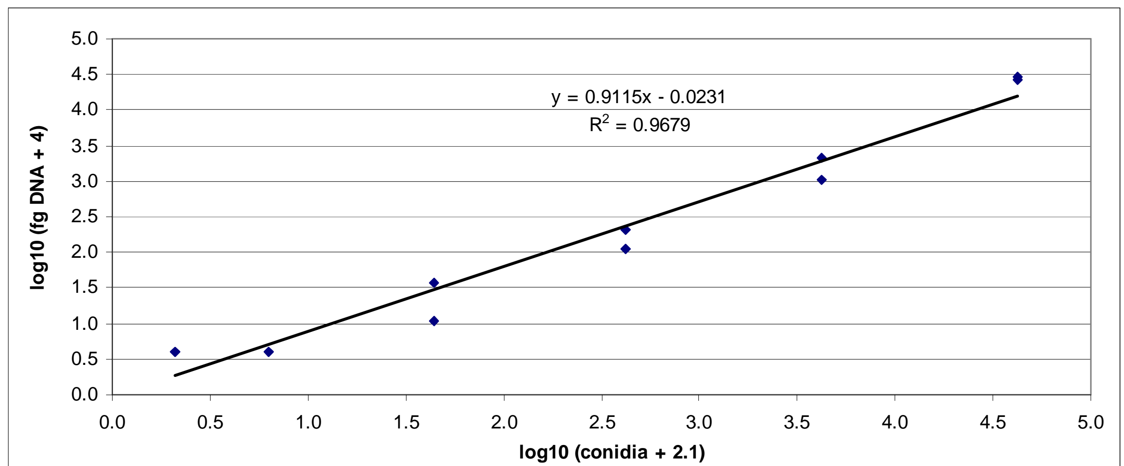

Figure 3-2 DNA yield from *L. maculans* conidia applied to Melinex tape segments with Tanglefoot® adhesive at concentrations of 0 – 4.2 x 10^5 conidia/ml

Table 3-6 DNA yields from *L. maculans* conidia applied to spore trap tape segments at various concentrations (mean and standard error, SE)

Conidia/ml	**0**	**42**	**420**	**4,200**	**42,000**	**420,000**
Conidia/sample	**0**	**4**	**42**	**420**	**4,200**	**42,000**
DNA (fg)	0	0	20	155	1,560	27,919
SE	0	0	9	35	372	635

3.3.6 Variability in calculated DNA values from conidia between qPCR assays

3.3.6.1 Experiment 9

When qPCR assays on *D. rabiei* DNA extracted from conidia in three different previous experiments were repeated, the calculated DNA values were lower than had been obtained in the original experiment in two cases and slightly higher in the third (Table 3-7). This resulted in a narrower range of mean calculated DNA yields on a per-conidium basis from the three different experiments than obtained when the three original sets of calculated DNA values were compared with one another.

Table 3-7 Reproducibility of calculated DNA yields from *D. rabiei* conidia from original and repeat PCRs from three different experiments

Original Experiment	Calculated DNA value (fg/conidium)		Repeat Ct - Original Ct[1]
	Original qPCR	Repeat qPCR	
	Mean (SE)	Mean (SE)	Mean
Experiment 4	52.6 (3.54)	15.93 (1.94)	1.85
Experiment 6	9.09 (1.05)	5.02 (0.68)	0.93
Additional experiment (data not shown)	2.95 (0.33)	3.71 (0.32)	-0.42

[1] Section 1.4.3.1

3.3.7 Spore disruption and DNA extraction from ascospores

3.3.7.1 Experiment 10

Yield of DNA from conidia of *D. pinodes* ranged from 13 to 24 fg/conidium. There was a significant ($P < 0.001$) linear relationship between log number of conidia and log DNA (Figure 3-3A), with no contamination of samples, as indicated by zero yield from the controls. Yield of DNA from ascospores ranged from 0 to 55 fg/ascospore, including zero or near-zero fg/ascospore from four out of seven samples, and thus was considerably more variable than from conidia (Figure 3-3B). However the regression relationship was still significant ($P < 0.05$). Mean DNA yield per spore from ascospores was not significantly different from that from conidia (Figure 3-4).

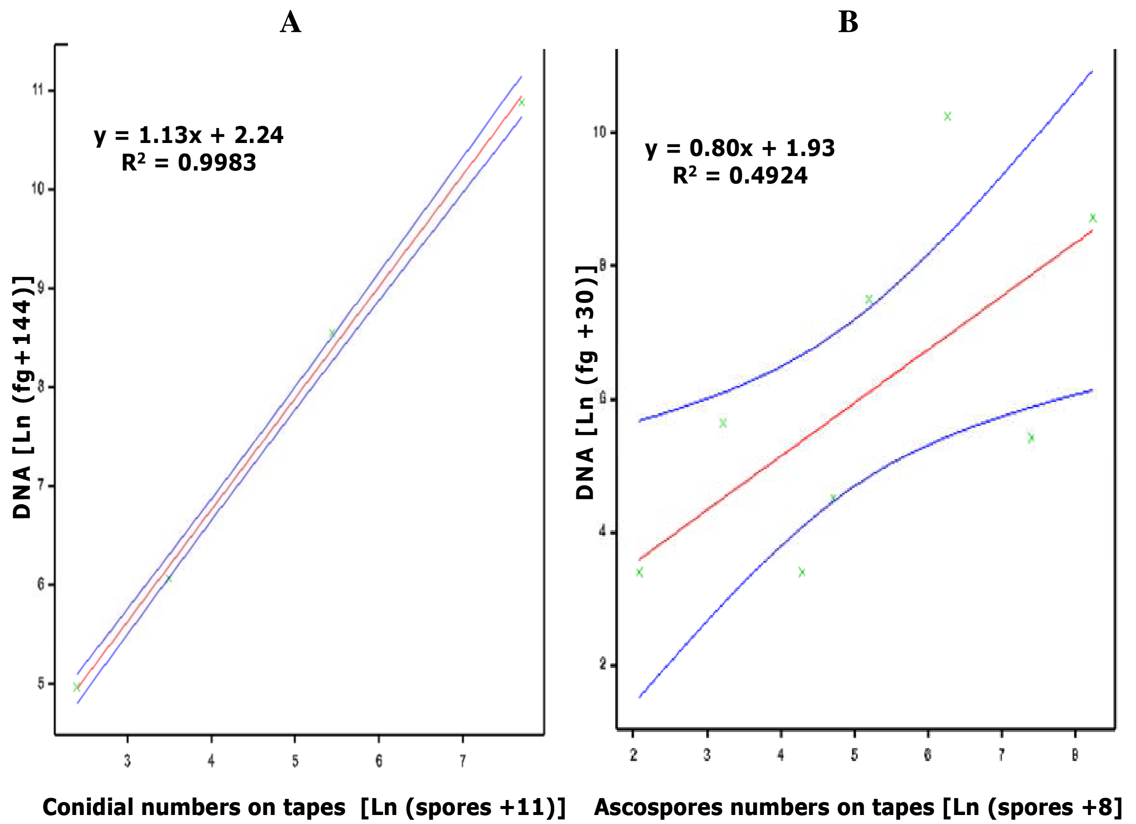

Figure 3-3 Regression lines with 95 percentile confidence limits for DNA yield from *D. pinodes* conidia (A) and ascospores (B) on Melinex tape with Tanglefoot® adhesive

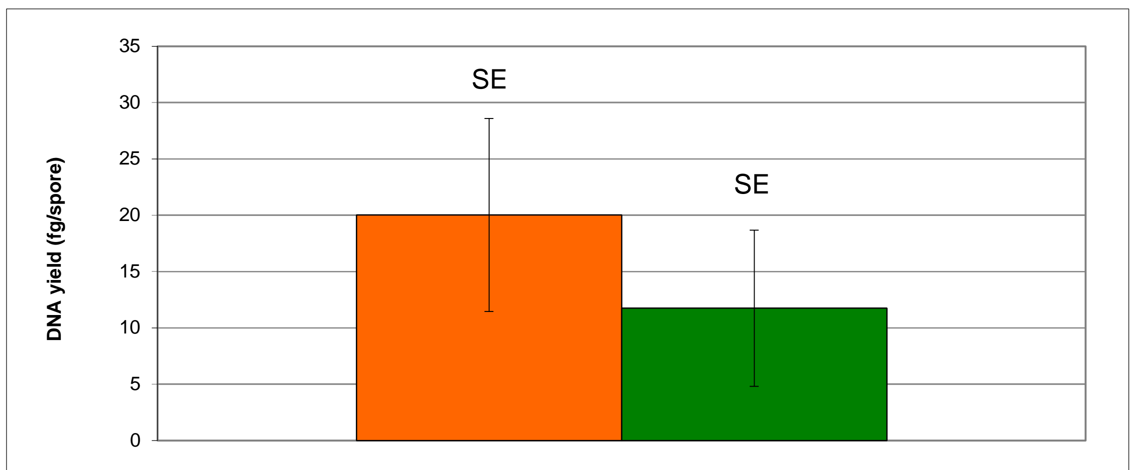

Figure 3-4 DNA yield (mean and SE) from conidia (■) and ascospores (■) of *D. pinodes* on Melinex tape with Tanglefoot® adhesive

3.3.7.2 Experiment 11

Yield of DNA ranged from 0.40 to 1.50 fg/spore from conidia and from 0.63 to 33.25 fg/spore from ascospores. Yield of DNA from ascospores of *L. maculans* was significantly greater than that from conidia, and was more variable (Figure 3-5), including zero or near-zero fg/ascospore from two out of five samples. No DNA of *L. maculans* was detected in the control without conidia or ascospores.

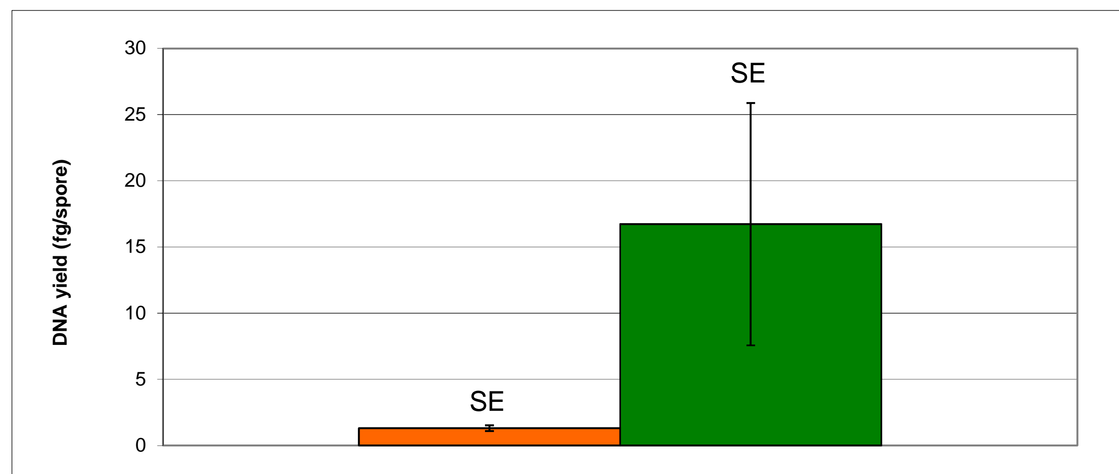

Figure 3-5 DNA yield (mean and SE) from *L. maculans* conidia (■) and ascospores (■) on Melinex tape with Tanglefoot® adhesive

3.3.8 Investigation of ascospore disruption

3.3.8.1 Experiment 12

Various pre-treatments and variations in the homogenisation step were compared to determine which was best for disrupting *L. maculans* ascospores.

A few ascospores were broken during preparation of the suspension (Table 3-8). These broken or "part-spores" consisted of two or more identifiable *L. maculans* ascospore cells. Approximately 67% of whole ascospores were disrupted by Treatment 1 ("fewer beads"), which also resulted in a significantly higher proportion of part-spores (Table 3-8). More than 99% of the ascospores were disrupted by Treatment 2 ("more beads"), with a similar number of part-spores remaining as were in the original suspension. Few ascospores were disrupted by either Treatments 3 or 4 (ceramic beads), with more part-spores resulting from Treatment 4 than from Treatment 3. Freezing spores prior to bead

beating (Treatment 5) did not significantly increase the number of disrupted ascospores compared with not freezing the spores (Treatment 1).

Table 3-8 Mean number of whole and part *L. maculans* ascospores counted at 100 x magnification in 600 µl of ascospore suspension following various bead beating treatments[1]

Treatment	Treatment Name	Whole spores	Part-spores
0	**No bead beat**	$1{,}233^{A}$	55^{Y}
1	**Fewer beads**	423^{B}	280^{X}
2	**More beads**	10^{C}	55^{Y}
3	**Ceramic BT**	Many	Some
4	**Ceramic MoBio**	Many	Many
5	**-80°C, fewer beads**	294^{B}	335^{X}

[1] Means with the same superscript letter are not significantly different from one another ($P < 0.05$)

3.3.9 Sensitivity of DNA extraction/qPCR system on ascospores

3.3.9.1 Experiment 13

There were significant ($P < 0.001$) positive correlations between the number of *L. maculans* ascospores and the DNA yield for both treatments (square root-transformed data). Greater number of beads gave significantly ($P < 0.01$) higher DNA yield than fewer beads, as indicated by comparison of the slopes of the regression lines for square root-transformed data (Figure 3-6). The limit of detection of ascospores using the greater number of beads (calculated by solving the regression equation $y = 0.6028x - 0.9043$, where y is the square root of the calculated value of DNA (pg) and x is the square root of the number of ascospores) was three ascospores, equating to 0.0195 pg (19.53 fg) DNA, which was detectable at threshold cycle 32. Because the intercept of the fitted line was less than zero it was not possible to estimate pg/ascospore from the regression equation but the mean DNA yield from the greater number of beads on a per-ascospore basis was 241.7 fg.

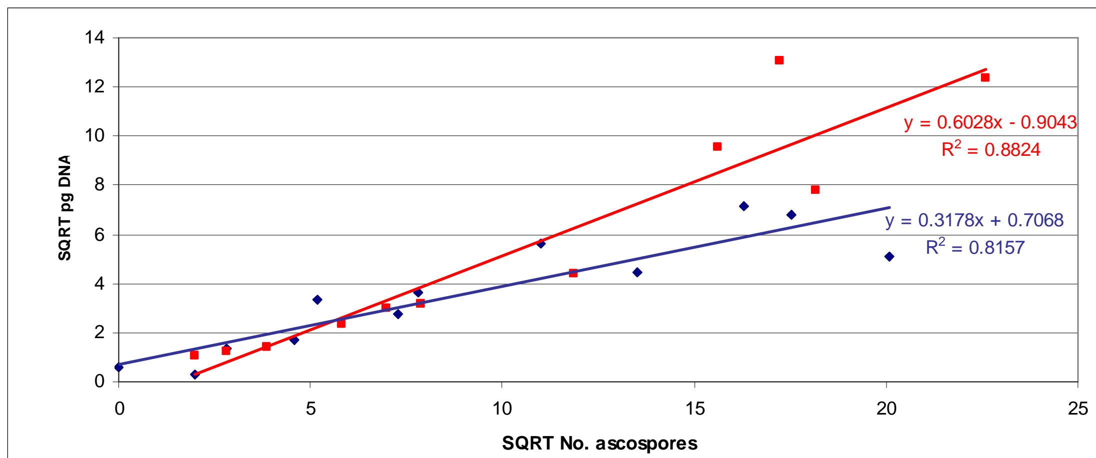

Figure 3-6 DNA yields from *L. maculans* ascospores on Melinex tape following DNA extraction procedure using 0.2 g (♦) or 0.6 g (■) of glass beads at the homogenisation step (square root-transformed data)

3.3.10 Effect on DNA yield of applying Tanglefoot® dissolved in hexane to spore trap tapes

3.3.10.1 Experiment 14

When DNA was extracted from tape samples with Tanglefoot® applied directly and applied dissolved in hexane, the mean DNA yield was 6.86 and 6.42 fg/conidium respectively. There was no significant difference between the two treatments ($P < 0.05$).

3.4 Discussion

An optimised DNA extraction and quantification method was developed for spores on tapes in spore traps. The method was based on the commercial PowerPlant® kit with modifications to the spore disruption step and the initial DNA extraction buffer. DNA was quantified using qPCR assays previously developed for all target fungi. A number of impediments were encountered and resolved in order to produce a robust and repeatable methodology for use with field samples.

3.4.1 Sensitivity

The limit of detection of conidia on spore trap tapes was one for *D. rabiei* and 10 for *L. maculans* (Experiments 7 and 8). The limit of detection and mean DNA yield of *L. maculans* ascospores on Melinex tape segments were three ascospores and 241.7 fg/ascospore, respectively (Experiment 13). This was less than that obtained by Kaczmarek et al. (2009), who estimated that each ascospore of *L. maculans* or *L.*

biglobosa contained 1-4 pg (1,000–4,000 fg) DNA. The reasons for this difference are not clear but could relate to differences in quantification relative to the standards used during the PCR assays, or differences in DNA extraction efficiencies. Kaczmarek (2009) used a DNA extraction method based on that of Graham et al. (1994), with a homogenisation step similar to the one used in the present study, but different buffers.

3.4.2 DNA extraction efficiency – spore removal and disruption

Initial studies focussed on using readily-available commercial DNA extraction kits. However it was found that they were not suitable and required modifications in order to remove spores from tapes and optimise the extraction of DNA. Homogenisation using either the PowerPlant® kit bead tubes or bead tubes with the non-ionic detergent Nonidet P40 and Ballotini glass beads, resulted in removal and disruption of most conidia; homogenisation using the UltraClean® kit bead tubes, on the other hand, did not remove conidia from spore trap tapes effectively.

Yield of DNA was consistently lower from conidia on tape segments than from conidia in suspension. An experiment was conducted to determine whether this difference was due to the presence of the tape, the adhesion of the conidia to the tape, or the presence of the adhesive. The results showed that presence of the tape did not interfere with extraction of DNA from conidia in suspension, and that conidia were not consistently removed from tapes, whether or not the tapes had been spread with Tanglefoot® adhesive. The lower yield of DNA from conidia on tape segments than in suspension, approximately equivalent to one PCR cycle, was consistent. The extraction from tapes, however, was sufficiently effective to be useful for comparisons of spores captured on tapes in the field.

In initial experiments to extract DNA from ascospores on spore trap tapes, approximately half of the samples yielded zero or near-zero DNA yields, suggesting that ascospores had not been consistently disrupted. Increasing the number of glass beads at the homogenisation step led to a significant improvement, with over 99% of ascospores disrupted. Increasing the number of glass beads at the homogenisation step led to higher mean yields of DNA from ascospores of *L. maculans* than had previously been achieved.

Increasing the number of beads used at the homogenisation step brought the ratio of beads to fluid in the bead tubes to 0.6 g : 600 μl i.e. 1 mg : 1μl. This was in line with

the proportion found by Calderon et al. (2002a) to be optimal for removal of wax and *P. brassicae* conidia or *L. maculans* ascospores from Melinex tape segments (0.2 g : 220 µl i.e. 0.9 mg : 1 µl). A similar ratio of beads to fluid has been used by other researchers (Driessen, 2005; Carisse et al., 2009; Rogers et al., 2009).

Freezing of tape segments with ascospores has been reported by some other researchers. For example, Rogers et al. (2009) froze ascospores of *Sclerotinia sclerotiorum* prior to artificially inoculating them onto plastic tape segments and extracting DNA from them. Kaczmarek et al. (2009) stored field-collected spore trap samples at -20°C prior to extraction of DNA and performance of qPCR assays to detect *Leptosphaeria* spp. It is possible that freezing spores assists the subsequent disruption and DNA extraction process, by making the cell walls more brittle, but no experimental evidence was found, either in the papers cited above, nor in any other literature to support or refute this suggestion. One benefit of freezing the spores prior to DNA extraction is that spores do not germinate during storage. It is also likely that freezing spores inhibits deterioration of DNA; this possibility warrants further investigation.

3.4.3 Spore types

DNA yield was significantly greater from ascospores than from conidia for *L. maculans*. This was most likely related to the difference in size between the two spore types for this species (35-70 x 5-8 µm for ascospores; 3-5 x 1.5-2 µm for conidia), and the greater number of cells (up to six) in the *L. maculans* ascospores compared with the conidia, which are unicellular. DNA yield was not significantly different between ascospores and conidia of *D. pinodes*, which are of similar size to one another. No comparison was possible between conidia and ascospores of *D. rabiei* because ascospores of this species were not available.

3.4.4 Variability

Comparison of results from different experiments revealed considerable variability in calculated DNA values from conidia on tapes. The variation was attributed to limits in the accuracy of conidial counts that was achievable with a haemocytometer, variations in pipetting the spore suspension, differences in DNA extraction efficiency from one experiment to another and variability between batches or "runs" of qPCR assays. An experiment was conducted to quantify the latter. When qPCR assays were conducted at the same time on DNA of *D. rabiei* previously assayed on three different occasions, the

variation in mean DNA values from the three experiments was reduced from 18-fold to 4.3-fold. Thus PCR variability between "runs" of qPCR assays accounted for approximately half the difference in mean calculated DNA yields between experiments.

Variability in qPCR assays can arise from a number of factors, including variation between PCR machines, variability in techniques such as pipetting, run-to-run variation in the standards, and differences between batches of reagents. The effects of variability resulting from different PCR machines, pipetting variability and run-to-run variability in standards can be reduced by conducting duplicate PCR assays and using the average of the two results. Duplicating PCR assays to increase the accuracy of results is a common practice. For example Rogers et al. (2009) performed qPCR assays in duplicate to detect *S. sclerotiorum* from spore trap samples. Likewise, Fraaije et al. (2005), in performing qPCR to quantify ascospores of *Mycosphaerella graminicola*, tested all spore trap samples in duplicate. Carisse et al. (2009) replicated all PCR assays to detect DNA from *B. squamosa* conidia in air samples in triplicate. Duplication of PCR assays was adopted when determining the sensitivity of the techniques in detecting DNA from ascospores of *L. maculans* (Experiment 13) and, thereafter, was incorporated into the methodology developed in this study.

Initial experiments with ascospores resulted in highly variable DNA yields for both *D. pinodes* and *L. maculans*. In both cases some ascospores had begun germinating, and the additional cells resulting from that may have contributed to the variability. Other contributing factors may have been the accuracy of spore counts on the tapes, and variation between samples in DNA extraction and qPCR 'runs', as discussed above. In subsequent experiments in which ascospores were frozen within a few days of deposition onto spore trap tapes the yields of DNA were far more consistent, thus confirming the benefits of freezing, as previously discussed.

3.4.5 PCR inhibition

Yeast was added to samples prior to DNA extraction as an exogenous control to check for PCR inhibition. Because of ongoing problems with variability of results it was decided to use the yeast values to standardise results for test organisms and thereby correct for sample-to-sample variation. This practice was adopted for Experiment 13 in which the sensitivity of the DNA extraction / PCR system on ascospores of *L. maculans* was determined, and in subsequent experiments described in this thesis. The use of

exogenous control sequences has been explored by other researchers. Carisse et al. (2009) added cDNA pSH-poly at 5×10^4 copies/µl to DNA extraction buffer prior to extraction of DNA from field samples, and amplified that sequence as an exogenous internal positive control to monitor the DNA extraction and PCR amplifications and check for possible false negatives. However, they did not use the results of the qPCR assays for the exogenous controls to adjust the results for their test organism (*Botrytis squamosa*). Haugland et al. (1999) explored the use of *Geotrichum candidum* conidia added to both test and calibrator samples to normalise *Stachybotrys chartarum* target sequences from airborne conidia, and thereby correct for sample-to-sample variation, but found that this method did not improve the accuracy of the quantification of *S. chartarum* conidia. Nevertheless they considered that inclusion of *G. candidum* may be a useful indicator of PCR inhibition. The use of exogenous controls has not been a common feature in the literature relating to application of PCR to air samples.

3.4.6 Effect on DNA yield of applying Tanglefoot® dissolved in hexane to spore trap tapes

Application of Tanglefoot® adhesive to spore trap tapes, which was convenient in terms of time required and consistency of spread on the tape surface, did not affect the amount of DNA detected on tape segments and therefore was considered to be a method suitable for use on spore trap samples.

3.5 Conclusions

A system suitable for quantifying DNA of the three model pathogens from spore trap samples was developed. The system was deemed sufficiently robust to proceed to field studies. However, further experimentation was needed to determine the potential impact of environmental factors and methods for tape preparation and sample storage on yields of DNA from field samples.

4 FACTORS AFFECTING EFFICACY OF DNA-BASED TESTS

4.1 Introduction

Methods developed to extract DNA from spore trap samples and apply qPCR assays to quantify the DNA of three pathogens (*D. rabiei*, *L. maculans* and *D. pinodes*) on spore trap tapes were reported in Chapter 3. Before using the assays in epidemiological studies of the three pathogens, there was a need to examine the potential effects of a number of factors which might affect the efficacy of these DNA-based tests; specificity of the assays, effect of climatic variables and effect of storage of samples prior to DNA extraction.

In applying qPCR assays to spore trap samples, an important consideration is that the assays do not generate false positive results (Ma et al., 2003; Williams et al., 2001). This is important to ensure the accuracy of results in any situation, but is particularly important if the assays are applied for biosecurity purposes, when a positive result may trigger the imposition of quarantine restrictions or other potentially costly response measures (Schaad et al., 2003). The PCR tests used in this study had been designed using sequence information available on GenBank and tested on local isolates. They were tested for specificity against closely related species and have been routinely used to monitor soil samples (McKay, A., SARDI, personal communication, 2008). However, testing of the PCR assays has not been conducted on air samples.

Both temperature and relative humidity may affect the efficacy of the spore trapping system (McCartney et al., 1997). Heat may alter the retention efficiency of the adhesive used on spore trap tapes or may directly reduce viability of spores. High relative humidity may result in germination of spores on the tapes either directly, or indirectly following condensation of water droplets, which could lead to an increase in yield of DNA from spores in air samples. At the other extreme, drying of spores might result in a decline in DNA. Maximum daily temperature at the field site at Kingsford, South Australia can reach 46.3^{o}C and relative humidity ranges from 7 to 100% (Anonymous,

2009), so there was a need to ascertain whether these extreme conditions would impact on yield of DNA from spores captured at the field site.

To minimise variability between DNA extractions and between PCR batches or "runs" it was decided to store spore trap samples at -20°C for up to 6 months so that samples could be processed in a single batch. As noted in the previous chapter it was considered likely that freezing spores at -20°C would inhibit deterioration of DNA. However, research was needed to confirm this suggestion.

The aims of the experiments reported in this chapter were to ascertain the specificity of the PCR assays when used on spore trap samples in the field, to determine how temperature and relative humidity might affect the detection of the target pathogens, and to determine whether storage of ascospores on the tapes at -20°C for up to six months would result in any reduction in the amount of DNA as quantified by qPCR.

4.2 Materials and methods

4.2.1 Specificity

Two experiments were conducted to check the specificity of the PCR assays when applied to spore trap samples from the field. In both cases, the specificity was also tested by the addition of large numbers of conidia of the other two model pathogens.

4.2.1.1 Specificity Experiment 1

Two spore traps were operated side-by-side at a site on the University of Adelaide Waite Campus, Urrbrae, South Australia during the week 18–25 February 2008. In the vicinity of the spore traps were trial plots where various agricultural crops, including chickpeas, had been grown in previous seasons. Pea straw, which is commonly used as a mulch, may also have been present in suburban gardens in the vicinity of the site. Weather conditions were dry and so spores of any of the three model pathogens (*D. rabiei*, *D. pinodes* and *L. maculans*) were unlikely to have been present in the air. After 6 days, both spore trap tapes were brought back in airtight containers to the laboratory, where they were sectioned horizontally and vertically into daily sections (48 x 9.5 mm) as described in Section 2.3.5, making four sets of tapes for each day of sampling. Of these four tape segments, two were mounted and stained for microscopic examination as described in Section 2.3.7, and the remaining two were set aside for DNA analysis. An additional 12 tape segments with Tanglefoot® (48 x 9.5 mm) were prepared which had not been exposed in the spore trap. A conidial suspension of all three model pathogens

(3×10^4 conidia/ml of each pathogen) was prepared as described in Section 2.4.1.1. The suspension, or RO water for the controls (100 µl per sample), was pipetted into 2 ml bead tubes. One tape segment from each of the six duplicate pairs from the spore trap was placed into a tube with conidial suspension, the other into a tube with RO water. The 12 tape segments which had not been exposed in the spore trap were placed into the 12 remaining bead tubes with either the spore suspension or RO water, six tubes of each. DNA was extracted using Method 1 (Appendix 6). The amount of DNA in each sample was estimated using qPCR assays with primers specific to the three model pathogens (Section 2.5, Table 2-2).

4.2.1.2 Specificity Experiment 2

Two spore traps were operated side-by-side at the field site at Kingsford, South Australia during the period 16 – 23 April 2008. Weather conditions were dry, so spores of any of the three model fungi (*D. rabiei*, *D. pinodes* and *L. maculans*) were unlikely to have been present in the air during that time. Spore trap tapes were brought back in airtight containers to the laboratory, where they were sectioned horizontally and vertically into daily sections (48 x 9.5 mm) as described in 2.3.5, making a set of four tapes for each of six sampling days. The spore trap tapes, and a set of tapes with Tanglefoot® adhesive which had not been exposed in the spore trap, were either spiked with a mixture of conidia of all three model fungi or not, as described in 4.2.1.1, except that the concentration of the conidial suspension was 5×10^4 conidia/ml for each of the three model fungi. DNA was extracted and qPCRs conducted as described in Section 4.2.1.1.

4.2.2 Effect of heat and relative humidity on DNA yield

Temperature was measured inside the spore trap while it was in operation, using a Tinytag® data logger (Gemini Data Loggers, UK) on a day when the maximum temperature recorded by the Bureau of Meteorology was 45.7°C. The maximum temperature recorded inside the trap, which was located in full sun, was 51°C.

The following experiments were conducted in humidity chambers consisting of sealed containers containing saturated salt solutions (Greenspan, 1977), a Tinytag® data logger to record temperature and relative humidity, and a platform on which to place samples on microscope slides (Figure 4-1).

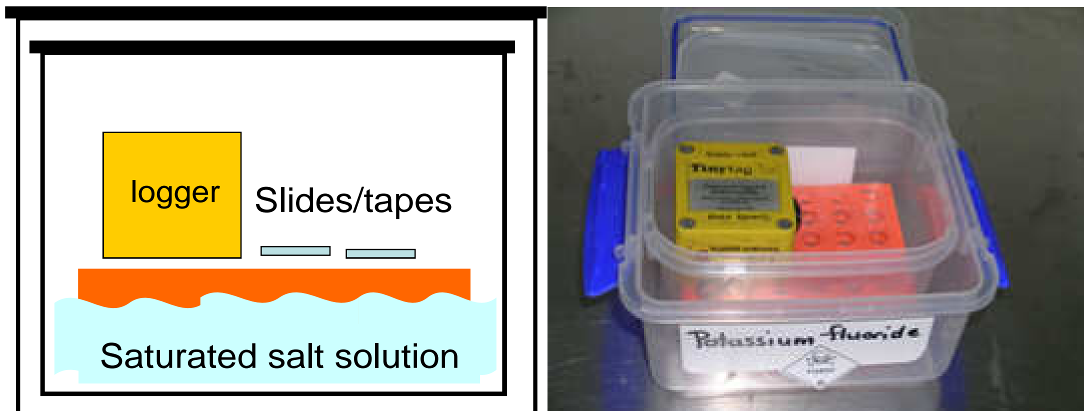

Figure 4-1. Humidity chamber used in temperature and relative humidity experiments; plan (left) and example (right)

4.2.2.1 Effect of temperature

The aim of these experiments was to determine whether high temperatures affected the yield of DNA from ascospores of *L. maculans* on segments of Melinex tape with (first experiment) and without (second experiment) Tanglefoot® adhesive.

In the first experiment a suspension of 1,000 *L. maculans* ascospores/ml was pipetted onto segments of Melinex tape with Tanglefoot® adhesive (100 µl each segment), and allowed to dry, as described in Section 2.4.1.2 Method 2. Each tape segment was then immediately placed into one of four humidity chambers (three or four samples per chamber), which were placed inside incubators at 25°C, 35°C, 45°C or 55°C for 24 hours. The relative humidity in each chamber was maintained at approximately 22% by the presence of saturated salt solutions (Table 4-1). After 24 hours the tapes were removed from the humidity chambers, inserted into 2 ml bead tubes and frozen at -20°C. The experiment was conducted in two batches, with a total of seven replicates of the 25°C and 35°C treatments, and eight replicates of the 45°C and 55°C treatments. DNA was extracted from the samples using Method 2 (Appendix 6) and DNA quantified using qPCR specific to *L. maculans* (Section 2.5). The experiment was arranged as a randomised complete block and data were analysed using Regression in Excel 2003 and ANOVA in Statistix 8.

The experiment was repeated without Tanglefoot® to determine whether an observed decline in DNA yield of *L. maculans* ascospores on tapes exposed to 55°C in the

previous experiment would also occur in the absence of adhesive on the tape. In the repeat experiment 60 µl of elution buffer was used at the end of the DNA extraction.

Table 4-1 Salt solutions used to produce relative humidities of approximately 22% in humidity chambers at various temperatures, and the actual relative humidity, recorded using a Tinytag® data logger, at each temperature

	25°C	**35°C**	**45°C**	**55°C**
Salt	Potassium acetate	Potassium fluoride	Potassium fluoride	Potassium fluoride
RH	22.5%	24.6%	21.5%	20.6%

4.2.2.2 Effect of relative humidity

The aim of this experiment was to determine whether high relative humidity affected the yield of DNA from ascospores of *L. maculans* captured onto tapes with Tanglefoot® adhesive.

In order to capture dry *L. maculans* ascospores, segments of Melinex tape (48 x 9.5 mm) with Tanglefoot® adhesive were attached to the arms of a Rotorod (whirling arm) air sampler (Lacey & West, 2006) using double-sided sticky tape. Ascospores were released from canola stubble and captured on the tapes as described in Section 2.2.5. Tape segments were removed, dissected into sections with similar numbers of ascospores, and the number of ascospores on each counted under 200 x magnification. The tape segments were then exposed to relative humidities of approximately 25%, 50% and 97% in humidity chambers with saturated salt solutions (Figure 4-1; Table 4-2) at 35°C for 24 hours. There were 4 replicates of the 25% treatment and 5 replicates of the other two treatments. DNA was extracted from the samples using Method 2 (Appendix 6) and quantified using qPCR with primers specific to *L. maculans* (Section 2.5). ANOVA was performed using Excel 2003.

4.2.3 Sample storage

The aim of this experiment was to determine if freezing of *L. maculans* ascospores on Melinex tape with Tanglefoot® adhesive, and storage at -20°C for 28 or 182 days, affected DNA yield.

Table 4-2 Salt solutions used to produce relative humidities of approximately 25%, 50% and 97% in humidity chambers, and the actual relative humidities recorded using a Tinytag® data logger, at 35°C

	25%	50%	97%
Salt	Potassium fluoride	Magnesium nitrate	Potassium sulphate
RH	24.6%	50.0%	96.7%

A suspension of approximately 1.5 x 10^5 *L. maculans* ascospores/ml was prepared as described in Section 2.4.1.2, and 100 µl pipetted, at the start of the experiment, onto each of 28 Melinex tape segments (48 x 9.5 mm) with Tanglefoot® adhesive prepared as described in Appendix 2. The tapes with ascospores were placed into purpose-built containers as described in Section 2.3.6, and subjected to one of the following four treatments;

1. not frozen, not stored
2. frozen at -20°C overnight
3. frozen at -20°C for 28 days
4. frozen at -20°C for 182 days

There were seven replicate tapes in individual tubes for each of the four treatments.

To check for any differences in subsequent DNA extraction efficiency among treatments, conidial suspensions of *L. maculans* (labelled as C0, C1, C28 and C182), each at concentration of 5 x 10^4 conidia/ml, in chilled (4°C) RO water, were freshly prepared as described in 2.4.1.1, on each of Days 0, 1, 28 and 182 respectively, and pipetted into 2 ml bead tubes (100 µl per tube). Three replicate tubes of the freshly prepared conidial suspension were included at each time of DNA extraction. As well, a further nine replicate tubes of conidial suspension C0 were prepared at the start of the experiment, frozen and held at -20°C, and included at each time of DNA extraction. This was done because of the potential for variation in the concentration of conidia between preparation times. As usual, yeast was added to each sample (ascospores and conidia) immediately prior to DNA extraction to check for differences in DNA yield between samples. DNA was extracted using Method 2 (Appendix 6). The amount of DNA in each sample was estimated using qPCR assays with primers specific to *L. maculans* (Section 2.5).

ANOVA was conducted on the calculated *L. maculans* DNA values, adjusted for calculated yeast DNA values as a covariate, using Genstat 11.1.

4.3 Results

4.3.1 Specificity

4.3.1.1 Specificity Experiment 1

Little to no DNA of target pathogens was detected on spore trap tapes which had been exposed in the field and to which no conidia had been added, and there was no significant difference ($P < 0.05$) between those tapes and the controls, which received no conidia and had not been exposed in the field (Figure 4-2). For the samples to which conidia had been added, yields of DNA from all three pathogens were slightly higher from field-exposed tapes than from tapes which had not been exposed in the field but the difference was not significant ($P < 0.05$).

The amount of DNA of all three pathogens was significantly higher ($P < 0.05$) from field-exposed tapes with conidia added, than from tapes not exposed in the field and with conidia added (Figure 4-3). DNA yields from co-extracted yeast were not significantly different between the four treatments (data not shown). There was considerable dust on the field-exposed tapes (Figure 4-4).

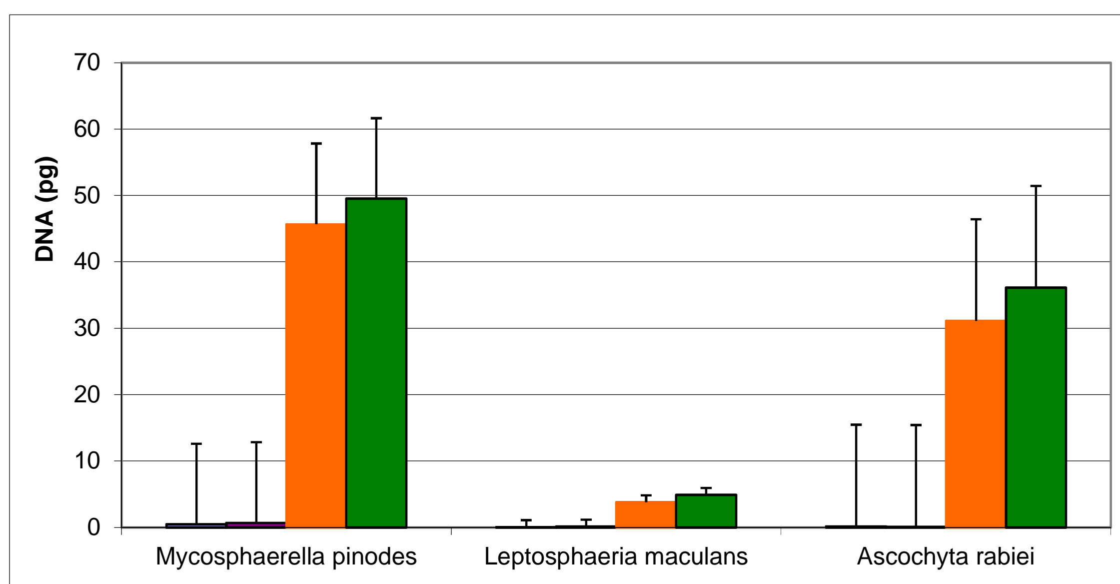

Figure 4-2 DNA yield from samples of spore trap tapes which had either been previously exposed or not in a spore trap at Waite Campus, Urrbrae, South Australia, during the week 18-25 February 2008, with or without conidia of *D. pinodes, L. maculans* and *D. rabiei* (3,000 conidia of each) in suspension added prior to DNA extraction (■ not exposed, no conidia added; ■ exposed, no conidia added; ■ not exposed, conidia added; ■ exposed, conidia added). Error bars show least significant difference ($P < 0.05$)

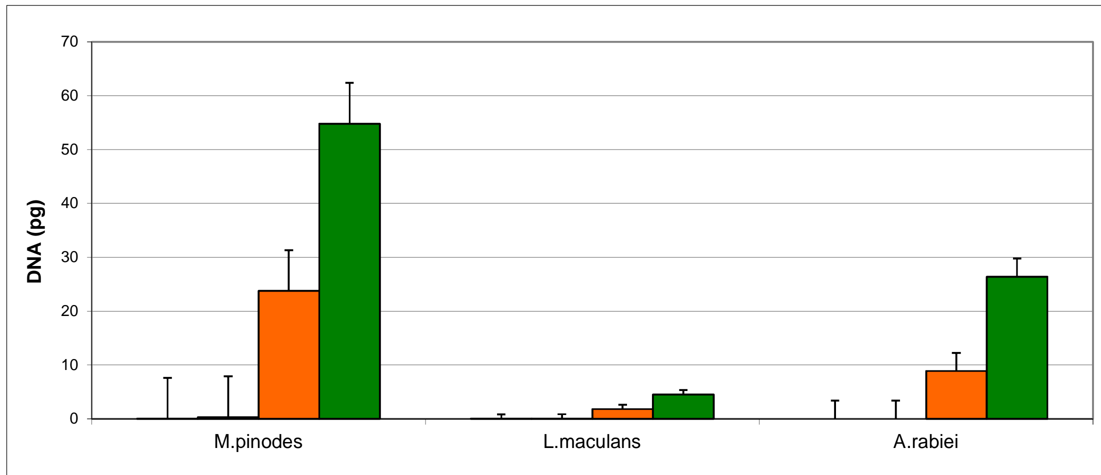

Figure 4-3 DNA yield from samples of spore trap tapes which had either been previously exposed or not in a spore trap at Kingsford, South Australia, during the period 16–23 April 2008, with or without conidia of *D. pinodes, L. maculans* and *D. rabiei* (5,000 conidia of each) applied to the tape segments prior to DNA extraction (■ not exposed, no conidia added; ■ exposed, no conidia added; ■ not exposed, conidia added; ■ exposed, conidia added). Error bars show least significant difference ($P < 0.05$)

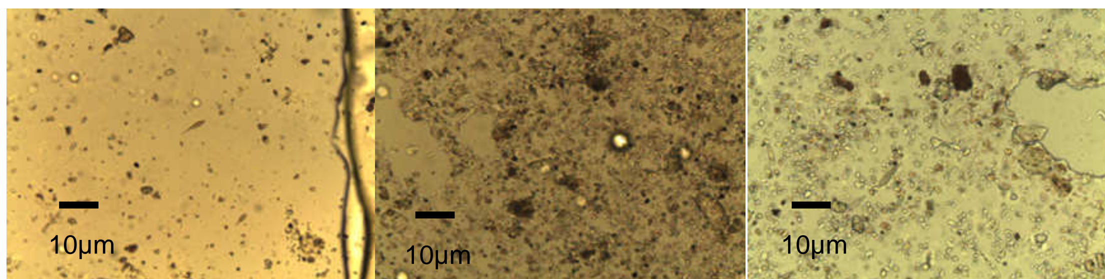

Figure 4-4 Micrographs of field-exposed tapes from specificity trial, showing dust, spores of various fungi, and other particles

4.3.2 Effect of heat and relative humidity on DNA yield

4.3.2.1 Effect of temperature

In the first experiment, using tapes with adhesive, there was a steady decline in yield of DNA from *L. maculans* ascospores on tapes with increasing temperature (Figure 4-5). The relationship, described by the equation $y = -0.0249 x + 2.1787$, where y is DNA (pg) and x is temperature (°C), was significant ($P < 0.01$; $R^2 = 0.29$). ANOVA indicated a significant difference ($P < 0.05$) in DNA yield between samples exposed to 55°C and those exposed to all other temperatures (Figure 4-5).

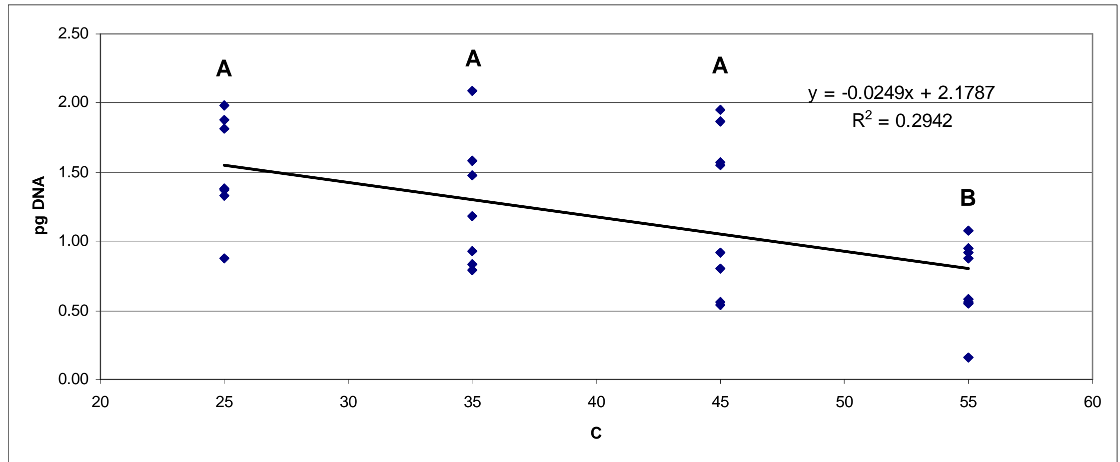

Figure 4-5 DNA yield from *L. maculans* ascospores on Melinex tape with adhesive, subjected to a range of temperatures for 24 hours. Sets of values with the same letter were not significantly different from one another ($P < 0.05$)

In the second experiment, using tapes without adhesive, there was a steady decline in yield of DNA from *L. maculans* ascospores with increasing temperature (Figure 4-6). The relationship, described by the equation y = -0.0554x + 3.7583, where y is DNA (pg) and x is temperature (°C), was significant ($P < 0.001$; $R^2 = 0.42$). DNA yield from ascospores exposed to 25°C was significantly higher ($P < 0.01$) than from those exposed to 55°C.

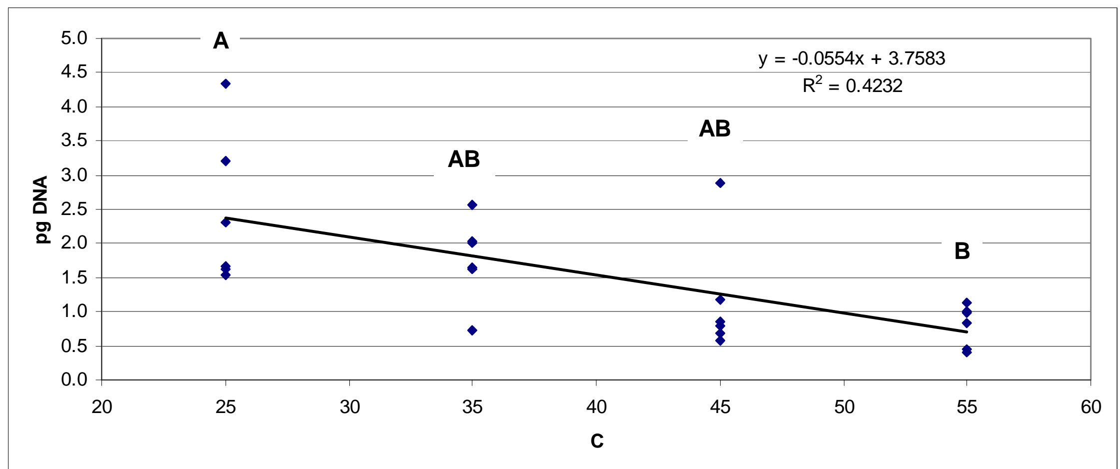

Figure 4-6 DNA yield from *L. maculans* ascospores on Melinex tape without adhesive, subjected to a range of temperatures for 24 hours. Sets of values with the same letter were not significantly different from one another ($P < 0.05$)

4.3.2.2 *Effect of relative humidity*

There was no significant difference ($P < 0.05$) among yields of DNA from ascospores exposed to relative humidities from 25% to 97% (Table 4-3).

Table 4-3 Mean yield of DNA from *L. maculans* ascospores on Melinex tape with adhesive, subjected to a range of relative humidities in a humidity chamber for 24 hours

RH	25%	50%	97%
DNA (fg/ascospore)	487	498	501

4.3.3 *Sample storage*

DNA yields both from yeast and from the fresh conidial suspension prepared on Day 182, were significantly less on Day 182 than on Days 0, 1 and 28, which were not significantly different from each other ($P < 0.05$) (Table 4-4). These results indicated that the DNA extraction efficiency was less on Day 182 than on the other days.

Covariance analysis indicated that yields of DNA from *L. maculans* ascospores varied in parallel with those from yeast. This was the case when the comparison was made across all treatments (Days 0, 1, 28 and 182) ($P < 0.001$) and when only Days 0, 1 and 28 were compared ($P < 0.05$). The latter covariance analysis was conducted to check that the lower yields of DNA on Day 182, due to lower DNA extraction efficiency, had not biased the covariate regression.

Table 4-4 Mean yield of DNA (pg) of yeast co-extracted with *L. maculans* spores, and of *L. maculans* conidia freshly prepared on each day of an experiment to determine the effect of storage at –20°C on DNA yield of *L. maculans* ascospores. Sets of values with the same superscript were not significantly different from one another ($P < 0.05$)

	Yeast	Fresh conidia
Day 0	$36{,}269^{A}$	9.6^{a}
Day 1	$51{,}083^{A}$	12.8^{a}
Day 28	$45{,}583^{A}$	24.5^{a}
Day 182	$12{,}049^{B}$	2.7^{b}

Yield of DNA was significantly ($P < 0.05$) higher (approximately double) from ascospores stored frozen for 28 days than from ascospores subjected to each of the other treatments, which were not significantly different from one another (Table 4-5).

Table 4-5 Mean yield of DNA (covariate-adjusted; log transformed) from *L. maculans* ascospores after storage at –20°C for various time periods. Sets of values with the same superscript were not significantly different from one another (P < 0.05)

Days of storage	0	1	28	182
Adjusted mean DNA (ln pg)	3.36^{A}	3.37^{A}	3.98^{B}	3.29^{A}

Covariance analysis indicated that yield of DNA from stored *L. maculans* conidia varied in parallel with variation in yield of DNA from yeast (P < 0.05).

Analysis of covariate-adjusted DNA yields indicated that there was a steady decline in yield of DNA from *L. maculans* conidia with storage (Figure 4-7). For this reason stored conidial suspensions were not used as a check for differences in DNA extraction efficiency between extraction days, as had been intended.

4.4 Discussion

The aims of these studies were three-fold: to determine specificity of the PCR assays when applied to air samples; to determine the impacts of heat and relative humidity on yield of DNA from spores on spore trap tapes; and to determine whether storage of samples at -20°C for up to 182 days (6 months), prior to DNA extraction, would affect yield of DNA from the spores.

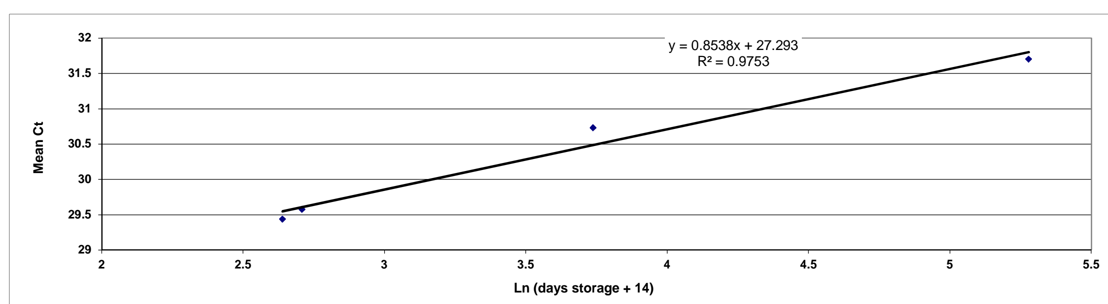

Figure 4-7 Threshold cycle (Ct) of *L. maculans* conidia stored at -20°C for various time periods (log-transformed data)

4.4.1 Specificity

The presence of large numbers of conidia of the two other model pathogens on tape segments did not interfere with the qPCR assays used for *D. rabiei*, *D. pinodes* and *L.*

maculans. The qPCR assays were shown to be specific to the target pathogens when applied to air samples collected from two field sites, including the site to be used for epidemiological studies (Kingsford, South Australia), shortly before commencement of the ascospore release season for these fungi. Very small amounts of DNA of target pathogens detected in the assays was assumed to have been due to small amounts of pathogen material in dust at the field sites. This assumption requires further testing. A large increase in DNA of target pathogens from field-exposed tapes with conidia, compared with that from control (non-exposed) tapes with conidia, was attributed to the presence of dust on the tapes, which may have assisted in spore disruption and/or removal of conidia from the tapes. Less DNA of *L. maculans* was detected than of the other two pathogens, most likely due to the smaller size and number of cells in *L. maculans* conidia compared with the other two (3-5 x 1.5-2 µm, one cell for *L. maculans* (Punithalingam & Holliday, 1972b), 8-16(-18) x 3-4.5(-5) µm, 2(-4) cells for *D. pinodes* (Punithalingam & Holliday, 1972b) and 12 (10-17.5) x 4.5 (3.5-5) µm, 1-2 cells for *D. rabiei* (Galloway & Macleod, 2003; Punithalingam & Holiday, 1972a).

The increase in yield of DNA from samples with dust has not been reported by other authors. On the contrary, other researchers have reported interference in PCR assays arising from the presence of dust in air samples. For example, in studies to monitor the spread of the sugarcane smut pathogen, *Ustilago scitaminea,* Magarey et al. (2008) found that additional DNA purification measures were required to overcome inhibition of PCR assays when applied to spore trap tape samples contaminated with dust or ash. In later studies, contamination of some sampling sites with dust generated by haulage equipment prevented detection of DNA of *U. scitaminea* altogether (Magarey et al., 2009). Driessen (2005) reported that some samples of DNA from *Puccinia boroniae,* extracted from spore trap samples, had to be diluted 1:100 before a PCR product was obtained. She attributed this to the presence of PCR inhibitors in dust and debris on the spore trap tapes. The effect in the present study may have been related in some way to the application of conidia, either to tapes or in solution, after the tape samples had been exposed in the field, and may not be manifested in a field situation. The impact of increased yield of DNA from spores on dusty tapes than from tapes without dust is likely to be minor for the model pathogens used in this study, which are released only in moist conditions. However, it should be taken into consideration during field studies on

those occasions that dry, dusty conditions immediately precede moist conditions suitable for ascospore release.

The specificity experiments were conducted during dry conditions because moist weather would most likely have triggered release of ascospores of the target pathogens from inoculum sources in the field, and thus interfered with the experimental results. However, it is likely that the air spora would be different during moist conditions, and might include particles which could either cross-react with, or inhibit, the qPCR assays. Other researchers have addressed the question of specificity of PCR assays for use in air samples, in various ways, as discussed in Section 1.4.5). These include checking the specificity of the initial selection of primers during development of the assay, testing assays against DNA from a panel of other fungi either closely related to the target fungus (Carisse et al., 2009), or not (Fraaije et al., 2005), or by spiking samples with spores of other fungi likely to be collected in air samples (Williams et al., 2001). The most rigorous way to check for specificity of PCR assays which are to be applied to air samples, is to check the PCR results using some other method, such as microscopy. As noted in Section 1.4.5, Calderon et al. (2002a) reported good agreement between PCR assays and microscopic observations when used to detect *L. maculans* on oilseed rape stems infested with the pathogen using a Burkard Hirst-type spore trap.

The specificity testing reported in this chapter was a preliminary study aimed at determining whether there was any obvious or ubiquitous source of cross-reactivity in air samples at the field sites, since the assays had been designed for testing samples from soils or plants, rather than from the air. However, because such testing could not exclude the possibility of cross-reactions or inhibition in samples collected in wet weather, it was decided that a selection of samples collected during field studies to be conducted in this project, would be examined microscopically to check for correlation with DNA results.

4.4.2 Effect of heat and relative humidity on DNA yield

Exposure of ascospores on Melinex tape to 55°C, at a constant relative humidity of approximately 22%, resulted in a significant reduction in yield of DNA, to approximately half that following exposure to 25°C, and this was the case whether or not Tanglefoot® adhesive had been applied to the tapes. There was also a trend towards

a reduction in yield of DNA with increasing temperatures between 25 and 45°C, but these differences were not statistically significant.

The trend towards reduction in DNA recovered from spores exposed to higher temperatures was consistent with that reported for viability of fungal spores, as discussed in Section 1.7. Reduced spore viability following exposure to heat has been reported by numerous authors, including Kochman (1979), who reported that decline in viability of urediniospores of *Phakopsora pachyrhizi*, the soybean rust pathogen, increased with exposure to increasing temperatures ranging from 11 to 42.5°C for 8 hours. Likewise Twizeyimana & Hartman (2010) reported a decline in viability of *Phakopsora pachyrhizi* urediniospores exposed to temperatures ranging from 25 to 50°C. The decline was fastest at 40 and 50°C, with zero germination following 4 or more hours of exposure, and slowest at 25°C, at which temperature it took 18 hours for germination to decline to zero. In addition to showing that spore viability was inversely proportional to temperature, these studies also indicated that some decline in spore viability may occur over time at a range of temperatures, down to at least 11°C. In the present study, yield of DNA was not checked at the start of the experiment, so it was not possible to determine whether the amount of DNA decreased over time at the lower temperatures (25 to 45°C). This, or at least viability of spores on tapes at the end of a sampling period, may warrant further investigation.

The effect of heat on spore viability depends on various factors including the moisture content of the spores, metabolic activity (particularly whether or not spores have commenced germination), nutrient availability, and the species of fungus (Barkai-Golan & Phillips, 1991). Moist heat is more damaging to spores than dry heat. In the present study, ascospores of *L. maculans* were kept dry before and during the study, and there was no indication from the appearance of the spores that germination had commenced. High temperatures are generally associated with dry weather in southern Australia, which is why the experiment was conducted at low relative humidity (22%). The impact of heat on DNA recovered from spore trap samples may be greater in moist conditions than was found in this experiment.

The effect of heat on spore viability increases with exposure time (Barkai-Golan & Phillips, 1991), and this is also likely to be the case for the effect on DNA within the spore. In the present study, extremely hot temperatures (sufficient to cause 55°C inside

the spore trap) were shown to require air temperatures of at least 45.7^{o}C. Such temperatures are unlikely to be maintained in the field for more than a few hours on any one day in cropping regions of southern Australia. However, if very hot weather were to continue for several days, the cumulative effect may be sufficient to cause a reduction in yield of DNA from spores in field samples. While ascospores of the fungi used as models in this study are unlikely to be released under such hot conditions, it is possible that hot weather may occur after release and capture of ascospores, but before trap clearance. The potential for reduced yield of DNA needs to be considered in assessing qPCR results from spore trap samples collected during periods of extremely hot weather. The finding that the effect of temperature on yield of DNA from spore trap tapes was independent of the presence of Tanglefoot® adhesive on the tape suggests that this adhesive is suitable for use in hot weather. This was further borne out by the fact that there was no observed change in consistency of the adhesive from spore trap tapes following exposure to 55°C. This is in contrast with Vaseline, which has a melting point between 38 and 60°C and is therefore considered unsuitable for use in hot weather (Gálan & Dominguez-Vilches, 1997).

Exposure of ascospores on Melinex tapes to relative humidities of approximately 25%, 50% and 97% at a constant temperature of 35°C did not affect the yield of DNA. The main factor likely to affect yield of DNA from spores exposed to different relative humidities is commencement of germination. This might occur at high humidity and would be expected to increase the amount of DNA. Another potential impact is from drying, which can reduce the viability of spores (Beyer & Verreet, 2005). It is not clear from the literature whether either *L. maculans* or *D. pinodes* ascospores can germinate at high relative humidity without free moisture, as might occur in a Hirst-type spore trap. There was no indication, from the experiment reported here, that germination of *L. maculans* ascospores had commenced, or that either high or low humidity had any effect on the amount of DNA. However, the experiment was conducted at a temperature higher than optimal for *L. maculans* ascospore germination (20°C; (Biddulph et al., 1999)), and the possibility that the spores would germinate at high relative humidity in more favourable temperatures cannot be ruled out. Time did not permit a repeat of the experiment reported here at 20°C. It was decided that, should DNA yields from field studies planned in this project, be higher than expected, duplicate tape samples would be examined for evidence of spore germination.

Relative humidity at the field site at Kingsford, South Australia can fall below 25%, to as low as 7%. It is possible that such dry conditions could impact on yield of DNA from ascospores, but the effect is not likely to be of great magnitude given that there was no apparent decline in DNA at 25% relative humidity.

Investigation of the impact of high temperature on DNA of other fungal species, such as *D. pinodes*, was not undertaken in the present study, due to time constraints, but this warrants further investigation. Further research on the impact of relative humidity on yield of DNA from both *L. maculans* and *D. pinodes* ascospores at temperatures optimal for spore germination is also required.

4.4.3 Sample storage

There was no indication of a decline in yield of DNA from *L. maculans* ascospores on Melinex tape with Tanglefoot® adhesive stored at -20°C for 182 days (6 months), but yield of DNA from conidia in suspension did decline over that period. An apparent increase in yield of DNA from both ascospores and fresh conidia on Day 28 was difficult to explain, as it was not matched by an increase in co-extracted yeast DNA. The DNA extraction efficiency may have been higher, and the concentration of yeast applied on that day may inadvertently have been less than on other days. The difference in yield of DNA represented a difference of one PCR cycle and was deemed to be within a range of variability acceptable for comparisons of spores captured on tapes in the field.

The difference in effect of storage on *L. maculans* conidia compared with ascospores may have been due to their smaller size and thinner cell walls. Alternatively, the difference may have been related to different storage conditions. Ascospores on tapes were stored at the rear of a large freezer room, and were unlikely to have been subjected to major temperature fluctuations. Conidial suspensions, on the other hand, were stored in a smaller freezer which was frequently opened, and may have been subjected to partial defrosting on occasions.

Time did not permit testing the effects of storage at -20°C on ascospores of *D. pinodes*. Given the decline in yield of DNA from *L. maculans* conidia, it is possible that storage may cause a reduction in DNA from spores morphologically different from *L. maculans* ascospores, such as ascospores of *D. pinodes*. The effect of storage at -20°C on spores of other species requires further investigation.

Few researchers have reported storage of samples obtained from air at -20°C prior to DNA extraction, as noted in Section 1.6, and none have commented on potential impacts of freezing spores on yield of DNA. Most researchers, for example Calderon et al. (2002a), Carisse et al. (2009) and Fountaine et al. (2010), appear to have extracted DNA from spore trap samples without prior storage. However, in many situations, there is a need to store samples prior to DNA extraction due to time constraints or if scaling up from research to more extensive application.

Some researchers have checked viability of spores following freezing and storage, but most studies have related to lyophilisation (freeze-drying) or storage in liquid nitrogen (Ryan & Smith, 2004). Freezing at -20°C is cheaper and more convenient than either of these methods and the studies reported here suggest that spores can be stored for at least 6 months without degradation of DNA.

4.5 Conclusions

The qPCR assays were shown to be specific to the target pathogens, in the presence of large numbers of spores of other fungi, and particles from air samples collected in the field in dry conditions. However, should yields of DNA from field samples planned for this project be unexpectedly high, specificity of the assays would be checked by microscopic examination of duplicate spore trap samples.

High temperatures were found to reduce DNA yields, but this was considered unlikely to have a major effect during the main ascospore release periods for the model pathogens in this study (late autumn and winter), when temperatures are seldom over 25°C. They may however cause a decline in yields from samples collected in the spring, summer and early autumn, and this needs to be considered in analysing data from field trials planned for this project. The impact of high temperature on DNA yields should be considered if applying the system described in this thesis to trap spores of other species in hot weather, particularly those species which release spores in dry conditions, when temperatures may be high.

There was no evidence that relative humidity affected yield of DNA from ascospores on Melinex tape with adhesive at 35°C. Further studies are required to confirm that relative humidity does not affect DNA yield at the lower temperatures more commonly encountered during times of ascospore release of the model pathogens in this study.

Storage of spore trap samples at -20°C for 182 days prior to DNA extraction was shown to have no impact on DNA yield from ascospores of *L. maculans*. The effect of storage at -20°C on spores of other species requires further investigation.

In summary, the system for detection of airborne ascospores of *D. rabiei, D. pinodes* and *L.maculans* has been demonstrated to be generally robust, fast and accurate, and to be suitable for epidemiological studies of these three model pathogens.

5 COMPARISON OF FIELD DATA WITH PREDICTIONS FROM EPIDEMIOLOGICAL MODELS

5.1 Introduction

Plant disease epidemiology can be described as the study of plant pathogens in populations, of environmental factors that influence the amount and distribution of the disease they cause, and of rates of change of disease in time and space (Zadoks & Schein, 1979; Zadoks, 1999). Understanding how a plant pathogen operates in any particular environment is key to management of plant disease, whether to eliminate a newly introduced pathogen, to monitor its spread and the success of any measures to limit that spread, or to minimise crop losses caused by the pathogen when it is established in an area.

Fungal plant pathogens often have complex life cycles involving the release of spores when environmental conditions, particularly temperature and moisture, are suitable. This ensures that spore release coincides with availability of suitable host plant tissue to colonise. Knowledge of the specific conditions that lead to spore release can enable the development of targeted control measures which are precise and accurate, and which minimise the resources required for effective control.

In order to achieve precision in the description of factors which determine the timing of particular phases in the life cycle of a plant pathogen, the epidemiology of a disease is sometimes represented by means of mathematical models. As previously discussed, (Section 1.8), epidemiological models may be based on an understanding of the mechanisms driving disease dynamics, or they may be empirical, with the values of model parameters determined by the best mathematical fit (Tivoli & Banniza, 2007).

The predictive models, Blackleg Sporacle and G1 Blackspot Manager, have been developed for blackleg of canola and blackspot of field peas respectively (Salam et al., 2003; Salam et al., 2011a). The purpose of these models is to predict the timing of release of airborne ascospores, which, as discussed in Sections 1.9.1 and 1.9.2, are the main source of primary inoculum for both these diseases. This information is then used

to generate crop disease forecasts to allow informed decision-making by farmers about disease management options, such as sowing date or fungicide usage.

The timing of onset of ascospore release differs between seasons at a location and between locations within a season (Salam et al., 2007). Blackleg Sporacle was developed using data from four locations in Western Australia but has not previously been calibrated or tested for any location in South Australia. Blackspot Manager was calibrated and tested using data from a number of locations in southern Australia, including data from the Kingsford site (Section 2.1) in 2007 and 2008. However, the data were not collected by means of spore trapping, but rather through release of ascospores in a wind tunnel from stubble incubated at the site (Section 2.2.5). Ascospore release of *D. pinodes* has not previously been measured using a spore trap combined with PCR diagnostics.

In the previous two chapters, development of methodology was reported for spore trapping combined with PCR diagnostic assays, for three pathogens, *L. maculans*, *D. pinodes* and *D. rabiei*. In this chapter the application of that methodology in the field, and the verification of the methodology through microscopy and use of trap plants to monitor aerial inoculum, are described.

The aims of the work were:

1. To test the spore trap/PCR-based diagnostic system in the field and compare it with a trap plant system

2. To determine the sensitivity of the system in detecting three model plant pathogens, *L. maculans*, *D. pinodes* and *D. rabiei*, which may be present at different aerial concentrations of ascospores

3. To use the system to test predictions of spore release generated by the epidemiological models Blackleg Sporacle and G1 Blackspot Manager

5.2 Materials and methods

5.2.1 Spore trap

2008 season: The slit-type volumetric spore trap (Section 2.3.2) was operated on a 7-day cycle at the trial site at Kingsford from 27 February 2008 till 28 January 2009, with the exception of the period 4 July to 12 August when the trap malfunctioned. The trap

was positioned with the aperture at a height of approximately 60 cm above ground. Spore trap tapes were replaced weekly. After removal from the trap the tapes were processed as described in Section 2.3.5.

2009 season: The spore trap was placed at the field site on 12 February 2009 in the same location as in 2008, and operated on a 7-day cycle till 17 March 2010. Spore trap tapes were replaced weekly and samples processed as described above.

5.2.2 Inoculum source

2008 season: Canola stubble, of unknown cultivar, heavily infested with *L. maculans* was collected on 28 February 2008 from a commercial property near Struan in the south-east of South Australia, which had been windrowed in the last week of November 2007. Stubble (0.9 kg), consisting of canola stem and root pieces of length 40-80 cm with blackleg cankers, was placed around the spore trap in a circle of radius 1.5 m, on 5 March 2008.

Field pea stubble was left *in situ* at the site of a 2007 disease trial at the Kingsford site in a plot approximately 200 m by 30 m running north-south, approximately 10 m to the west of the spore trap. The trial had been sown with cultivars susceptible to blackspot (Kaspa, Alma, OZP0602 [now released as Gunyah], and breeding line WA2211) in May 2007, and harvested in November 2007. The field peas had been rated as having up to 57% of the plant affected by blackspot (McMurray et al., 2011).

Chickpea stubble was left *in situ* at the site of a 2007 disease trial at the Kingsford site in a plot 12 m by 12 m at distance of 350 m west-southwest of the spore trap (Coventry 2011a; Coventry 2011b). The plots had been sown in June 2007 with three cultivars differing in their susceptibility to ascochyta blight, including cultivar Howzat which had become severely diseased, Almaz and Genesis 90. The trials were harvested in late November 2007.

The prevailing wind direction during rain periods in autumn through to spring at the field site is west-southwest, so the placement of the spore trap was downwind from inoculum sources.

2009 season: Canola stubble infested with *L. maculans* (4.5 kg), field pea stubble infested with *D. pinodes* (3.75 kg), and chickpea stubble infested with *D. rabiei* (1.5 kg), were placed around the trap in a circle of radius 4 m on 12 February 2009 (Figure

5-1). The canola stubble was cultivar ATR-Beacon collected from a 2008 crop severely infected with *L. maculans*, on a commercial property at Mayhall near Struan, on 2 February 2009. The field pea stubble was a mixture of cultivars Kaspa and Alma gathered from a 2008 field pea disease trial at SARDI's Turretfield Research Station near Kingsford on 12 February 2009, which had been rated as having up to 53% of internodes affected by blackspot. The chickpea stubble was a mixture of cultivars Howzat and Almaz collected from a 2008 chickpea Ascochyta blight disease trial at Turretfield Research Station on 14th December 2008 which had moderate to high disease severity.

Figure 5-1 Stubble of canola infested with *L. maculans*, field pea infested with *D. pinodes* and chickpea infested with *D. rabiei* in a circle of radius 4 m around the spore trap and trap plants at Kingsford in February 2009. Arrows indicate the location of the stubble

5.2.2.1 Assessment of fruiting bodies on stubble in early spring 2009

On 2 September 2009 the area around the spore trap, where stubble had been placed at the start of the season, 7 months previous, was searched for stubble. Three randomly-selected stem sections each of canola and field pea stubble were collected and the number and maturity stage of fruiting bodies was assessed as described in Section 2.2.4. No stubble readily identifiable as chickpea was detected.

5.2.3 DNA diagnostics

At the end of each season PCR analyses were conducted on spore trap samples. It was not possible to analyse all samples due to resource constraints. To decide which samples to select, model predictions were generated by Blackleg Sporacle and G1 Blackspot Manager to determine the predicted start, peak and end of ascospore release for *L. maculans* and *D. pinode*s, respectively. An assumption was made that if ascospores of *D. rabiei* were released at the field site, the timing would be similar to that of *D. pinodes* since no model was available to predict timing of *D. rabiei* ascospore release in the Australian environment. Duplicate spore trap samples (Section 2.3.5) for the period before, during and after the predicted start and end of ascospore release for each pathogen, were examined microscopically, and the number of ascospores counted as described in Section 2.3.7. Once the timing of the start and end of the ascospore release season had been verified, several samples were selected at a number of points on the model prediction curve, starting before the beginning, and finishing after the end, of the predicted ascospore release. Most samples were selected from days on which rain had been recorded at the trial site, but non-rain days were also included, in order to check for false positive DNA results.

DNA was extracted using Method 2 (Appendix 6). DNA yields were quantified by qPCR as described in Section 2.5. DNA yields of model pathogens were standardised to the mean yeast yields for all samples as described in Section 2.5.

5.2.4 Microscopy

Samples for microscopic examination were selected to correspond with low, medium and high yields of DNA from matching spore trap samples and, in the case of *D. pinodes*, samples collected in both winter and summer were included. In order to determine whether the relationship of DNA yield to ascospore numbers was the same for both seasons, samples from both the 2008 and the 2009 seasons were counted for *L. maculans*. For *D. pinodes*, ascospores were counted only for the 2009 season, although several summer samples from the 2008 season were examined microscopically to confirm the presence of *D. pinodes* ascospores, without counting.

Ascospore counts were conducted as described in Section 2.3.7. In each case, at least every fourth vertical transect was counted. For portions of tape where there was a transition between large and small numbers of ascospores, at least every second

transect, and in some cases every transect, was counted, depending on spore numbers and variability. For each portion of tape, ascospore numbers were estimated by multiplying the number of ascospores counted by the inverse of the proportion of transects counted. Estimated total ascospore numbers per sample were calculated by adding the estimates for each portion of tape.

To determine the relationship between DNA and ascospore numbers, regression lines were fitted using Microsoft Excel® 2003.

5.2.5 Trap plants

In order to validate the spore trap results and to determine how aerial concentration of ascospores related to infection of host plants, trap plants were placed alongside the spore trap on a weekly basis as described in Section 2.3.1.

5.2.5.1 Trap Plants in 2008

In 2008, trap plants were placed in the field from the week ending 30 April until the week ending 5 November. The numbers of lesions were counted approximately 15 days (for field pea and chickpea) or approximately 18 days for canola days after return from the field. Canola cotyledons, which had abscised by the time of counting, were not included in the count.

5.2.5.2 Trap Plants in 2009

In 2009, trap plants were placed in the field from the week ending 5 May until the week ending 4 November. Trap plants were assessed approximately 5 days (field peas) or 14 days (canola and chickpeas) after being brought back from the field. The reason for the earlier counting of lesions than in 2008 was that there were many more lesions on field pea and canola trap plants than in 2008, and these tended to coalesce if left too long. In contrast to 2008, lesions on canola cotyledons were included in the count, as cotyledons had not yet abscised.

5.2.6 Epidemiological models

The mathematical models, Blackleg Sporacle (Salam et al., 2003) and G1 Blackspot Manager (Salam et al., 2011a), developed for blackleg of canola and blackspot of field peas, respectively (Section 1.8), incorporate the effects of temperature and rainfall on pseudothecial development and ascospore release. G1 Blackspot Manager is based on Blackleg Sporacle, and the models are essentially identical apart from the values of some parameters, which reflect differences in the biology of the two pathogens.

In the models, each day from harvest of previous season's crop is considered to be either favourable for pseudothecial maturity (FPM) or not. A day is deemed to be FPM when rainfall for a specified period (R-threshold-duration) is greater than or equal to a threshold value (R-threshold), and the temperature for a specified period (T-threshold-duration) is above a threshold (T-threshold). Once sufficient FPM days have elapsed, ascospore release can start, provided that the daily rainfall exceeds a threshold (RAD-threshold). R-threshold-duration, R-threshold, T-threshold-duration, T-threshold and RAD-threshold, are all parameters of the models. The date on which sufficient FPM days have elapsed is also a parameter (PM-begin). Cumulative ascospore release follows a beta distribution curve, with the start and end value of the range designated as the start and end of the process of ascospore maturation, PM-begin and PM-end respectively. Two additional parameters specify the shape of the beta distribution, PM-peak (the mode of the distribution) and PM-peak sharpness (Salam et al., 2003). Both these parameters are estimated by comparison with measured distributions. The fraction of ascospores released during an ascospore discharge event, AD-fraction, is another parameter of the models and is assumed to be constant throughout the period of ascospore discharge.

The value of parameters used in each of the models is presented in Table 5-1. The main difference in the biology of the two pathogens leading to differences in parameter values is the response to temperature of pseudothecial maturity (Salam et al., 2011a).

5.2.7 Generating model predictions

The Blackleg Sporacle model was run with start date as 1 January using original parameter values (Table 5-1) in the first instance, and using patch point weather data sets for Struan, South Australia, supplied by Queensland Department of Environment and Resource Management (the procedure for computing the interpolated data has been described by Jeffrey et al. (2001)) for the period 1 January till date of collection of stubble from the field, and thereafter using weather data recorded by the automated weather station (AWS) at Kingsford, supplemented by Australian Bureau of Meteorology (BOM) weather data for Rosedale, South Australia during instances when the AWS malfunctioned.

Table 5-1 List of parameters and their values used in the Blackleg Sporacle and G1 Blackspot Manager models for predicting onset of pseudothecia maturity and seasonal ascospore showers in relation to blackleg in canola and blackspot of field peas, respectively (Salam et al., 2003), (Salam et al., 2011a)

Parameter	Definition	Unit	Value	
			B'Leg	**B'spot**
AD-fraction	Fraction of mature ascospores released during an ascospore discharge event	unitless	0.40	0.40
PM-begin	Number of days favourable for pseudothecia maturation before the onset of pseudothecia maturity	days	43	10
PM-end	Number of days favourable for pseudothecia maturation from PM-begin until pseudothecia maturation ends	days	125	90
PM- peak	Number of days favourable for pseudothecia maturation from PM-begin until the rate of pseudothecia maturation reaches a maximum	days	30	45.1
PM-peaksharpness	Dimensionless number that affects the shape of the pseudothecia maturation function	unitless	2	1.25
R-threshold	Lower limit of total accumulated rain required for conditions to be favourable for pseudothecia maturation	mm	4	4
R-threshold-duration	Time period used in calculating the value of R-threshold	days	7	7
RAD-threshold	Amount of daily rainfall on and above which an ascospore discharge event can take place	mm	0.20	0.20
T-threshold	Limit of the mean daily temperature required for conditions to be favourable for pseudothecia maturation (upper limit for Blackleg Sporacle; lower limit for G1 Blackspot Manager)	°C	22	9
T-threshold-duration	Time period used in calculating the value of T-threshold	days	10	10

Blackspot Manager was run with start date 1 January using original parameter values (Table 5-1) in the first instance, and using weather data recorded by the AWS at Kingsford, supplemented by Australian Bureau of Meteorology (BOM) weather data for Rosedale, South Australia during instances when the AWS malfunctioned.

5.2.8 Model calibration

To calibrate the models Blackleg Sporacle and G1 Blackspot Manager for the Kingsford site, pattern analysis was carried out for each data set (2008 and 2009) by plotting daily spore trap DNA data against daily model predictions, each expressed as fraction of the total. A regression line was fitted with the intercept set at the origin. Points of major

deviation from the regression line were investigated to determine a likely explanation for the discrepancy.

In order to make comparisons with model predictions, the spore trap DNA data were converted to percentage of the total measured yield of DNA. Since not all ascospores released were captured, and since the yield of DNA was not determined for every day throughout the season, these percentages were multiplied by a "conversion factor". The conversion factor was arrived at by calculating the value which maximised the overall fit between the observed and predicted values, using the SOLVER function in Microsoft Excel® 2003. The SOLVER function is designed to optimise a value for a formula including a number of parameters (Salam et al., 2007).

Based on the results of these investigations, the models were modified where appropriate, for example by incorporating the effect of relative humidity on ascospore release.

For each model, sensitivity analysis of model parameters was carried out by changing the value of each parameter separately, to determine which had the greatest impact, measured as root mean squared deviation (RMSD), when the resulting model predictions were compared with spore trap DNA data. Several values for each of the most sensitive parameters were tested in the model in all combinations, using a factorial design. For each combination of parameter values, the resulting model predictions were compared with the spore trap data using RMSD. Calibration was achieved by selecting the set of parameter values giving the lowest RMSD.

In the absence of a full spore trap data set for 2008 for blackleg, trap plant data were compared with model predictions, using regression analysis.

5.3 Results for *L. maculans*

5.3.1.1 Spore trap

In 2008, spore trap samples gave low amounts of *L. maculans* DNA (less than 2 pg/tape) from late March till early June when the amount of DNA began to increase (Figure 5-2 A and B). Of the samples tested, the amounts of DNA were greatest on 9 June (35 pg/tape) and on 30 and 31 August (57 and 50 pg/tape respectively). It was not possible to determine whether these were the highest DNA yields for the season because of the gap in the spore trap data from 4 July to 12 August. After early October, yields

of DNA were generally low (less than 2 pg/tape), although some slightly higher amounts (2-5 pg/tape) were detected in late November and mid-December. The yield of DNA from a single sample coinciding with a rain event in late January 2009 was negligible.

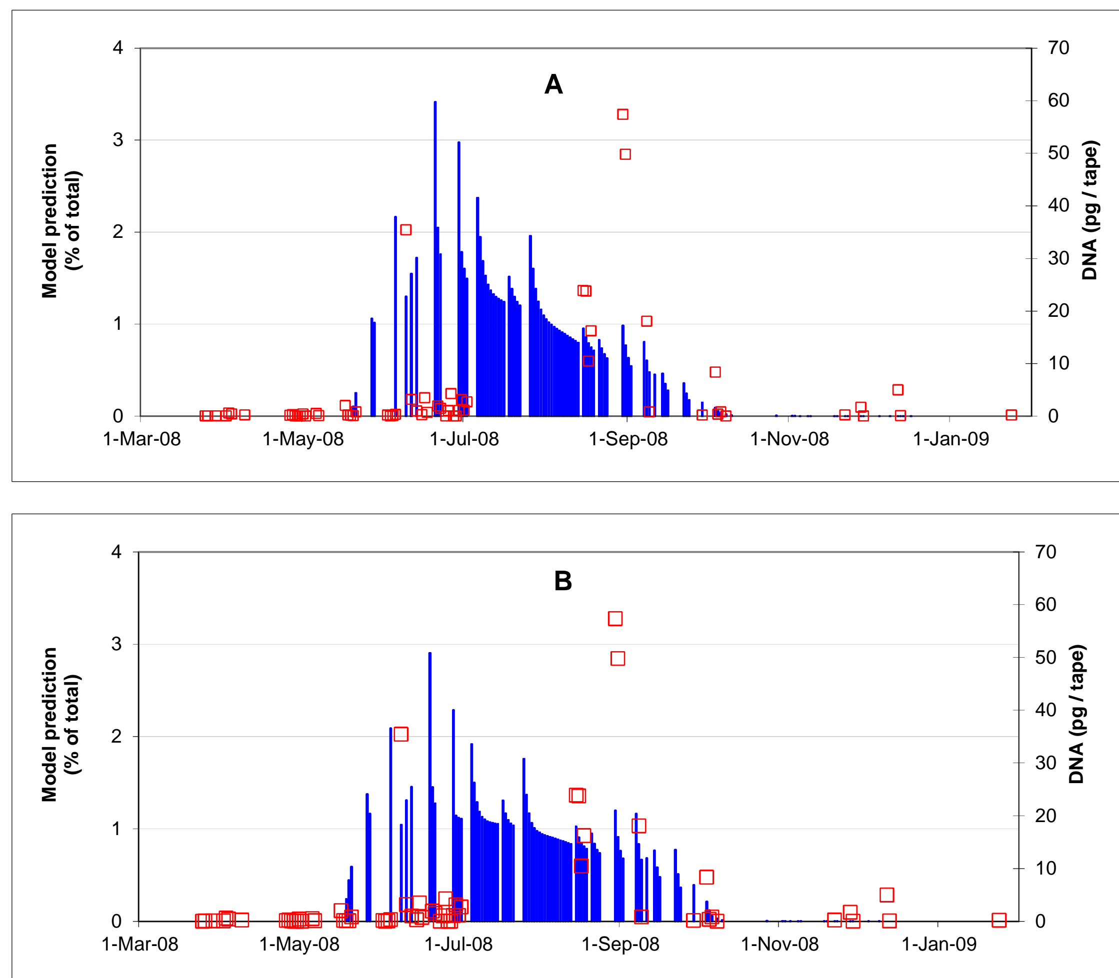

Figure 5-2 Yield of *L. maculans* DNA (☐) from a spore trap, and predicted ascospore release from the Blackleg Sporacle model (▌) at Kingsford in 2008 and early 2009 A. before and B. after model calibration

Yields of DNA in 2009 remained low (less than 2 pg/tape) from early March till early June. They then increased sharply to mid-July (highest yields being 22 and 28 pg/tape on 10 and 11 July, respectively) before dropping to low levels (less than 2 pg/tape) by mid-October (Figure 5-3 A or B). Yields remained low during rainy days in the summer.

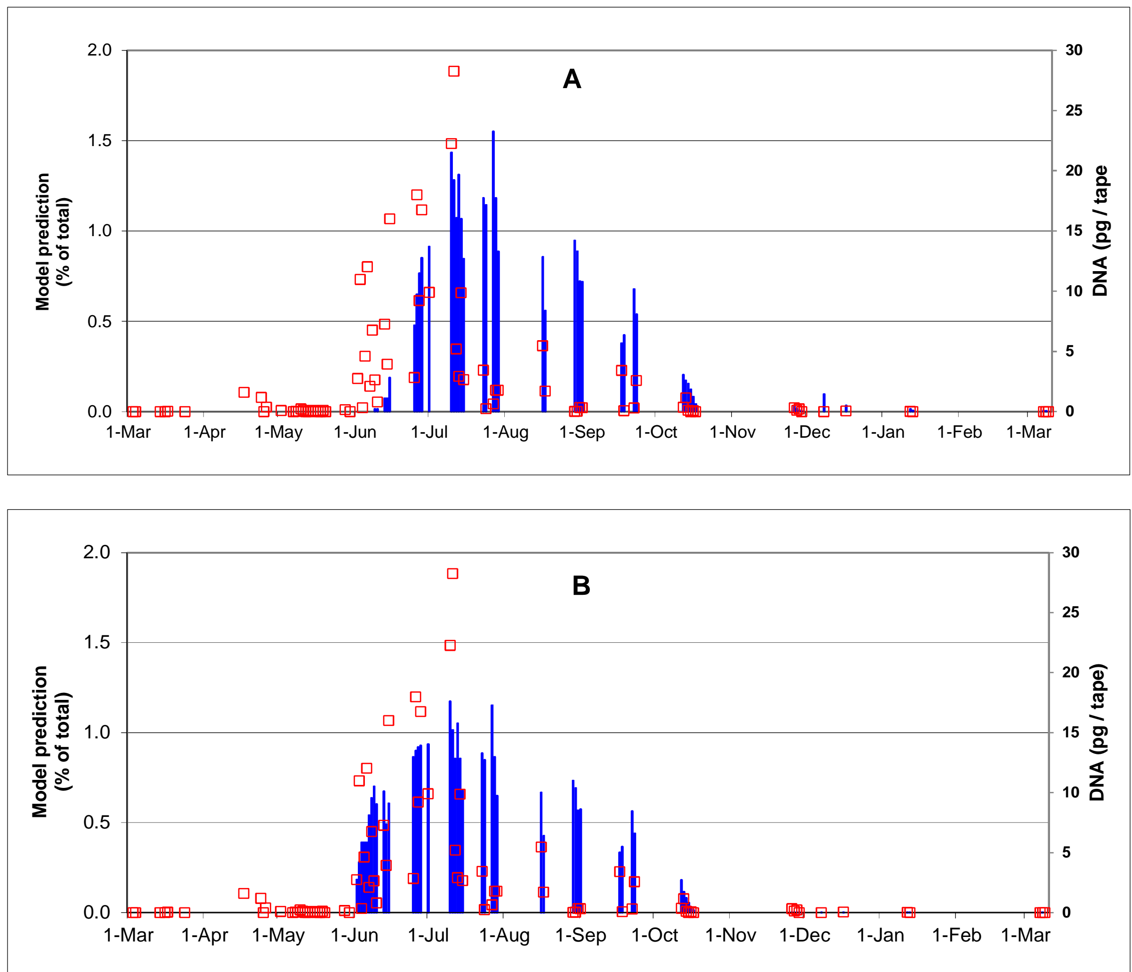

Figure 5-3 Yield of *L. maculans* DNA (□) from a spore trap, and predicted ascospore release from the Blackleg Sporacle model (▌) at Kingsford in 2009 and early 2010 A. before and B. after model calibration

5.3.1.2 Assessment of fruiting bodies on stubble in early spring 2009

The numbers of fruiting bodies counted on stem sections (0.5 x 1 cm) on three canola stems collected from around the spore trap on 2 September 2009 were 30, 42 and 2 respectively. Of 15 fruiting bodies mounted and examined at 100 x magnification, eight were old fruiting bodies without spores (stage E) and the remaining seven were pseudothecia at varying stages of maturity; one at stage B, one at stage C and five at stage D1.

5.3.1.3 Microscopy

Table 5-2 shows the estimated number of *L. maculans* ascospores on daily spore trap tape samples and the corresponding DNA yield of *L. maculans* on duplicate tape segments for selected 2008 samples. There were 12 instances where no ascospores were counted but DNA was detected on duplicate tape segments. In six of those cases,

including the two with the highest DNA yields, the entire tape segment had been examined microscopically, so it was likely that the microscope counts on matching tape segments were correct. It was concluded there were some false positive DNA results. However, in all instances the yield of DNA was low (highest value 0.58 pg/tape). There was a significant ($P < 0.001$) linear relationship between DNA yield and number of ascospores counted ($R^2 = 0.9295$; $y = 0.3617x + 0.7225$ where y = DNA (pg) and x = number of ascospores; Figure 5-4 A or B). Solving the regression equation with number of ascospores = 1 gave 1.084 +/- 1.181 (95% confidence limits) pg DNA, which was detectable at cycle threshold 31.9. Thus the limit of detection was one ascospore.

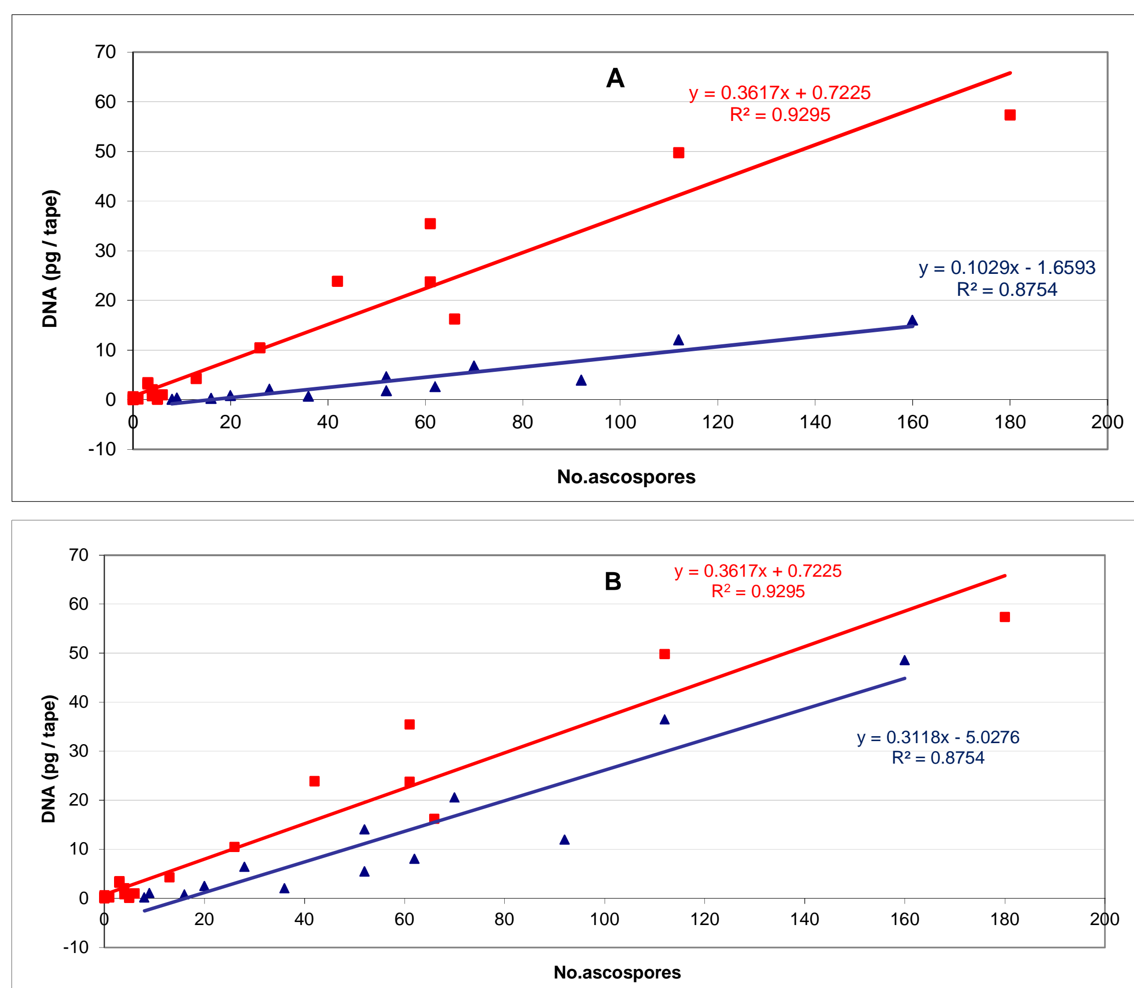

Figure 5-4 Correlation between the number of *L. maculans* ascospores counted on spore trap tapes viewed at 200 x magnification, and yield of *L. maculans* DNA from duplicate spore trap tapes from a range of samples from a spore trap at Kingsford in 2008 (■) and 2009 (▲). A. 2009 data not adjusted to 2008 DNA yields; B. 2009 data adjusted to 2008 DNA yields

Table 5-2 Number of *L. maculans* ascospores counted on spore trap daily tape samples (48 x 9.5 mm) viewed at 200 x magnification, and yield of DNA from duplicate spore trap tapes from a range of samples from a spore trap at Kingsford in 2008

Date	DNA (pg / tape)	No. ascospores	Proportion of tape examined
29-Mar-08	0.05	0	0.5
02-Apr-08	0.04	0	0.5
03-Apr-08	0.58	0	All
04-Apr-08	0.41	0	All
09-Apr-08	0.23	0	0.2
26-Apr-08	0.15	1	All
27-Apr-08	0.25	0	All
28-Apr-08	0.05	0	All
29-Apr-08	0.03	0	All
30-Apr-08	0.02	0	All
01-May-08	0.41	0	0.2
02-May-08	0.29	0	0.2
17-May-08	2.01	4	0.5 for areas with spores, 0.25 for remainder
18-May-08	0.16	0	0.2
19-May-08	0.11	5	0.2
20-May-08	0.10	5	0.2
21-May-08	0.80	4	All
09-Jun-08	35.43	61	All
11-Jun-08	3.16	3	All
13-Jun-08	0.99	6	All
16-Jun-08	3.45	3	All
26-Jun-08	4.27	13	All
15-Aug-08	23.86	42	0.5 for areas with spores; 0.25 for remainder
16-Aug-08	23.76	61	0.5 for areas with spores; 0.25 for remainder
17-Aug-08	10.44	26	0.5 for areas with spores; 0.25 for remainder
18-Aug-08	16.22	66	0.5 for areas with spores; 0.25 for remainder
30-Aug-08	57.36	180	0.5 for areas with spores; 0.25 for remainder
31-Aug-08	49.76	112	0.5 for areas with spores; 0.25 for remainder

In 2009 there were no instances where no ascospores were counted but DNA was detected on duplicate tape segments (Table 5-3). Again, it was possible to fit a significant ($P < 0.001$) linear relationship between DNA yield and number of ascospores counted, but both the slope and the intercept of the line differed significantly from that

obtained from 2008 data (R^2 = 0.8754; y = 0.1029x - 1.6593 where y = DNA (pg) and x = number of ascospores; Figure 5-4A). The DNA yield per ascospore was considerably lower than in 2008, with the ratio of slopes being 3.5. When the 2009 DNA data were standardised to the 2008 data, using the ratio of mean yields of co-extracted yeasts for each of the data sets (3.03), the difference in yield of DNA was much reduced (new regression equation y = 0.3118x - 5.0276; ratio of the 2008 and 2009 regression line slopes 1.16; Figure 5-4B), but there was still a significant difference ($P < 0.05$) between both the slopes and the intercepts of the two regression equations. Solving the regression equation y = 0.3118x - 5.0276, with number of ascospores = 1 gave -4.7158 +/- 5.333 (95% confidence limits) pg DNA. The upper end of this range (0.6175 pg) was detectable at cycle threshold 33.9, thus there was a chance of being able to detect a single ascospore. The smallest number of ascospores at which solving the regression equation gave a calculated DNA amount above the detection threshold was 17, which was equivalent to 0.273 +/- 4.691 pg (273 +/- 4,691 fg) DNA, which was detectable at threshold cycle 35.6. Therefore the calculated limit of detection was 17 ascospores.

Table 5-3 Number of *L. maculans* ascospores counted on spore trap daily tape samples (48 x 9.5 mm) viewed at 200 x magnification, and yield of DNA from duplicate spore trap tapes from a range of samples from a spore trap at Kingsford in 2009

Date	DNA (pg / tape)	No. ascospores	Proportion of tape examined
4-Jun-09	0.35	9	All of areas with spores; 0.25 for remainder
5-Jun-09	4.64	52	0.25
6-Jun-09	12.05	112	0.25
7-Jun-09	2.13	28	0.25
8-Jun-09	6.78	70	0.5 for areas with spores; 0.25 for remainder
9-Jun-09	2.66	62	0.5 for areas with spores; 0.25 for remainder
10-Jun-09	0.82	20	0.25
14-Jun-09	3.95	92	0.25
15-Jun-09	16.02	160	0.25
24-July-09	0.27	16	0.25
27-July-09	0.67	36	0.25
28-July-09	1.80	52	0.25

Ascospores viewed on tape segments from both 2008 and 2009 showed no sign of germination.

5.3.1.4 Trap plants

In 2008 no lesions were detected on trap plants from the weeks ending 30 April to 25 June 2008, with the exception of the week ending 11 June when a few were recorded (Figure 5-5). The number of lesions increased from early July to a peak in mid-July and remained high till mid-August after which it declined to relatively low levels. After 24 September canola trap plants placed in the field were affected by hot, dry conditions and it was not possible to ascertain accurately the number of lesions.

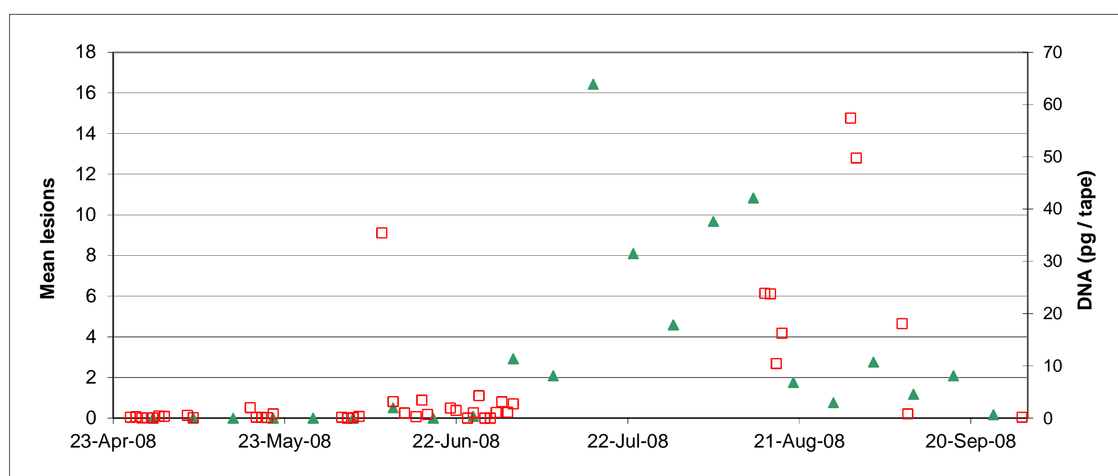

Figure 5-5 Yield of *L. maculans* DNA (□) from a spore trap, and number of leaf lesions on canola cultivar ATR-stubby trap plants (▲) at Kingsford in 2008

In 2009 the trap plant data followed a very similar pattern to the spore trap DNA data, with lesions first forming on plants placed in the field during the week ending 27 May, the highest mean number of lesions recorded in mid-July and no further lesion development from early October (Figure 5-6).

5.3.1.5 Comparison with model predictions

The assumption was made that the small number of ascospores released early in the season (before 30 May), which occurred in both years, originated from stubble from the season before the last. These data points were removed from further comparisons between model predictions and field data.

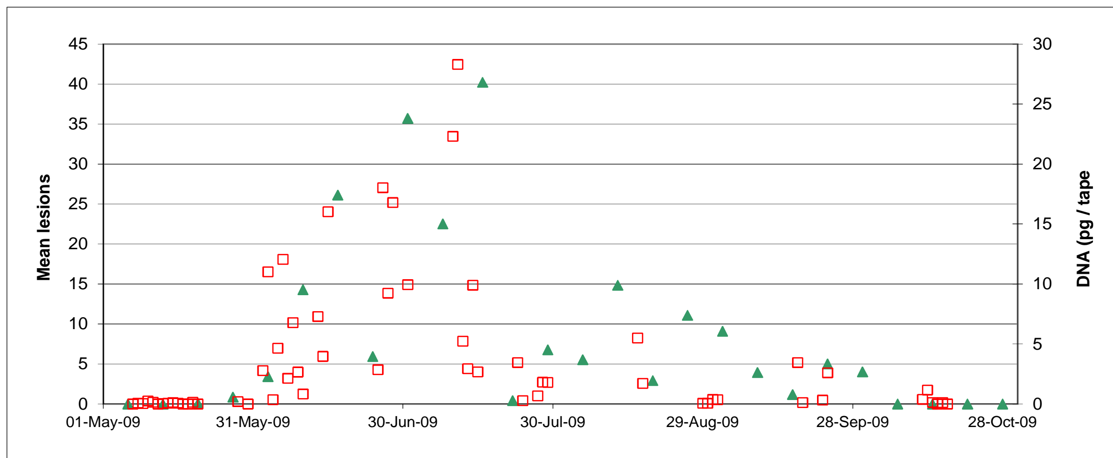

Figure 5-6 Yield of *L. maculans* DNA (□) from a spore trap, and number of leaf lesions on canola cultivar ATR-stubby trap plants (▲) at Kingsford in 2009

Overall comparison of 2008 spore trap data with model predictions was not feasible due to trap malfunction from 4 July to 12 August 2008, which coincided with the period of peak ascospore release as indicated by trap plant data (Figure 5-2A).

Regression analysis indicated a significant linear relationship between model predictions and number of lesions on trap plants ($P < 0.001$) (Figure 5-7A).

Although there was a good match in the overall pattern of ascospore release between 2008 trap plant data and model predictions there was considerable discrepancy between the weekly values, with the model predicting earlier ascospore release than indicated by the trap plant data (Figure 5-8A).

The overall pattern of ascospore release predicted by the model for 2009 was very similar to that observed from DNA assays of spore trap samples. However, in contrast to 2008, the model predicted a slightly later commencement of ascospore release (9 June compared with 2 June for spore trap), and a later peak ascospore release, than suggested by the spore trap data (Figure 5-3A).

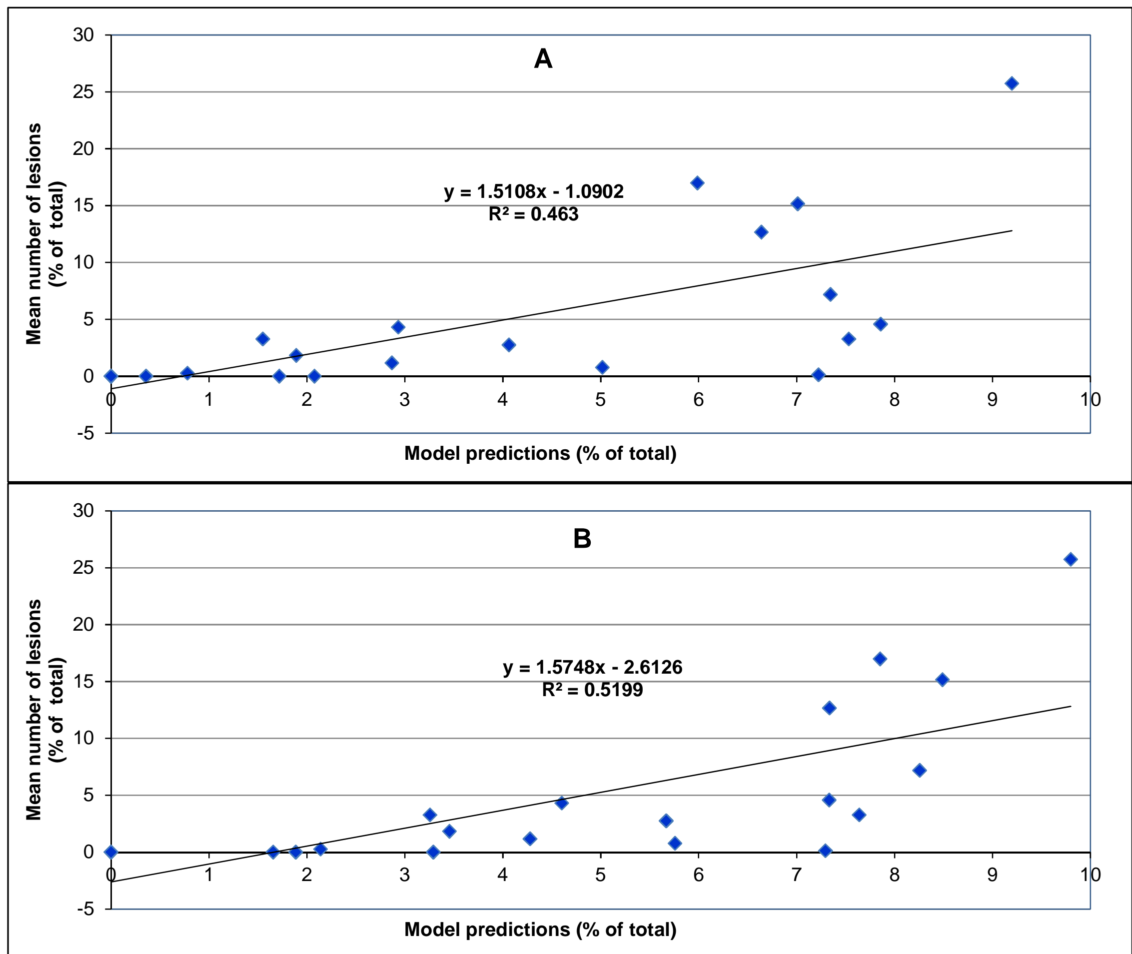

Figure 5-7 Correlation between the number of leaf lesions on canola trap plants after exposure to inoculum at Kingsford in 2008, and predictions from the Blackleg Sporacle model A. before and B. after modification and calibration for the Kingsford site

5.3.1.6 Blackleg Sporacle model modifications

To improve the fit between observed and predicted ascospore release, some modifications were made to the model, which was then calibrated to the Kingsford site. Firstly, in order to smooth the model prediction curve, a 7-day moving average was incorporated; the model prediction for any day was calculated as the average of the model predictions for that day and the following 6 days. Seven days was chosen because this fitted well with the 7-day (prior) rainfall built into model predictions of ascospore maturation. Secondly, spore trap DNA yields were multiplied by a conversion factor of 15.04, to maximise the fit between observed and predicted values.

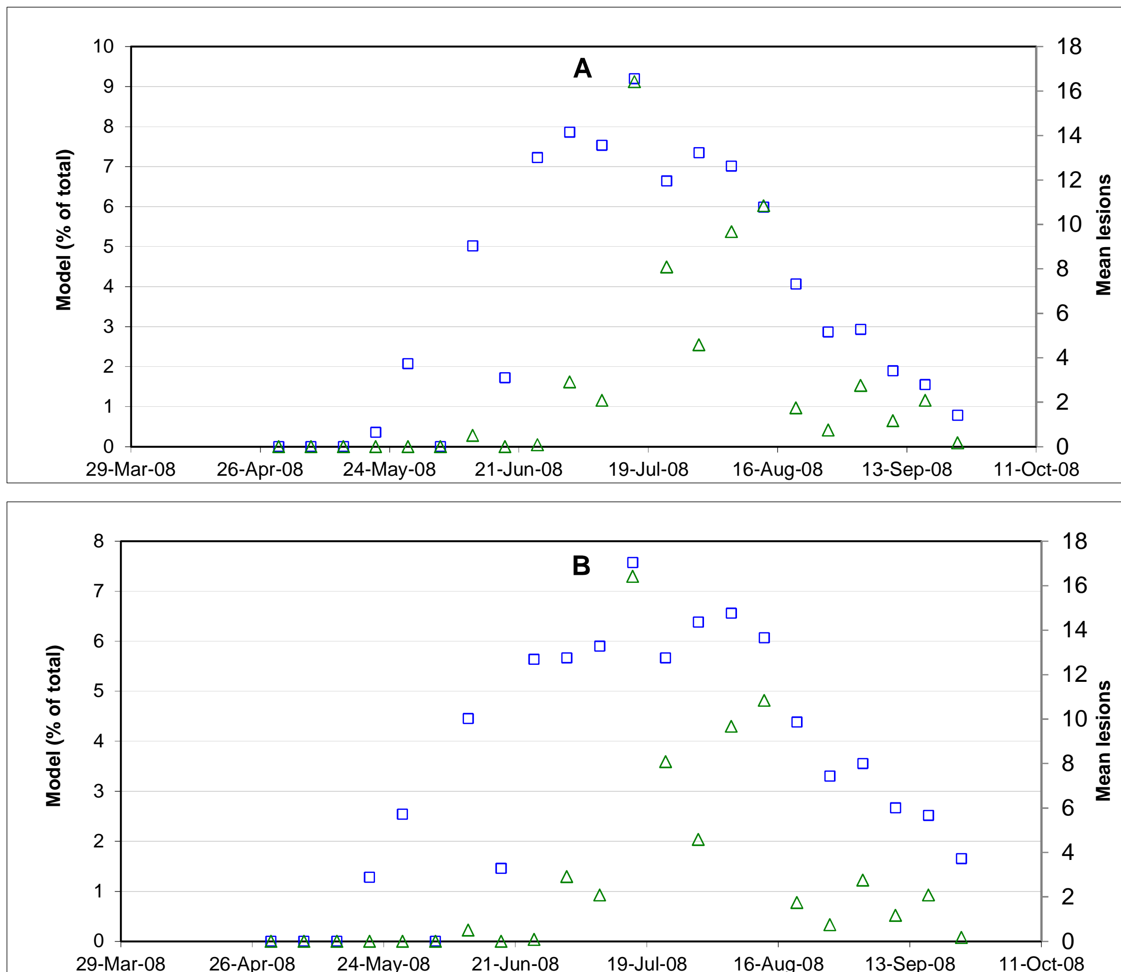

Figure 5-8 Mean number of leaf lesions on canola cultivar ATR-stubby (Δ) at Kingsford 2008 and predictions from the Blackleg Sporacle model (□) A. before and B. after model calibration

5.3.1.7 Blackleg Sporacle model calibration

Model parameter changes:

Model calibration was conducted by generating model outputs using different combinations of values for each of the most sensitive parameters. Two values of AD-fraction (**0.4**, 0.5), and three values each of PM-end (100, **125**, 150), PM-peak (10, 20, **30**), PM-peak sharpness (Gama) (1, **2**, 3), R-threshold (2, 3, **4** mm), and T-threshold (18, **22** and 26 ^{o}C), were used in all possible combinations, in a factorial design (486 combinations; values in bold print are the values used in the original [uncalibrated] model). Values to be tested were selected as follows: for AD-fraction and R-threshold, in addition to the original value, only values higher than the original were tested, since the data suggested earlier onset of ascospore release. For PM-peak the data suggested a shorter period between start and peak of ascospore release, so the original and two

values lower than the original value were chosen for testing. For the other chosen parameters, the original value, and one on either side of that value were tested.

Model outputs were compared with the 2009 spore trap data using RMSD. The individual contributions of the changed parameter values to the improved RMSD are shown in Table 5-4. These contributions were not additive, as there was interaction between parameters. The original set of parameter values gave an RMSD of 0.534. The set of parameter values (PM-peak 30; PM-end 125; PM-peak sharpness 1; T-threshold 26 ^{o}C; R-threshold 2 mm and AD-fraction 0.5) gave the lowest RMSD of 0.423, an overall improvement of 19.76%. (Table 5-4; Figure 5-3A and B).

When the calibrated model was run using 2008 Kingsford weather data, the correlation between the model predictions and the trap plant data was improved slightly, with an increase in the coefficient of determination (R^2) from 0.46 to 0.52 (Figure 5-7A and B; Figure 5-8B).

Table 5-4 Improvements in fit, measured by root mean squared deviation (RMSD), between Blackleg Sporacle model predictions and Kingsford 2009 spore trap data, due to changes made to the model and calibration of the model to Kingsford site

Parameter	Old value	New value	RMSD	% Improvement
Original parameter set			0.534	
AD-fraction	0.4	0.5	0.502	4.70
PM-peak-sharpness	2	1	0.498	5.58
R-threshold	4	2	0.468	11.13
T-threshold	22	26	0.527	1.30
Combination			0.423	19.76

5.4 Results for *D. pinodes*

5.4.1 Spore trap

5.4.1.1 DNA yield

In 2008 the selected spore trap samples gave low DNA yields (0-6 pg/tape) from early March till late April, after which the yields began to increase (Figure 5-9). The highest yields (4 to 40 pg/tape) occurred between 17 and 21 May; thereafter they declined. There was a gap in the spore trap data from 4 July to 12 August due to trap malfunction. From mid-August, DNA yields remained relatively low (less than 9 pg/tape) although

they did not decline to zero. From late November 2008 till late January 2009 DNA yields on the occasional summer rain days were generally higher than in winter, the highest yield (104 pg/tape), on 13 December, being more than double the highest yield recorded in May.

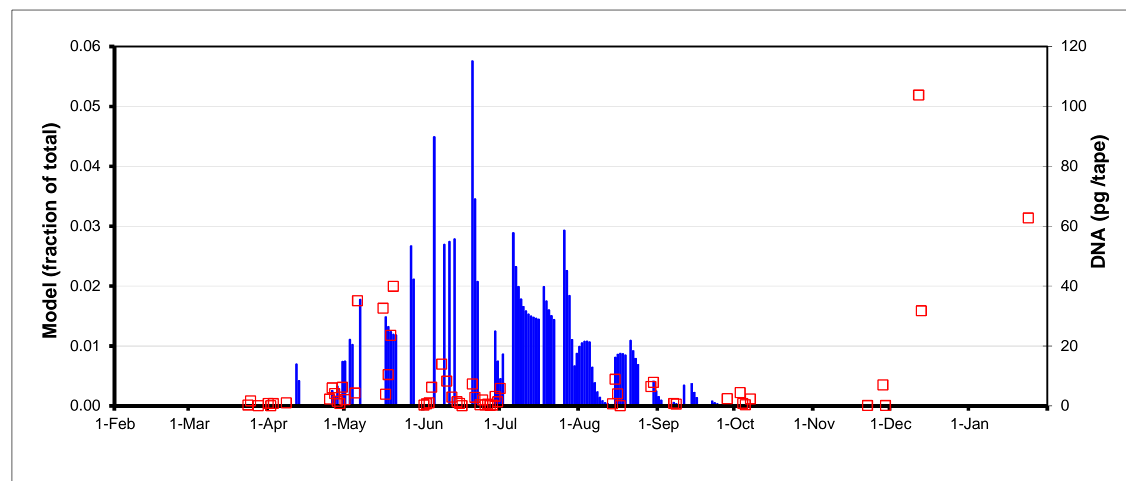

Figure 5-9 Yield of *D. pinodes* DNA (□) from a spore trap, and predicted ascospore release from the unmodified Blackspot Manager model (▌) at Kingsford in 2008 and early 2009

In 2009 a similar pattern occurred, with DNA yields remaining low (less than 2 pg/tape) till mid-April, high yields in mid-May and early June (up to 62 pg/tape), and low yields (less than 2 pg/tape) from late June to early September (Figure 5-10). DNA yields increased from mid-September and were mostly high (up to 82 pg/tape) from October through the summer (sometimes higher than the winter peak), with a few exceptions (0-1 pg/tape) in October and January 2010. There were low to moderate yields of DNA (1-14 pg/tape) in early March 2010.

5.4.1.2 Assessment of fruiting bodies on stubble in early spring 2009

Little field pea stubble remained around the spore trap on 2 September 2009. Only a few fruiting bodies were found on the three selected field pea stems and none contained spores.

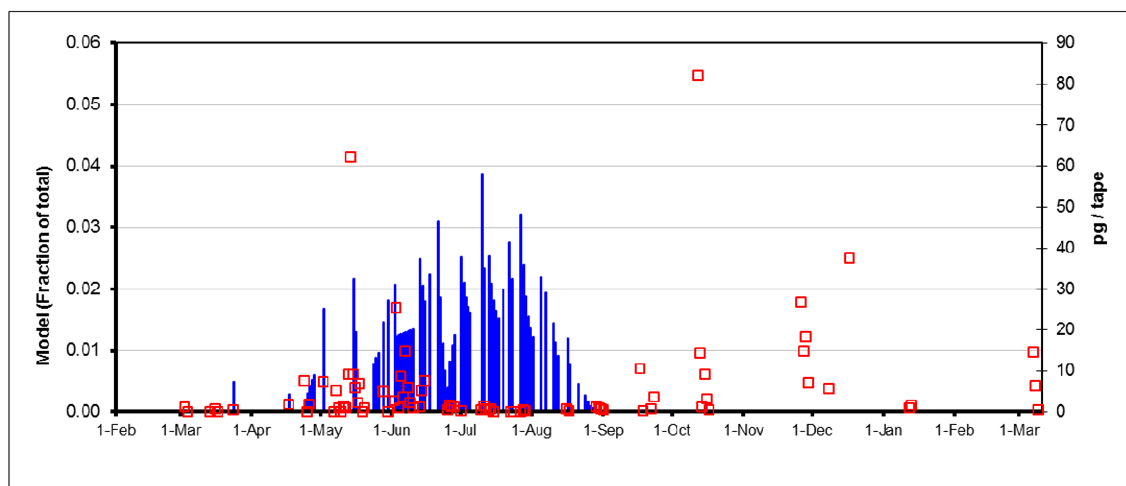

Figure 5-10 Yield of *D. pinodes* DNA (□) from a spore trap, and predicted ascospore release from the unmodified G1 Blackspot Manager model) (▌) at Kingsford in 2009 and early 2010

5.4.1.3 Microscopy

Microscopic counts of *D. pinodes* ascospores were not undertaken for 2008 samples but examination of spore trap tapes on selected summer days (December 2008 to February 2009) of the 2008 stubble incubation season indicated that many ascospores were caught on the days with high DNA values (12 and 13 December 2008 and 24 January 2009).

Table 5-5 shows the estimated number of *D. pinodes* ascospores on daily spore trap tapes and the corresponding yield of *D. pinodes* DNA on duplicate tape segments from the 2009 season. Only two samples were deemed to be false positives; in both cases the yield of DNA was low. One of these (12 January 2010) was most likely due to the presence of considerable amount of dust on the tape, which may have contained traces of *D. pinodes* DNA from fragments of infected plants. This data point was excluded from further analysis. There was no obvious explanation for the DNA on the other sample deemed to be a false positive and so it was included in calculations. The only other sample with a significant amount of dust was that of 8 December 2009. The yield of DNA from that sample on a per ascospore basis, however, was in keeping with that of other samples (Table 5-5). Yield of DNA per ascospore was not significantly different between samples collected in winter and those collected in late autumn and summer (analysis not shown). There was a strong linear relationship between square root DNA and square root ascospores ($P < 0.001$; $R^2 = 0.775$; Figure 5-11). The y-intercept was not significantly different from zero ($P < 0.05$), therefore the relationship

indicates the value of false positives was not significantly different from zero. Solving the regression equation y = 0.1109x + 0.9501, where y is the square root of the calculated value of DNA (pg) and x is the square root of the number of ascospores, for number of ascospores = 1 gave 1.12 pg DNA, which was detectable at threshold cycle 35.2. Thus the limit of detection was one ascospore.

Ascospores viewed on tape segments showed no sign of germination.

Table 5-5 Number of *D. pinodes* ascospores counted on spore trap tapes viewed at 200 x magnification, and yield of DNA from duplicate spore trap tapes, from a range of samples from Kingsford in 2009 and early 2010

Date	No. ascospores	DNA (pg)	DNA (fg/ ascospore)
4-Jun-09	0	1.11	-
5-Jun-09	200	10.28	51.4
6-Jun-09	232	4.15	17.9
7-Jun-09	400	17.76	44.4
8-Jun-09	278	7.07	25.4
9-Jun-09	108	2.74	25.4
10-Jun-09	48	1.03	21.5
14-Jun-09	208	6.1	29.3
15-Jun-09	304	8.83	29.0
24-Jul-09	0	0	-
27-Jul-09	0	0	-
28-Jul-09	12	0.33	27.5
26-Nov-09	2,434	31.95	13.1
27-Nov-09	54	17.71	328.0
28-Nov-09	738	21.86	29.6
8-Dec-09	202	6.81	33.7
17-Dec-09	4,619	44.95	9.7
12-Jan-10	0	0.91	-
13-Jan-10	71	1.93	27.2

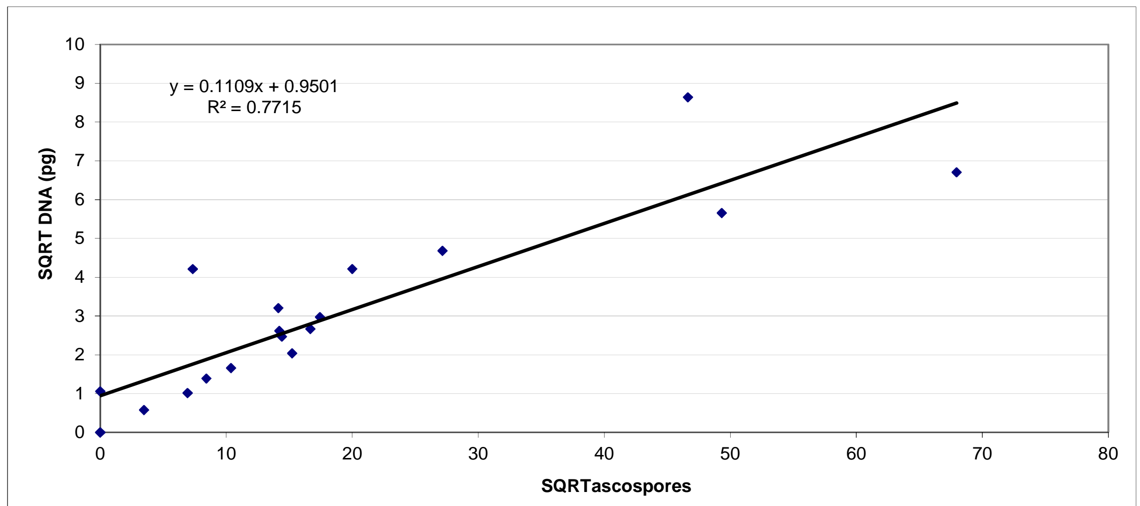

Figure 5-11 Correlation between the number of *D. pinodes* ascospores counted on spore trap tapes viewed at 200 x magnification, and yield of *D. pinodes* DNA from duplicate spore trap tapes from a range of samples from a spore trap operated at Kingsford in 2009 and early 2010 (square-root-transformed data)

5.4.2 Trap plants

In 2008, at the first time of placement in the field (week ending 30 April 2008), trap plants developed, on average, over 20 lesions each, which approached the highest number of lesions recorded on trap plants for the season. Trap plant and DNA data were in broad agreement (Figure 5-12). During the week ending 1 October only five out of the 12 trap plants survived, due to hot, dry conditions. In subsequent weeks trap plants survived but scorching was evident on some leaves.

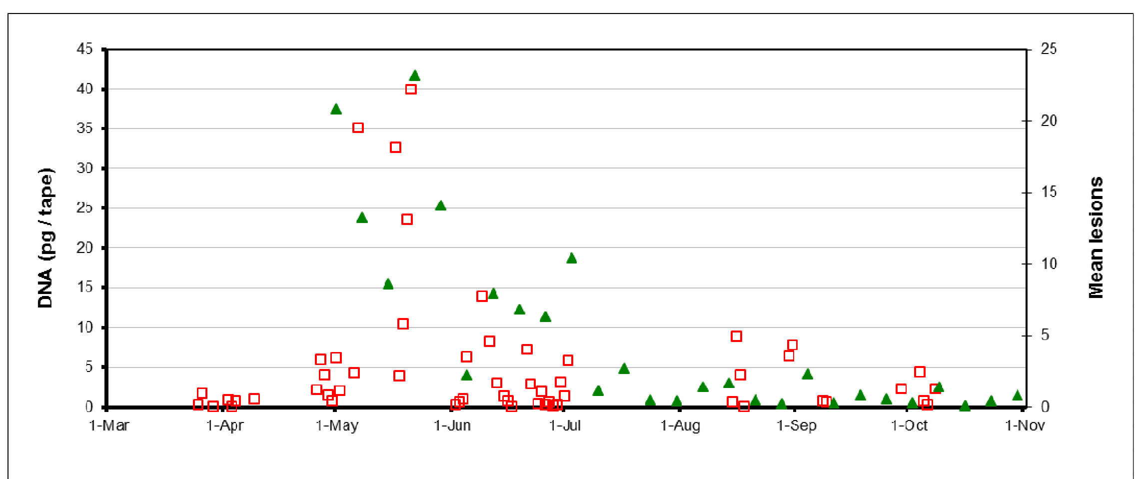

Figure 5-12 Yield of *D. pinodes* DNA (□) from a spore trap, and number of leaf lesions on trap plants of field pea cultivar Parafield (▲), at Kingsford in 2008

In 2009 trap plants developed moderate numbers of lesions during the first 2 weeks of placement (means of 113 and 285 lesions per plant in weeks ending 6 and 13 May 2009 respectively), during which rainfall was low (total 0.6 mm and 0 mm per week respectively). The highest mean number of lesions on trap plants (1,129) occurred during the week ending 20 May during which a total 3.8 mm rain was recorded. The mean number of lesions declined rapidly until early July and remained few (less than 25 lesions per plant per week), with slightly more on two occasions in late September (48 and 31 lesions per plant on weeks ending 23 and 30 September respectively). Trap plant and DNA data were in broad agreement until mid-September when DNA increased a great deal (up to 82 pg/tape as mentioned previously) but trap plant lesions only a little (Figure 5-13). Dry conditions in the field affected plant growth during the week ending 16 September and from mid-October onwards, with evidence of scorching on some leaves.

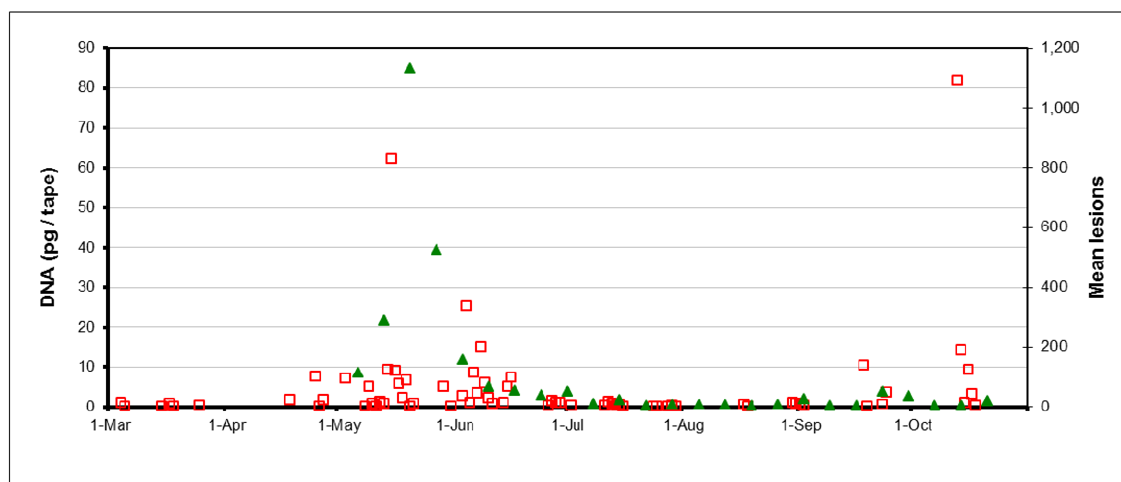

Figure 5-13 Yield of *D. pinodes* DNA (□) from a spore trap, and number of leaf lesions on field pea cultivar Parafield trap plants (▲), at Kingsford in 2009

5.4.3 Comparison with model predictions

In 2008 Blackspot Manager accurately predicted the start of ascospore release in late April, but continued to predict high ascospore numbers after the spore trap DNA data suggested numbers had begun to decline, in early June (Figure 5-9). The model predicted high ascospore release continuing into July but there was no DNA data from the spore trap at that time to compare with the model, due to trap malfunction from 4 July to 12 August. Trap plant data, however, indicated an earlier decline in ascospore

numbers than suggested by the model (Figure 5-12). The model predicted a decline in ascospore numbers to late September and this prediction was upheld by both spore trap and trap plant data.

In 2009, once again the model predictions and spore trap DNA data were in broad agreement at the start of the season, but the model continued to predict ascospore release after the spore trap DNA data indicated a decline. From late June to end August, the model predicted ascospore release but little to no DNA was detected in spore trap samples (Figure 5-10).

<u>Out-of-season ascospore release</u>. In both 2008 and 2009 the spore trap DNA data indicated that ascospores were released in late spring and summer. The model failed to simulate this ascospore release (Figure 5-9 and Figure 5-10).

5.4.4 G1 Blackspot Manager model modifications

5.4.4.1 Adding an in-crop cycle

Spore trap data indicated that ascospores of *D. pinodes* were released in two cycles; autumn/winter (March to August) and spring/summer (September to February) but G1 Blackspot Manager, when run with start date as 1 January, only predicted the release of ascospores in autumn through to early spring. To correct this, an additional "in-crop" cycle was incorporated into model predictions. The in-crop cycle was set to commence on 8 August and the out-of-crop cycle remained with start date as 1 January. August 8 was chosen as the start of the in-crop cycle to reflect earliest observations of pseudothecia on leaves and petioles in the crop at the Kingsford site in 2009 (Davidson, J.A., SARDI, personal communication, May 2010). Figure 5-14A shows the resultant model predictions for the period 1 August 2007 to 9 May 2010, together with spore trap DNA data for 2008 and 2009.

5.4.4.2 Incorporating a relative humidity component into the model

<u>Effect of relative humidity on ascospore release</u>

There were a number of anomalies in 2008 between the spore trap DNA data and the model predictions. In particular, on 5, 6 and 16 May, and on 4, 8 and 10 June, DNA was detected in spore trap samples on days when the model predicted no ascospore release. Examination of these data points showed that, for each of those days, although recorded rainfall was zero, maximum relative humidity was high (Table 5-6). The mean

of the maximum relative humidity on days without rain when little or no DNA was detected was 74.5% (SE 6.4).

Table 5-6 Yield of *D. pinodes* DNA and maximum relative humidity on sampling days on which the unmodified Blackspot Manager model predicted no release of ascospores but *D. pinodes* DNA was detected in spore trap samples at Kingsford in 2008

Date	DNA (pg/tape)	Max. RH (%)
5-May-08	4.25	82.5
6-May-08	35.06	95.3
16-May-08	32.59	85.4
04-Jun-08	6.22	93.0
08-Jun-08	13.90	88.8
10-Jun-08	8.24	94.9

It appeared that ascospores were released at high relative humidity, but this had not been factored into the model. The model was therefore modified, by introducing a threshold for maximum relative humidity as a trigger for ascospore release (provided mature pseudothecia with ascospores were available). Spore trap data were compared with predictions from the modified model using RMSD. When the relative humidity threshold was set at 90% the RMSD was reduced for both 2008 and 2009, resulting in improvements of 29.3% and 10.1% respectively, compared with model predictions without a relative humidity threshold (Table 5-7).

Effect of relative humidity on pseudothecial maturation

Following the incorporation of the effect of relative humidity on ascospore release into the model, the DNA data for both 2008 and 2009 were plotted against model predictions (Figure 5-15). The graph shows that some aberrant data points remained. For the most extreme of these, DNA yields were high (74.6, 98.3 and 103.7 pg) but model predictions zero or near-zero. These data points occurred on 14 May 2009, 12 October 2009 and 12 December 2008, respectively. In each case, examination of the model showed that ascospore release was not predicted because, according to the model, mature ascospores had been depleted and new ones not yet sufficiently matured to be

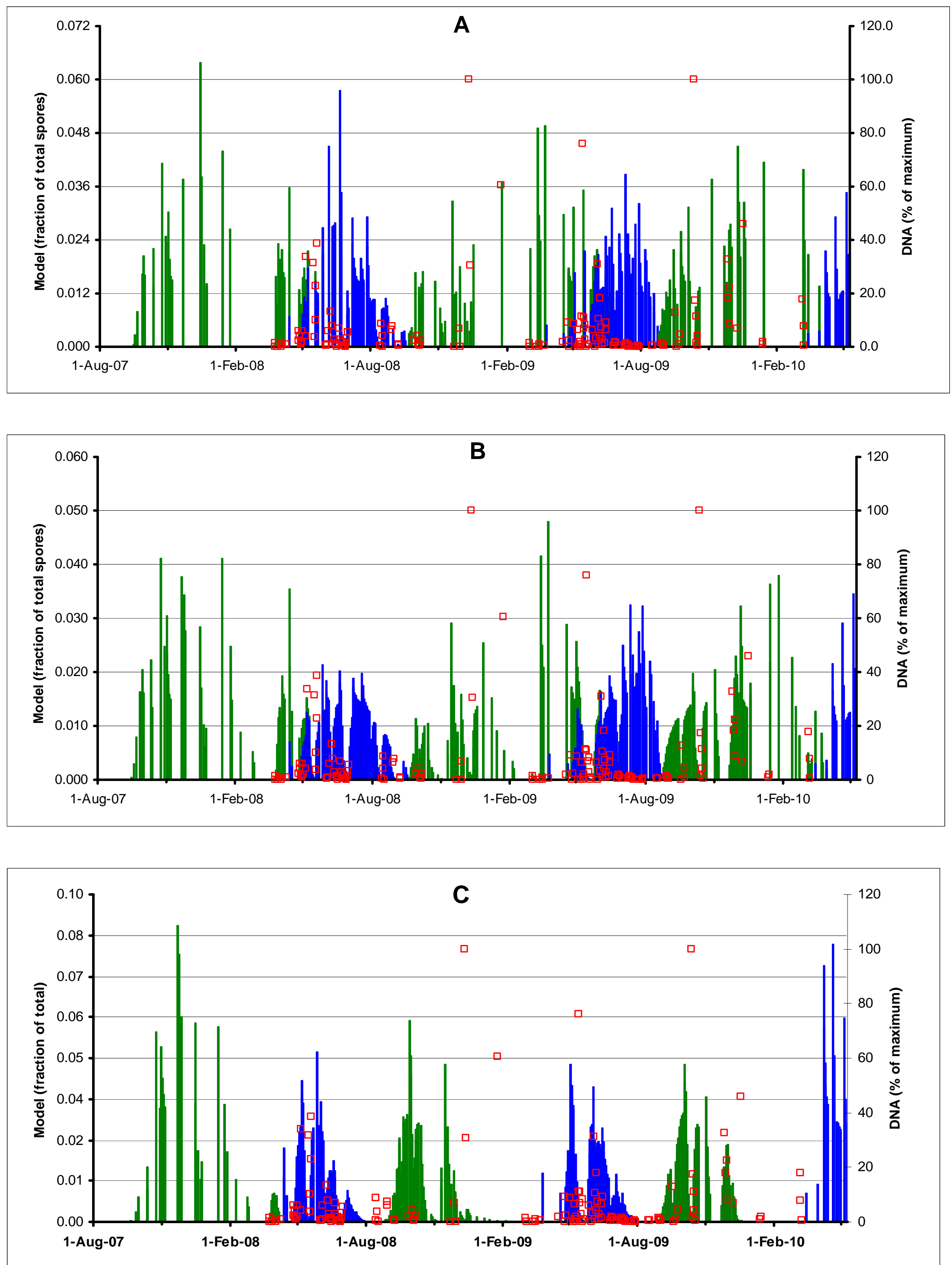

Figure 5-14 Yield of *D. pinodes* DNA (□) from a spore trap at Kingsford in 2008 and 2009, and ascospore release predicted by the Blackspot Manager model for both the in-crop (▌) and out-of-crop (▌) cycles from August 2007 to May 2010. A. Unmodified model. B. Model with relative humidity factor incorporated. C. Model with relative humidity factor incorporated, calibrated to the Kingsford site

released. Examination of weather data showed that in each case there had been high relative humidity (> 95%) during the week preceding ascospore release. This suggested that relative humidity may have had an impact on pseudothecial maturation which had not been incorporated into the model. Since pseudothecial maturation had not been measured directly in this study, it was deemed that there was insufficient evidence to warrant incorporating an effect of relative humidity on pseudothecial maturation into the model. Instead, a 7-day moving average for the model prediction was incorporated; the model prediction for any day was calculated as the average of the model predictions for that day and the following 6 days. This had the effect of smoothing the model prediction curve. Because it effectively brought forward the effect of rain that fell after the date of ascospore release, it brought forward predicted pseudothecial maturation on those occasions described above. The choice of 7 days in the moving average fitted well with the 7-day (prior) rainfall built into model predictions of ascospore maturation.

Table 5-7 Improvements in fit, measured by root mean squared deviation (RMSD), between Blackspot Manager model predictions and Kingsford 2008 and 2009 spore trap data, following modifications to the model, changes in the values of model parameters, and calibration of the model to the Kingsford site

Change	**Parameter value (old)**	**Parameter value (new) OUT-OF-CROP**	**Parameter value (new) IN-CROP**	**RMSD 2008 (%)**	**RMSD 2009 (%)**	**% Improve-ment in RMSD 2008**	**% Improve-ment in RMSD 2009**
Original (unmodified) model				0.075	0.149		
Original model modified with RH component				0.053	0.134	29.3%	10.1%
PM-end	90	90	65	0.124	0.104	-65.3%	30.2%
PM-peak	45	15	35	0.071	0.135	5.3%	10.1%
PM-peak sharpness	1.25	6	6	0.105	0.160	-40.0%	-7.4%
T-threshold	9	6	6	0.071	0.088	5.3%	40.9%
Combination of parameter changes				0.060	0.066	20.0%	55.7%

Figure 5-14B shows the predictions of the model after modification to account for the effects of relative humidity, plotted against spore trap data. The model predictions fitted the data better than before the modifications but anomalies remained, demonstrating the need to calibrate the model for the Kingsford site.

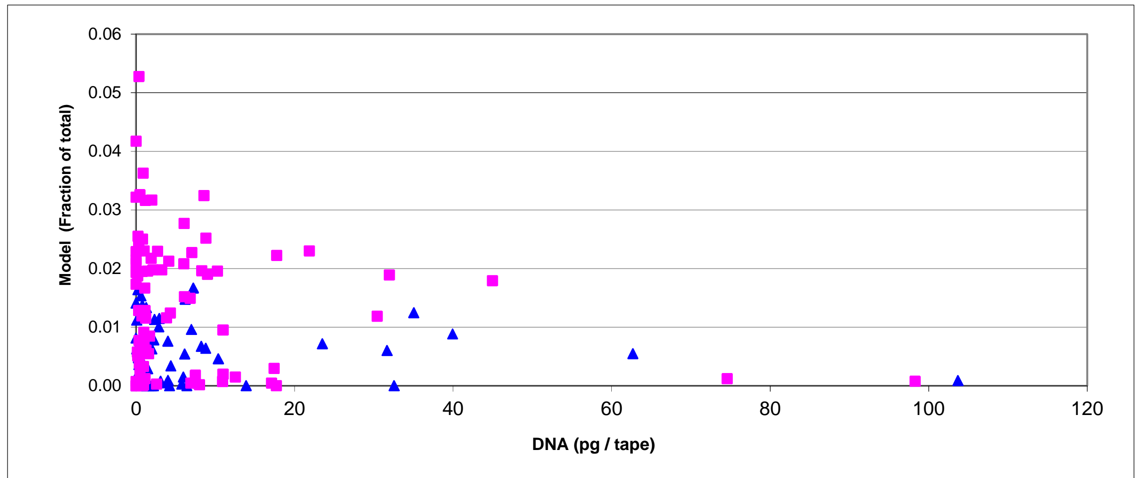

Figure 5-15 Comparison of yield of *D. pinodes* DNA from a spore trap, with predictions from the Blackspot Manager model modified by including a relative humidity component, for 2008 (▲) and 2009 (■)

5.4.5 G1 Blackspot Manager model calibration

Model parameter changes:

PM-begin

This parameter determines the number of days favourable for pseudothecial maturation before onset of pseudothecial maturity. It was not modified because there is already good evidence from the literature, and from previous studies at the Kingsford site, that onset of maturity occurs in approximately 10 favourable days (Davidson et al., 2009b; Salam et al., 2011a).

PM-end

This parameter determines the number of days favourable for pseudothecial maturation from PM-begin until pseudothecial maturation ends, and is a measure of availability of suitable substrate for pseudothecial maturation, largely reflecting breakdown of plant tissue. 2009 data suggested that this parameter needed to be lower than the original value (90) for the out-of-crop cycle (Figure 5-14B). In-crop pseudothecia and

ascospores are produced on senescent tissue in the crop, consisting mostly of soft tissue (leaves, petioles and tendrils). This tissue can be expected to break down more quickly than stubble, which consists largely of stems. PM-end was therefore hypothesised to be a smaller number of days for the in-crop cycle than for the out-of-crop cycle. In the calibration process, three values of PM-end (80, 90 and 100) were tested for the out-of-crop cycle, and three values (50, 70 and 90) for the in-crop cycle.

PM-peak

This parameter determines the number of days favourable for pseudothecial maturation from PM-begin until the rate of pseudothecial maturation reaches a peak. Comparing the spore trap data with model predictions (before calibration to the Kingsford site; Figure 5-14B), the actual peak in spore trap DNA for the out-of-crop cycle was clearly earlier than that predicted by the model, for both 2008 and 2009. This suggested that the value for PM-peak should be reduced from the original value of 45. PM-peak was hypothesised to be greater for ascospores produced in-crop than out-of-crop because the stubble on which pseudothecia are produced for the out-of-crop cycle is already abundant at the start of autumn, whereas the senescent plant tissue on which pseudothecia develop in the crop builds up over time during the in-crop cycle. On that basis the values of PM-peak chosen for testing for the in-crop cycle were higher than those chosen for the out-of-crop cycle. The values of PM-peak tested for the out-of-crop cycle were 15, 30, and 45, and for the in-crop cycle 25, 35 and 45.

PM-peak sharpness

This parameter determines peak sharpness of the pseudothecial maturation curve. The data suggested a much sharper peak than the model predicted (Figure 5-14B). An increase on the original value of 1.25 was therefore warranted. In the calibration process, three values of PM-peak sharpness (3, 6 and 9) were tested for both the out-of-crop cycle and the in-crop cycle.

R-threshold

The value of *R-threshold* has previously been determined based on the best fit between observed and predicted values for this parameter in combination with *PM-begin* (Salam et al., 2011a) and was not modified here.

T-threshold

Spore release during winter of both 2008 and 2009 was less than the model predicted (Figure 5-16A and Figure 5-17A). The temperature threshold was reduced from 9°C to 6°C to improve the match with winter conditions at the field site. This had an impact both on daily and seasonal total predicted number of ascospores released. The net result was to reduce the predicted proportion of ascospores released over winter in 2008 (Figure 5-16B). In 2009 there was very little change to model predictions (Figure 5-17B). Overall, changing the parameter T-threshold from 9°C to 6°C closed the gap between the curves for model predictions and spore trap data.

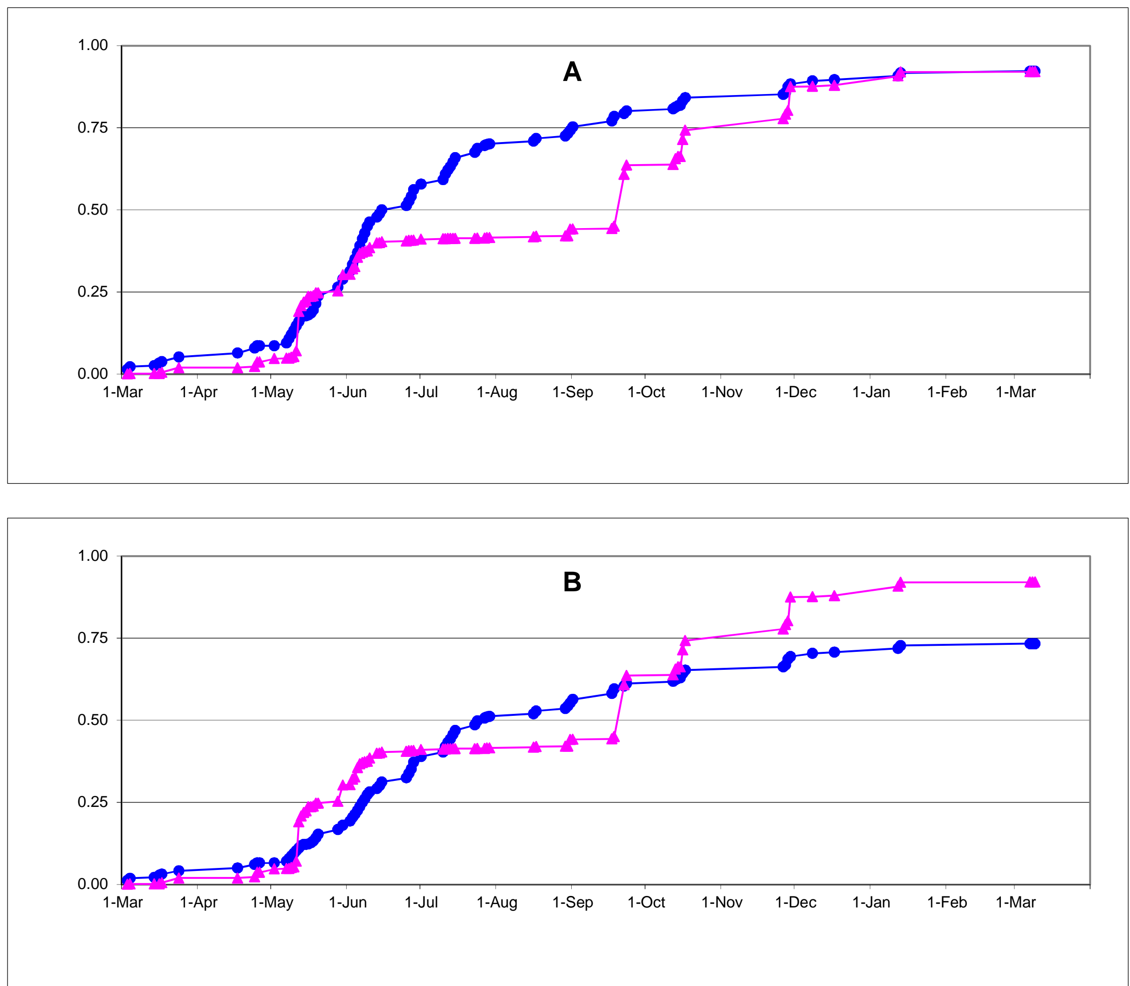

Figure 5-16 Blackspot Manager model predictions, expressed as cumulative fraction of spores released (●), and yield of *D. pinodes* DNA, expressed as cumulative fraction of DNA adjusted by a conversion factor [Section 5.2.8] (▲), from a spore trap at Kingsford in 2008, before and after changing the T-threshold parameter from 9 to 6. A. T-threshold = 9; B. T-threshold = 6

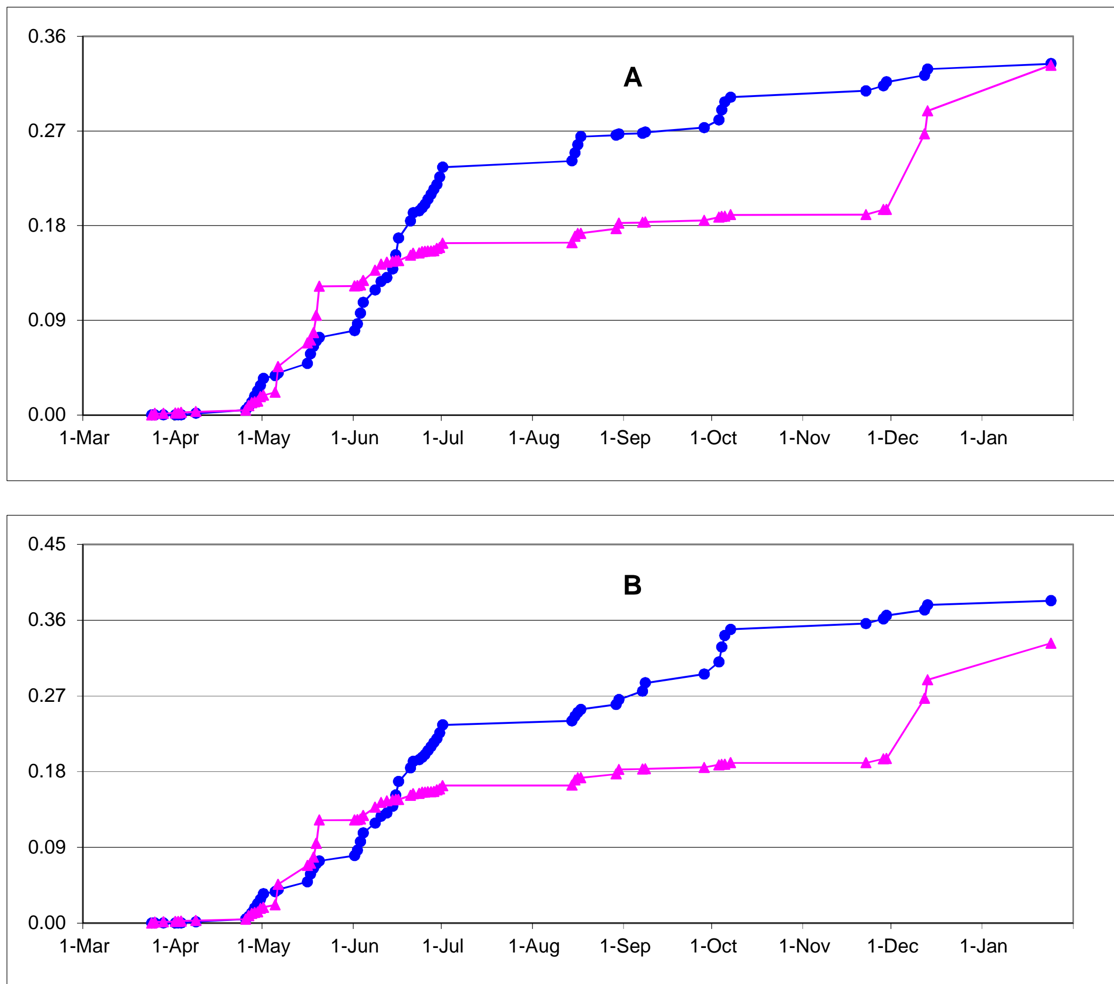

Figure 5-17. Blackspot Manager model predictions, expressed as cumulative fraction of spores released (●), and yield of *D. pinodes* DNA, expressed as cumulative fraction of DNA adjusted by a conversion factor [Section 5.2.8]) (▲), from a spore trap at Kingsford in 2009, before and after changing the T-threshold parameter from 9 to 6. A. T-threshold = 9; B. T-threshold = 6

Conversion factor

DNA yields were multiplied by a conversion factor of 6.3 to maximise the fit between the observed and predicted values.

5.4.6 Comparison with model predictions

The model was calibrated to the Kingsford site by incorporating into it the combination of parameter values which gave the lowest RMSD. The calibrated model predictions are shown in Figure 5-14C. The improvement in RMSD contributed by each change is shown in Table 5-7.

5.5 Results for *D. rabiei*

No DNA of *D. rabiei* was detected in any spore trap samples and no lesions attributable to *D. rabiei* developed on chickpea trap plants in either the 2008 or the 2009 season.

5.5.1.1 Assessment of fruiting bodies on stubble in early spring 2009

No identifiable chickpea stubble could be found around the spore trap in spring 2009.

5.6 Discussion

Spore trapping combined with PCR-based assays enabled testing of the epidemiological models Blackleg Sporacle and G1 Blackspot Manager to predict timing of ascospore release for two of the model pathogens, *L. maculans* and *D. pinodes*, respectively. The results of the PCR assays were validated by microscopic counting of spores, and by results of ascospore monitoring using trap plants.

5.6.1 L. maculans

Spore trap DNA assays showed that ascospore release began in mid-May to early-June. The 2009 data indicated a peak in mid-July (spore trap data were missing at the time of the peak in 2008), but some ascospores were detected until October in both seasons. There was little indication of ascospore release on days with rain in summer, although a small amount of DNA was detected in late-November and mid-December 2008.

Small amounts of DNA were also detected in the spore trap samples in April 2009. It was considered unlikely that ascospores would have been produced on stubble from the 2008 crop so early in the season, and so this was attributed to development of ascospores on 2007 stubble. The assumption that active pseudothecia would be still present on that stubble after the summer required further investigation.

Counting of ascospores on tapes showed a highly significant linear relationship between the number of ascospores and the DNA yields in both seasons. There were few false positive DNA results in the system. However, the relationship between ascospore numbers and yields of DNA differed more than three-fold between the two years, possibly due to alterations in DNA extraction methods. Variability between PCR runs may also have contributed to the differences (Section 3.4.4). Standardising the data across the two years using yeast DNA yields accounted for most, but not all, of this difference, possibly due to small differences in concentration of the yeast suspensions. It was possible to detect a single ascospore of *L. maculans* in 2008, but issues in the analysis, possibly relating to inaccuracies in spore counts at low ascospore numbers (discussed further below), and in DNA measurements near the measurement detection limits (Hospodsky et al., 2010), confounded that finding in 2009. Further studies are

required to define the true relationship between ascospore numbers and quantity of DNA.

The correlation between qPCR assays and microscopic ascospore counts was closer in the study reported here ($P < 0.001$; $R^2 = 0.93$ in 2008; $P < 0.001$; $R^2 = 0.88$ in 2009) than that obtained by Kaczmarek et al. (2009) ($P = 0.05$; $R^2 = 0.72$ and $R^2 = 0.69$) in comparing qPCR assay results and microscopic ascospore counts of *Leptosphaeria* spp. from 2 years of spore trapping of canola fields in Poland. Kaczmarek et al. (2009) combined the qPCR assay results and microscope counts for *L. maculans* with those of the closely related species *L. biglobosa,* both of which occurred in their study area, and the ascospores of which could not be distinguished from one another visually. *L. biglobosa* was not included in the study reported here because *L. maculans* has been shown to predominate in canola-growing areas in the state of South Australia (Sosnowski et al., 2001), as it does in other parts of southern Australia (Mengistu et al., 1991; Ballinger & Salisbury, 1996). Another factor that may have contributed to the closer fit of the results of Experiment 13 compared with those of Kaczmarek et al. (2009) is that they were standardised using yeast DNA yields to allow correction for sample-to-sample variation.

Comparisons between trap plant and spore trap data across the full data set were not possible for 2008 due to missing spore trap data, but there was good agreement between the two sets of data in 2009.

Blackleg Sporacle model predictions were compared with trap plant data in 2008. The overall patterns were very similar but there was considerable discrepancy between the weekly values, and the model predicted a much earlier start to ascospore release than suggested by the trap plant data. The sensitivity of the trap plant system may have been lower in 2008 than in 2009, firstly because there were fewer ascospores (based on microscope counts of ascospores on spore trap samples), and secondly because the number of lesions on cotyledons was not included in the count in 2008, whereas it was in 2009. The spore trap data (DNA and microscope counts) indicated that some ascospores were released in mid-May, and that considerable numbers were released in the week ending 11 June 2008, higher than suggested by the trap plant data. It is possible that the trap plant system did not allow accurate detection of the start of ascospore release in 2008 because its sensitivity was too low that season.

In contrast to 2008, the Blackleg Sporacle model predicted a slightly later commencement, and a later peak of ascospore release in 2009 than suggested by both the spore trap data and the trap plants. Calibration of the model for the Kingsford site resulted in a slightly closer correlation between the model prediction and the trap plant data in 2008, and a better fit to the 2009 spore trap data, with RMSD reduced by 19.6%. Due to the considerable differences in trap plant data between 2008 and 2009, and to the lack of spore trap data at a critical period in 2008, these calibrations need to be validated in further seasons.

5.6.2 D. pinodes

Ascospore production occurred in two periods over a yearly cycle. DNA yield from spore trap samples increased from mid- to late-autumn until early winter, then increased again in mid- to late-spring and on occasional rainy days in summer. There was good agreement between microscopic ascospore counts and yields of DNA, thus corroborating the DNA results. There were very few false positives in the system. Comparatively small amounts of DNA from samples with very high spore numbers were most likely due to inaccuracies in spore counts. Microscope counts were conducted only for 2009; further studies are required to confirm this relationship. The ascospores detected in spring and summer 2009 are unlikely to have originated on field pea stubble around the spore trap, which had mostly decomposed, and on the remainder of which, few if any, viable pseudothecia were left by early spring. They may have arisen from pseudothecia produced on senescent tissue in field pea crops grown in the neighbourhood in 2009.

Trap plants were first placed in the field at the end of April, as hot and dry conditions before then were likely to cause desiccation of the plants. Spore trap data indicated that some ascospores were released earlier, coinciding with small amounts of rainfall in March and April. In both seasons, data for trap plants and spore trap were in broad agreement until mid-spring. At that time yields of *D. pinodes* DNA from spore trap samples increased, but the number of lesions on trap plants did not, possibly due to wilting and death of trap plants caused by heat and drought. These results demonstrate the limitations of the trap plant system. McDonald & Peck (2009) were able to maintain a field pea trap plant system at Kingford for a slightly longer period - from early April till November each year from 1998 to 2001 - without desiccation of the plants. In their system water loss was minimised by the use of younger field pea

seedlings than in the present study (2 rather than 3 weeks old), and by the use of a sealed rather than an open container with water in which pots of seedlings were immersed. Regardless of the system, trap plants do not enable accurate identification of the start of the autumn/winter cycle of ascospore release, and are unsuitable for monitoring aerial inoculum through the summer, to determine the dynamics of the spring/summer cycle. Furthermore, use of trap plants introduces additional sources of variation into the system for measuring inoculum, compared with volumetric spore traps, as infection depends on the deposition of spores on susceptible plant tissue, successful spore germination, and penetration and invasion of that tissue. Also, deposition of spores may be affected by wind turbulence in the vicinity of the plant, and spores may be washed from the plants by rain after deposition, both of which can add further variability to the system.

Prediction of the start of ascospore release from stubble by the G1 Blackspot Manager model (Salam et al., 2011a), was confirmed by the spore trap results in both 2008 and 2009. However, the data for both the spore trap and the trap plants indicated an earlier decline in ascospore numbers than was predicted by the model. The model was calibrated, and minor modifications were made to it to account for these differences; (1) a cycle of spring and summer release of ascospores was incorporated and ascospore release predictions from this cycle added to the autumn cycle predictions; (2) relative humidity was factored into the predictions of ascospore release; and (3) a 7-day moving average was incorporated in order to smooth model predictions. Collectively the modifications and calibration of the model reduced the overall deviation between model predictions and spore trap data, as measured by RMSD, by 20% in 2008 and 56% in 2009. These changes need to be validated in further seasons and at other sites.

Modifications to the model were based on an understanding of the epidemiology of *D. pinodes* as outlined below.

(1) Spring and summer release of ascospores: The detection of ascospores in spore traps and on trap plants in late autumn and early winter was due to the release of ascospores from infested stubble (Carter & Moller, 1961; Bretag, 1991). The increase in DNA in spring was most likely related to in-crop development of ascospores on senescent plant material (Hare & Walker, 1944; Carter & Moller, 1961). The release of ascospores in summer is in agreement with the detection of ascospores from stubble

incubated on the soil surface at the Kingsford site and retrieved in mid-January to early February 2008 (Salam et al., 2011a). McDonald & Peck (2009), having analysed the results of monitoring aerial inoculum of field pea ascochyta blight over a period of 3 years at Kingsford and 1 year at nearby Roseworthy, suggested that ascospore release during rainfall events in summer and autumn leads to a reduced amount of aerial inoculum remaining at emergence of the subsequent field pea crop. Those authors postulated that aerial inoculum from infested field pea residues was depleted once 190-200 mm of rain had fallen after harvest, the date of which was nominally set as 1 December. The relationship built into the modified G1 Blackspot Manager model in the present study is relatively complex, taking into account, in addition to maturation and release of aerial inoculum on stubble over the summer and autumn, the production of aerial inoculum produced on senescent tissue in the crop and the more rapid decomposition of that inoculum compared with that produced on stubble. The 1 December may be a more logical start date for the out-of-crop cycle for Kingsford than 1 January as was used here, since field pea crops are usually harvested by early December. However, when the unmodified G1 Blackspot Manager model was run with a commencement date of 1 December for the out-of-crop cycle (data not shown) this resulted in little difference in the model predictions compared with a start date of 1 January, and no improvement in the match between model predictions and spore trap data, most likely because rainfall in December in South Australia is typically extremely low. However, the potential effect of high rainfall in December needs to be investigated.

(2) Relative humidity: A relative humidity factor was incorporated into the modified G1 Blackspot Manager model because *D. pinodes* DNA was sometimes detected in spore trap samples on days without rain. Model predictions were found to match airborne ascospore concentrations when a maximum relative humidity threshold of 90% was incorporated into model predictions of ascospore release. The exact moisture conditions leading to the release of *D. pinodes* ascospores appear to be complex. The largest concentration of airborne ascospores occurs following rainfall (Carter, 1963; Bretag, 1991; Zhang et al., 2005). However, ascospore release does not necessarily occur during rainfall. Zhang et al. (2005) found that the highest concentration occurred one to two days after rainfall between the hours of 1700 and 0400. Carter (1963) found that ascospores of *D. pinodes* could be released on days without rain but with dew, but

the release of ascospores preceded the dew by approximately 1.5 hours. This was related to a diurnal rhythm of ascospore release, with ascospores released in the late afternoon. Possibly ascospores are released providing the inoculum source (stubble or senescent field pea tissue) is moist and relative humidity is high. Schoeny et al. (2007) found that 70% relative humidity, measured at 1.4 – 2.2 m above ground level, provided the best predictions of onset of ascochyta blight of field peas in a disease onset model developed in France. The authors considered that this was probably associated with optimal moisture periods within the crop canopy due to dew formation. Disease onset requires a combination of prior ascospore release, spore germination and infection of the pea plant, and the moisture conditions conducive to spore germination and infection, as measured by Schoeny et al. (2007), may be different from those conducive to ascospore release, which was measured in the current study. This may explain the difference in optimum relative humidity in the model of Schoeny et al. (2007) compared with the studies reported here.

(3) Seven-day moving average: *D. pinodes* DNA was sometimes detected in spore trap samples on days when the model predicted that mature ascospores had been depleted and not yet replenished. These occasions were associated with high humidity in the preceding week, and rainfall either on the day of ascospore release or shortly thereafter, suggesting that pseudothecial maturity proceeds in conditions of high humidity. Because pseudothecial maturity was not measured directly in this study, no direct relationship was incorporated into the model, but the model prediction curve was smoothed by incorporating a 7-day moving average. This effectively brought forward model predictions of ascospore release on those occasions when there may have been high humidity just prior, but no rainfall until after, the day of simulation. It also compensated for any variability in the timing and duration of rainfall in the district, which may have led to ascospore detection at times other than when rainfall was detected at the weather station.

Deviations between model predictions and spore trap data may have arisen from a number of factors, including inaccuracies in quantifying DNA using qPCR (Section 3.4.4) and imprecision in model predictions. Additional research into some aspects of the epidemiology of *D. pinodes*, as follows, might enable further refinement of the model:

Firstly, more information relating to the timing of commencement of the in-crop cycle might enable improvements to model predictions of ascospore release in spring and summer.

Secondly, the rate and timing of breakdown of soft pea tissue on which pseudothecia are formed during the in-crop cycle is not well understood. Dickinson (1967) reported that pea leaves are rapidly colonised by saprophytic fungi following senescence, but noted that dead pea leaves are very thin and subject to drying out. In warm, dry conditions, such as those which prevail during summer in southern Australia, decay of soft tissue may take longer than occurs in Ireland, where the study of Dickinson (1967) was conducted. Better understanding of the rate and timing of breakdown of soft tissue may enable improved forecasting of the duration of the in-crop cycle.

Thirdly, the effects of relative humidity on pseudothecial maturation and ascospore release of *D. pinodes* are improperly understood. Hare & Walker (1944) reported that mature pseudothecia were produced on mature field pea plants inoculated in the greenhouse when the sand in which the plants grew was kept continuously moist, suggesting that free moisture on the plant surface is not necessarily required for pseudothecial development. Further research to confirm and quantify the effect of high humidity on pseudothecial maturation may enable this to be factored directly into the model.

Fourthly, as previously discussed, further research is required to determine more precisely the effect of high relative humidity on ascospore release.

Fifthly, more information on the lower temperature threshold for conditions to be favourable for pseudothecial maturity (T-threshold) may lead to further improvements in model predictions. Hare & Walker (1944) reported that the number of pseudothecia of *D. pinodes* formed *in vitro* at 4, 8, and 12°C was the same as at 16°C, but that the time taken for their formation was greater. No information was found in the literature regarding the impact of low temperatures on pseudothecial maturity *in vivo*. T-threshold was set at 9°C (Salam et al., 2011a), based on experimental data for the out-of-crop cycle, during which mean temperatures[1] did not drop below 9°C (Salam, M.U.,

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[1] Mean of maximum and minimum daily temperature, used in Blackspot Manager model

Department of Agriculture and Food, Western Australia, personal communication, 2010). Because mean temperatures at Kingsford for the in-crop cycle were sometimes as low as 6°C, the temperature threshold in the modified model was revised down to that temperature, but whether this is the best threshold value requires further investigation.

5.6.3 D. rabiei

No ascospores of *D. rabiei* were detected either on trap plants or in the spore trap samples. The amount of infected chickpea stubble in both 2008 and 2009 was less, and the distance from infected stubble in 2008 greater, than was the case for canola stubble infested with *L. maculans* and pea stubble infested with *D. pinodes*. Furthermore, the amount of disease on the stubble may have been less than for the other two model pathogens, and other variables such as the number of fruiting bodies per lesion and the number of ascospores per fruiting body may differ between the three pathogens. Although direct comparisons with the other model pathogens cannot be made, the results suggest that few or no *D. rabiei* ascospores were released from the stubble. This is consistent with results from Leo et al. (2011) and Phan et al. (2003) that all isolates of *D. rabiei* in Australia are of the one mating type (MAT 1-2) and no ascospores are produced. As previously discussed (Section 1.9.3), the fact that only one detection of *D. rabiei* ascospores, at very low numbers, has been reported for South Australia, suggests that either MAT 1-1 is present as a very small proportion of the population so far undetected, or homothallic production of ascospores occasionally occurs. A third possibility is that ascospores from stubble from South Australia were mis-identified as *D. rabiei*.

5.6.4 Accuracy of the spore trapping/qPCR methodology

For both *L. maculans* and *D. pinodes* the correlation between qPCR assays and ascospore counts was comparable to or better than that reported by other researchers, e.g. Fraaije et al. (2005), Carisse et al. (2009) and Rogers et al. (2009) (Section 1.4.5). The close correlation may have been because yields of DNA from co-extracted yeast were used to correct for sample-to-sample variation and/or because a greater proportion of the duplicate tape was viewed during spore counting in the study reported here than in some other studies (e.g. Rogers et al. (2009)). Minor differences may have been due to inaccuracies in the microscope counts resulting from subsampling, and/or to differences in the number of ascospores landing by chance on one or the other of the

two halves of the tape used for either microscopy or PCR assays. These factors would have had most effect when few ascospores were trapped. Variation in DNA extraction, and precision of DNA measurement, particularly near detection limits, may also have contributed to difficulties in defining the relationship between ascospore numbers and yields of DNA as discussed above in relation to *L. maculans*.

Other factors that might have introduced variability were identified in Chapter 4, i.e. presence of dust on tapes, exposure of spore traps to high temperatures and germination of ascospores on the tapes. In the field studies reported here, presence of dust on tapes appeared to cause an increase in the amount of DNA detected in only one of the samples tested, and there was no sign of ascospore germination on tapes. There was only one occasion throughout the two years of field sampling on which very high temperatures occurred after rainfall (potentially resulting in release and capture of ascospores), but before subsequent trap clearance. That was during the week ending 16 December 2009, when air temperature was between 35 and 40°C for the 3 hours immediately preceding trap clearance. It is unlikely that the temperature inside the spore trap exceeded 45°C at that time; temperature inside the spore trap when it is placed in the sun is higher than ambient air temperature, but only by approximately 5°C (Chapter 4). Since exposure of *L. maculans* ascospores to 45°C for 24 hours was shown not to significantly reduce yield of DNA compared with 25 and 35°C (Chapter 4), the exposure of the spore tape to high temperature for a few hours is unlikely to have reduced the yield of DNA. On the contrary, the very high yields of DNA of *M. pinodes* from samples collected during the summer, and the similarity in yields of DNA per ascospore samples collected in winter and in summer, suggest that exposure to high temperature did not reduce the amount of DNA in the spores.

5.7 Conclusions

The close relationship between the results from the trap plants and the spore trap, and between these and predictions generated by epidemiological models, indicate these are all reliable systems to detect or predict the timing of release of airborne ascospores. There are severe limitations with trap plants, which can only be grown when environmental conditions are suitable, and the use of spore traps to monitor ascospore release has overcome these limitations. In the past, the usefulness of spore traps have been limited by the time-consuming process of identifying and counting ascospores

using a microscope. The PCR assays have eliminated the need for this process and are a reliable means of quantifying ascospores caught in the traps. Care must be taken to quantify the relationship between ascospore numbers and DNA results, and ensure that this is consistent between PCR assays and between seasons.

The spore trapping system proved to be robust when used in field studies, with little suggestion of interference in the amount of DNA detected due to either the occasional presence of dust on tapes, sample storage or high temperature.

Epidemiological models that predict timing of ascospore release are an important tool for disease management by industry. For example, crop disease forecasts for various locations across southern Australia, generated using Blackleg Sporacle and G1 Blackspot Manager, and are published on the Department of Agriculture and Food, WA, website (http://www.agric.wa.gov.au/cropdisease), and updated weekly during the cropping season, to enable farmers to modify their disease management strategies based on up-do-date predictions for their locations. This study has used spore trapping combined with DNA analysis to improve performance of these epidemiological models.

6 EXAMINATION OF ASSUMPTIONS: RELEASE OF *L. MACULANS* ASCOSPORES AFTER DROUGHT AND DISPERSAL OF *D. PINODES* INOCULUM IN DUST

6.1 Introduction

In the field studies reported in Chapter 5, it was assumed that *L. maculans* ascospores detected early in the season originated on stubble from the season before the last. However there were no previous publications or experimental data available on which to base that assumption. Likewise, in the experiments reported in Chapters 4 and 5, small amounts of DNA of target pathogens were detected in spore trap samples collected from the field during dry conditions, which were assumed to have been due to presence of pathogen material in dust at the field sites. These assumptions were examined in two field experiments conducted at the Kingsford site in 2009 and 2010.

6.1.1 Release of L. maculans ascospores after drought

In Chapter 5 the need for further research was identified to clarify whether active pseudothecia might still be present in autumn on stubble from two seasons before, i.e. at least 15 months after harvest. This question arose in relation to the assumption that April and May were too early for mature pseudothecia to have developed on the stubble from the preceding season's canola crop (the Blackleg Sporacle model predicted the first appearance of mature pseudothecia in June in both years). A similar question had previously been posed in relation to the detection of ascospores in field trials at Wagga Wagga, New South Wales early in 2007 (Salam, M.U., Department of Agriculture and Food, Western Australia, personal communication, 2008). In 2006 there was good rainfall (73.6 mm) at the field site early in the season (January through April), but this was followed by repeated periods of drought during May, August, September and October, with only occasional rain. Canola was sown in April 2007 and the crop became severely affected by blackleg. This could not readily be explained by inoculum arising on crop residues from the 2006 season, because that crop had little disease due to the low rainfall. The Blackleg Sporacle model was run twice, using rainfall and temperature data for the Wagga Wagga site, and with unlimited ending period (Salam, M.U., Department of Agriculture and Food, Western Australia, personal

communication, 2008). The first run had a commencement date of 1 January 2006, and modelled release of ascospores from 2005 stubble. The second had a commencement date of 1 January 2007, and modelled the release of ascospores from 2006 stubble. With the first run, the model predicted the release of a large number of ascospores from 2005 crop residues in March and April of 2007 (Figure 6-1A). With the second run, ascospore release was not predicted to commence until July 2007 (Figure 6-1B). Thus the model predictions matched field observations only when ascospore release from 2005 stubble was factored in. However, biological evidence was required to test the assumption that mature pseudothecia could recommence ascospore release following a prolonged dry period.

Several researchers have investigated release of *L. maculans* ascospores from stubble after prolonged dry periods. McGee (1977) studied ascospore release from canola stubble collected in April from crops in Victoria, Australia, that had been severely affected by blackleg in the previous season, and held in the open under shelter. Ascospore release in a wind-tunnel was measured following a 20-hour preconditioning period at 90% relative humidity. Ascospore numbers, from batches of stubble removed weekly from the shelter, increased between April and May, remained high until August, and then declined to zero by late January. The same pattern was repeated the following year, although the numbers of ascospores released were much smaller. This indicated that pseudothecia on infested stubble were able to release ascospores in the second year after the crop was grown. However, ascospore release was measured only after a preconditioning period favourable for pseudothecial maturation.

Marcroft et al. (2003) showed that canola stubble left on the soil surface at three sites in Victoria, Australia, was still able to release ascospores 18 months after and, in the case of low rainfall sites, up to 42 months after, harvest, following a preconditioning period of 24 hours at 100% relative humidity. Although in all cases mean ascospore numbers were less from 18-month-old than from 6-month-old stubble, the difference was significant only for the high rainfall site.

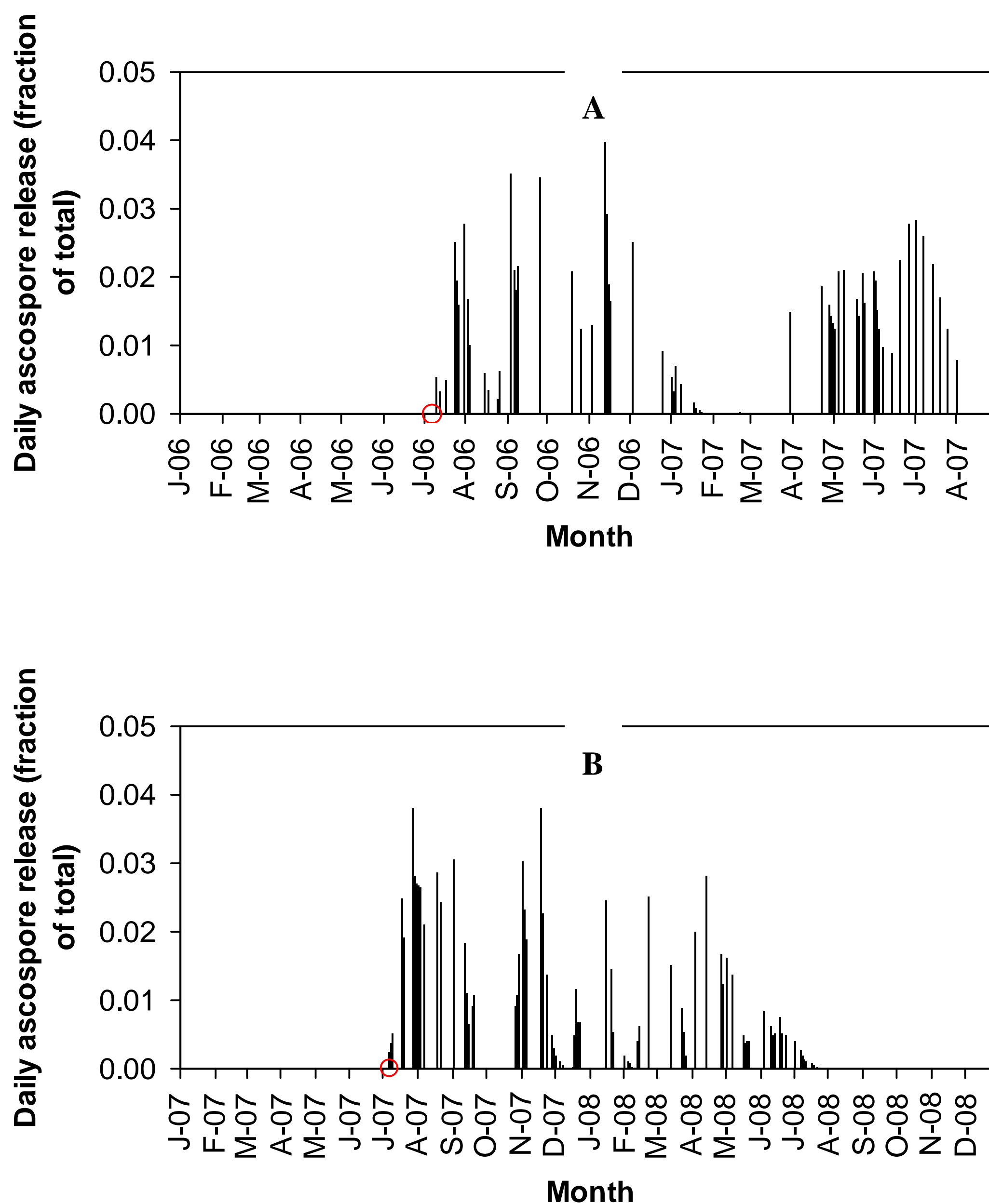

Figure 6-1 Blackleg Sporacle model predictions of *L. maculans* ascospore release from infested residues of a canola crop grown at Wagga Wagga, New South Wales, in 2005 (A) and 2006 (B). Graphs courtesy M. U. Salam (unpublished data)

In Kentucky Hershman & Perkins (1995) collected stubble from recently harvested crops in May or June and stored it outside in a semi-protected area, partly shaded but otherwise exposed to ambient temperature and rainfall. Ascospore discharge from wetted stubble collected from several sites in Logan County, Kentucky in 1989, 1990

and 1991, was monitored after preconditioning at 100% relative humidity for 24 hours. For most sites and years, stubble discharged most ascospores within the first year after harvest, but for two different sites in different seasons, significant numbers of ascospores were released 15-19 months after harvest. Release of ascospores at that time had the potential to infect newly emerging crops during autumn and winter, although in practice this was unlikely to occur because little canola stubble survives in the field in Kentucky beyond the twelfth month.

It is clear from these studies that ascospores can be released from canola stubble in the second year after harvest, but in each case this occurred after a preconditioning period of 20 or 24 hours at high relative humidity (>90%), in which maturation of pseudothecia could proceed (Bernard et al., 1999). Naseri et al. (2009) reported that maturation of *L. maculans* pseudothecia requires prolonged moist conditions. At 15°C in a controlled environment, pseudothecia on canola stubble which had previously been thoroughly wetted by soaking in water for 12 hours, needed to be moistened at least twice a day for pseudothecial maturation to proceed. Pseudothecia matured most quickly during continuous wetness, and maturation did not proceed at all when stems were wetted only once per day, indicating that short wetness periods were insufficient.

In southern Australian field conditions in autumn, high relative humidity (>90%) is rarely maintained for 20 or more hours continuously. For example, examination of weather data for Kingsford in April 2008 indicated that it took from 1 till 26 April before a total of 24 hours of relative humidity greater than 90% had occurred, and the longest period of high humidity in that time was 9.50 hours, followed by 3.75 hours. Therefore, in the field, pseudothecial maturation is unlikely to proceed quickly until the advent of prolonged wet weather.

Once pseudothecia are mature, they release ascospores, upon wetting, in discrete events. Salam et al. (2003) noted that, in any one ascospore discharge event, only a portion of the available ascospores in mature pseudothecia is released. The number of ascospores released during the next suitable rainfall period is a proportion of the sum of the remaining ascospores and the number of new ascospores that have been produced in the pseudothecia in the interim. It is not clear whether mature pseudothecia are still viable and able to release ascospores upon wetting following disruption of maturation by

prolonged dry conditions, or whether release of ascospores requires maturation of immature pseudothecia.

6.1.2 Dispersal of D. pinodes in dust experiment

If pathogen DNA could be detected in dust this may provide added opportunities to monitor for pathogens of biosecurity concern. Indeed there would be some additional advantages to this approach in comparison with monitoring spore release. In the first instance, as was illustrated in the Chapter 5, ascospores are only released at specific times in response to environmental triggers, which are dependent on the biology of the pathogen. These triggers differ between sites and years, and may not be readily predictable for pathogens newly arrived in an area, necessitating longer periods of monitoring than is desirable. Furthermore, because timing of spore release differs from one pathogen to another, opportunities to monitor for more than one pathogen at a time through detection of airborne spores may be limited. If the techniques developed in this study could be applied to detection of pathogens in dust, it might minimise these difficulties. Possibly the major advantage of this approach however, is that it may enable the detection of plant pathogens that do not release airborne spores.

If it were possible to monitor for presence of pathogens in dust, the most opportune time to collect samples would be during harvest, when a considerable amount of dust is generated from crop residues. A previous study has shown that, not only can plant pathogen material be present in dust generated at harvest, this material can also constitute a source of inoculum for the following crop. Buchwaldt et al. (1996) demonstrated that *Colletotrichum truncatum*, which causes anthracnose on lentils, can be dispersed to a distance of at least 240 m from the edge of a lentil crop in dust generated during harvest, and that lentil plants inoculated with this dust, both shortly after harvest and at the start of the following season, developed anthracnose. *C. truncatum* produces two kinds of inoculum on infected lentil plants; conidia and microsclerotia, the latter of which survive between crops on plant debris or in soil (Buchwaldt et al., 1996). The pathogen is also seedborne, but transmission from seed to seedling does not readily occur (Buchwaldt et al., 1996).

In addition to the value of detecting pathogen DNA in dust for the purpose of monitoring, it would be very useful, from an epidemiological perspective, to know whether the model pathogens in this study can be spread in dust generated at harvest.

The most suitable model pathogen was *D. pinodes*, since commercial crops were available for the study and the disease is widespread and severe in South Australia. Unlike *C. truncatum*, *D. pinodes* does not form microsclerotia on infected plant tissue but mature pseudothecia with ascospores are present in large numbers on infested portions of pea plants at harvest, and ascospores are able to remain viable over summer (at least within pseudothecia), as described in Chapter 5. In a moderately infected crop, plant material infested with *D. pinodes* could be expected to constitute a significant portion of the dust, along with uninfested plant matter and soil. Another potential inoculum source in dust is sclerotia or chlamydospores, particularly the latter, which have been shown to form from conidia or ascospores *D. pinodes* within 2 weeks in soil (Carter & Moller, 1961).

6.2 Aims

The overall aim of the drought experiment was to determine whether *L. maculans* pseudothecia on canola stubble from the season before the last could release significant numbers of ascospores immediately at the start of rains in the autumn. Accordingly, the objectives were to determine the effect of exposure of *L. maculans*-infested stubble to prolonged dry conditions, on:

1. number of ascospores released upon wetting
2. pseudothecial maturity
3. ascospore viability
4. number of pseudothecia on the stubble

The aims of the dispersal experiment were to determine whether DNA of *D. pinodes* could be detected in dust collected during harvest of an infected pea crop, and whether the dust was able to act as a source of inoculum of blackspot disease on pea seedlings.

6.3 Materials and methods

6.3.1 Release of L. maculans ascospores after drought

The 2008 canola stubble was gathered from a commercial property at Mayhall near Struan in late February 2009, placed on the soil surface in full sun at the University of Adelaide Waite Campus, Urrbrae, South Australia, and monitored for pseudothecial maturation (Section 2.2.4) and commencement of ascospore release (Section 2.2.5).

Once approximately 50% of pseudothecia had reached maturity stage D1 (fully mature, see Table 2-1), and ascospores were consistently released from batches of stubble upon wetting, canola stem and root pieces with blackleg cankers were trimmed to 15 cm and placed into nylon mesh bags (8 stems per bag). The bags with stubble were placed under a shelter (Figure 6-2) in full sun at the Kingsford site on 8 July 2009. The shelter had been purpose-built to exclude rain but allow transmission of light, and was constructed of a wire cage with a sheet of polycarbonate attached as a roof.

Figure 6-2 Shelter with mesh bags containing canola stubble with mature pseudothecia, which was used to shelter the stubble from rain during exposure to ambient temperature and sunlight at Kingsford

At intervals of 1 or 2 months, three bags, selected at random, were removed from the shelter and brought back to the laboratory. Three stems were removed from each bag for assessment of pseudothecial numbers, pseudothecial maturity and ascospore germination. The remaining five stems were left in the mesh bags and used for ascospore release assessments.

Ascospore release was measured as described above. When ascospore numbers were high (at least 250 ascospores per rotorod arm: Weeks 4, 8 and 16), subsampling was employed; every fourth transect was counted and the numbers adjusted by multiplying by four.

The stubble collected from the shelter on Week 28 (20 January 2010) was preconditioned for 24 hours in conditions optimal for pseudothecial maturation (100% relative humidity, 15°C, 12 hour light) (Naseri et al., 2009), in a growth cabinet, in order to determine whether pseudothecia were able to produce new ascospores. After preconditioning, ascospore release was measured again as described above. The number and maturity stage of *L. maculans* pseudothecia on stubble were determined as described above.

To assess viability, ascospores were collected from each of three stems of canola stubble as described in Section 2.2.3.1, except that the ascospores were released onto water agar in the base of the Petri dish. After 24 hours at room temperature (approximately 22°C), the number of ascospores germinated out of 100 ascospores, observed under 100x magnification, was counted.

Weather data were recorded by the AWS at Kingsford as described in Section 2.1.

6.3.2 Dispersal of D. pinodes in dust experiment

Dust was collected during harvest of a commercial pea crop at Kingsford, South Australia on 14 December 2009. The severity of blackspot disease in the crop was assessed just before harvest by rating 50 plants in a semi-circle of radius approximately 200 m from the edge of the crop. The number of infected nodes was counted on each plant. The area of peas harvested was approximately 13 ha (400 m x 325 m).

Wind speed and direction were measured at the site using a Kestrel 3500 Delta T. Pocket Weather Meter and recorded manually approximately every 15 minutes from start of harvest at 10.00 am till completion at 1.00 pm. At each 15-minute interval five readings were taken over the course of one minute and the mean of the five readings was calculated.

Plastic sheets 1 m x 1 m were placed, immediately before harvest, at 20 m intervals, in a transect at right angles to, and starting 4 m from, the crop edge, in a west-south-westerly direction, to a distance of 200 m away from the crop. At completion of harvest, the

sheets were folded to contain the dust that had landed on them, and brought back to the laboratory where dust was removed into glass vials using a vacuum suction device (Figure 6-3), weighed and held at room temperature prior to inoculation of plants. Dust collected from the header filter, which had been cleaned prior to harvest of the crop, was also collected and used for preliminary inoculation studies.

Figure 6-3 Suction device used to remove dust, collected onto plastic sheets placed in a transect from the edge of a field pea crop in Kingsford during harvest, into glass vials

Rotorod samplers (Figure 2-1), with double-sided tape on the leading edge of each arm, were placed in two transects at right angles from the crop edge in a west-south-westerly direction, at distances 0, 50, 100, 200, 400 and 500 m from the crop edge (Figure 6-4). At completion of harvest, the sampler arms were removed, placed in an airtight container and brought back to the laboratory. The double-sided tapes were removed from one arm of each sampler, placed on a microscope slide and cut into four sections, of length approximately 15 mm. The four sections were inserted into a 2 ml screw-top tube containing 0.6 g Ballotini glass beads. Care was taken to ensure that each tape segment was covered in glass beads as they were inserted into the tubes, to prevent adhesion of tape segments to each other or the side of the tube. DNA was extracted from the samples using Method 2 (Appendix 6), from Step 4 onwards, and quantified using a qPCR assay specific to *D. pinodes* (Section 2.5).

Figure 6-4 Sampling of dust generated during harvest of a field pea crop at Kingsford, December 2009. A. Transect of plastic sheets and rotorod samplers at right angles from the edge the field B. Rotorod sampler with battery mounted on post C. Transect of plastic sheets and rotorod samplers with harvester generating dust visible in the crop behind

In an initial experiment to determine the minimum amount of dust for inoculation of seedlings, 21-day-old pea seedlings were inoculated on 11 January 2010 using dust removed from the header filter. Differing amounts of dust (0, 10, 20, 50, 100 and 200 mg) were suspended in 0.5 ml of sterile RO water with 0.1% Tween 20, and painted onto the upper surface of two leaves at each of nodes 2 and 3 on each plant (i.e. 4 leaves per plant), using a paintbrush. The plants were covered to maintain high humidity, and grown in conditions favourable for disease development (15°C; 12 hour light; Roger et al., 1999) in a growth room. Presence of lesions was recorded after 3 days, and lesions were examined for presence of fruiting bodies after 7 days. Isolation was not attempted as it was considered likely that fruiting bodies would form on pea leaves if infected with *D. pinodes*.

On 15 January 2010 field pea seedlings of age 22 days were inoculated using the dust collected onto the plastic sheets. For each sample, a slurry was made by mixing 20 mg dust into 225 µl sterile RO water with 0.1% Tween 20, and painted onto the upper surface of each of two leaves at node 3 on each plant, using a paint brush (Figure 6-5). The plants were covered, grown and examined as described above.

Figure 6-5 Field pea seedlings inoculated with a slurry of dust, collected during harvest of a field pea crop affected by blackspot, in RO water with 0.1% Tween 20. Inoculum was applied to the upper surface of two leaves of each seedling at node 3

6.4 Results

6.4.1 Release of L. maculans ascospores after drought

The number of ascospores captured by a rotorod sampler in the wind tunnel from 2008 canola stubble which had been held under shelter at Kingsford, and then wetted for 5 minutes, is shown in Figure 6-6. At the start of the experiment (7 July 2009), small numbers of ascospores were released from each batch of stubble. The numbers were higher after 4 weeks and remained high for at least a further 4 weeks before declining. Data were extremely variable (Figure 6-6) and analysis of the log-tranformed data showed that the decline in the number of spores released was not statistically significant ($P < 0.05$) until Week 23 (16 December 2009) at which time the numbers had declined to approximately the same as at the start of the experiment. Spore release continued to decline and, from Week 28 (20 January 2010) onwards, few ascospores were released.

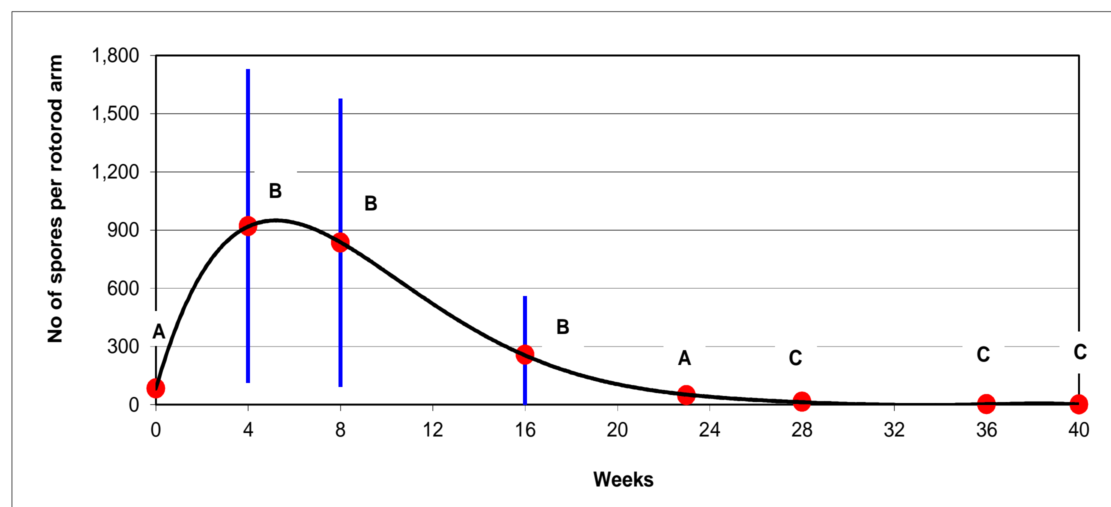

Figure 6-6 Mean number of *L. maculans* ascospores captured onto one arm of a rotorod sampler in a wind tunnel, from canola stubble held under shelter at Kingsford for up to 40 weeks, and then wetted for 5 minutes. Error bars represent standard deviation. Data sets (representing week from start of sampling) with the same letter (A-C) were not significantly different from one another when log-transformed data were analysed by ANOVA ($P < 0.05$)

After preconditioning the stubble collected from the shelter on Week 28, for 24 hours in conditions optimal for pseudothecial maturation, the mean number of ascospores captured onto one arm of the rotorod sampler in the wind tunnel, following 5 minutes wetting, was 1.5. This was not significantly different from the number of ascospores released from the same stubble before preconditioning ($P < 0.05$).

The proportion of pseudothecia which were mature (Stage D1) was close to 50% at the start of the experiment. Analysis of the arcsin-transformed data for Weeks 0 – 28 indicated that this proportion did not change significantly (P = 0.55; n = 9) for the first 28 weeks (Figure 6-7). However, a qualitative change was observed, starting at Week 23, in that fewer ascospores were released upon application of gentle pressure to the cover slip, and some intact asci containing mature ascospores were ejected from the pseudothecium. When no ascospores were released from asci the pseudothecia were assigned to "Stage D2" (Section 2.2.4). By Week 36, all pseudothecia which had ascospores were at Stage D2, i.e. all ascospores remained inside the intact asci, most of which had been ejected from the pseudothecium.

It was often difficult to discriminate between Stages B and C, as asci within the pseudothecium usually overlapped, making it difficult to count the number of ascospores in the asci. However, the numbers of pseudothecia at Stages A, B, C and E were each generally low throughout the experiment. Statistical analysis of the proportion of pseudothecia at these maturity stages was not attempted due to frequent zero values.

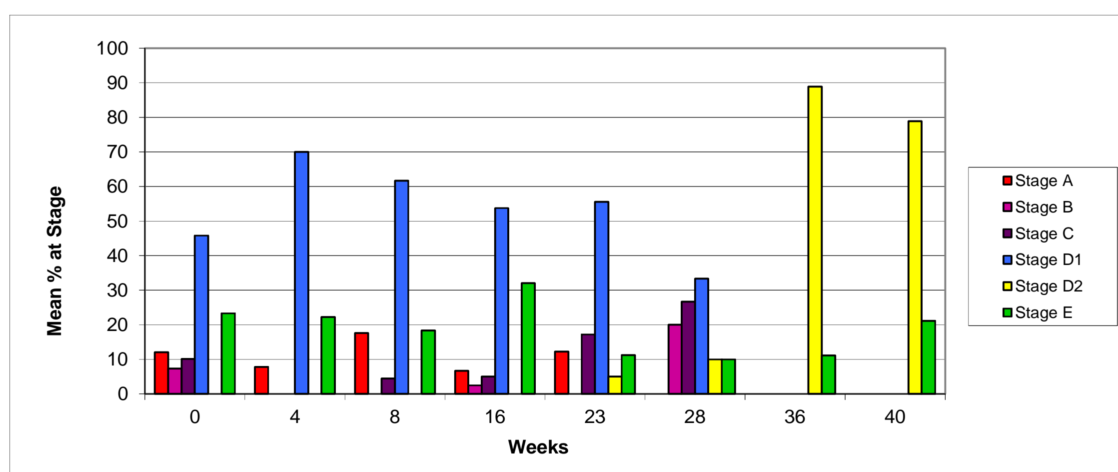

Figure 6-7 Mean proportions of *L. maculans* pseudothecia, on 2008 canola stubble placed under shelter at Kingsford for up to 40 weeks, at various stages of maturity (Section 2.2.4)

The number of pseudothecia per 0.5 cm^2 of canola stubble did not change significantly throughout the course of the experiment. The data were variable, but analysis of log-transformed data indicated oscillation in the numbers, rather than a decline over time (Figure 6-8).

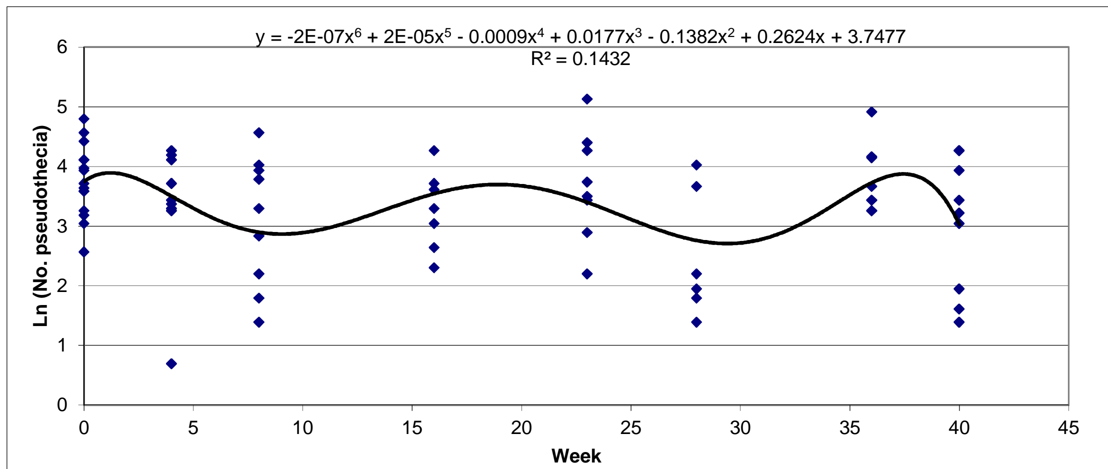

Figure 6-8 Number of pseudothecia per 0.5 cm^2 of 2008 canola stubble placed under shelter at Kingsford (log-transformed data).

Spore germination was high at the start of the experiment and remained high for the first 16 weeks; thereafter germination rates declined to a mean of approximately 64% by Week 40 (Figure 6-9).

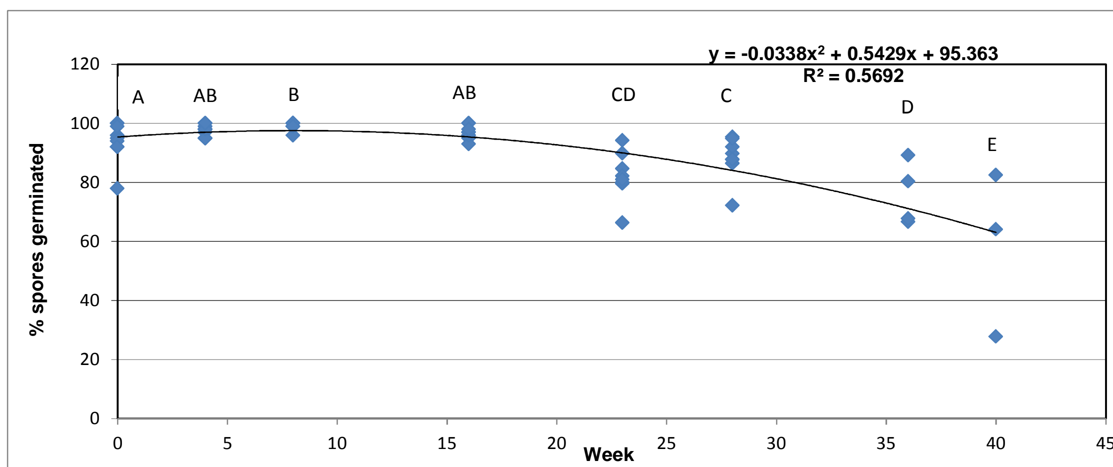

Figure 6-9 Percentage of *L. maculans* ascospores released from 2008 canola stubble placed under shelter at Kingsford that were viable. Data sets (representing week of sampling) with the same letter (A-E) were not significantly different from one another when arcsin-transformed data were analysed by ANOVA ($P < 0.05$)

Weather data were compiled for the Weeks 8-16, 16-23 and 23-28, during which changes in pseudothecia, ascospore release and ascospore germination were recorded. The mean daily maximum temperatures for Weeks 8-16, 16-23 and 23-28 were 20.0,

30.1 and 31.7°C, respectively. The mean daily relative humidity values (average of 9.00 am and 3.00 pm relative humidity) were 74.5, 51.2 and 43.2% for the same time periods.

6.4.2 Dispersal of D. pinodes in dust experiment

Disease severity in the crop was moderate, with a mean of 11.2 (standard error 0.3) infected internodes per plant (i.e. disease severity rating of 37.3%). Wind direction was easterly throughout, with a mean wind speed of 6.5-10.9 m/second.

There was an exponential decline in the amount of DNA extracted from rotorod spore trap samples with distance from the edge of the crop (Figure 6-10). At distance 504 m from the crop, the mean amount of *D. pinodes* DNA detected on one arm of a rotorod sampler was 744.3 pg. The detection limit for *D. pinodes* in the PCR test was 16 fg (at a cycle threshold of 40; Section 1.4.3.1).

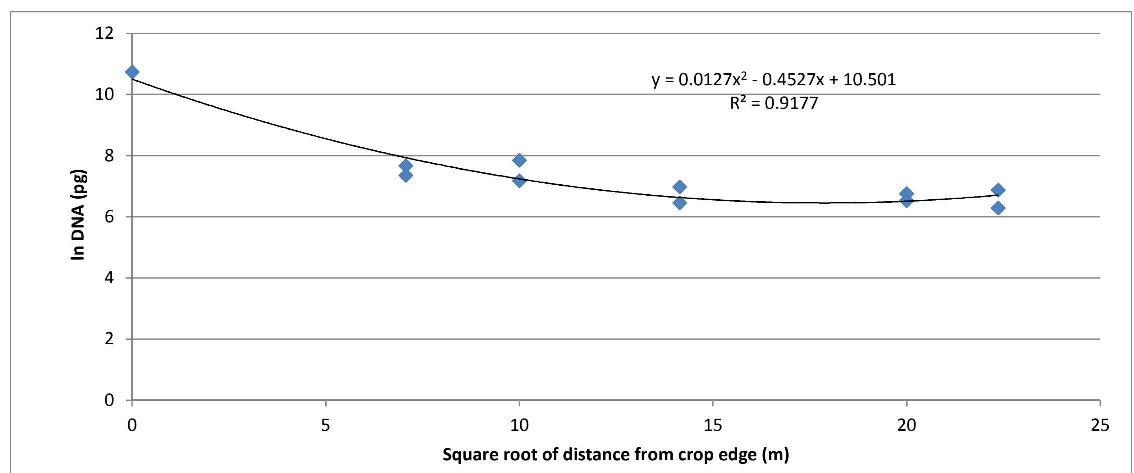

Figure 6-10 Amount of *D. pinodes* DNA on adhesive tape from rotorod samplers placed by a commercial pea crop at Kingsford during harvest in December 2009

There was an exponential decline in the amount of dust, collected onto plastic sheets placed on the ground during harvest of field peas, with distance from the edge of the crop (Figure 6-11).

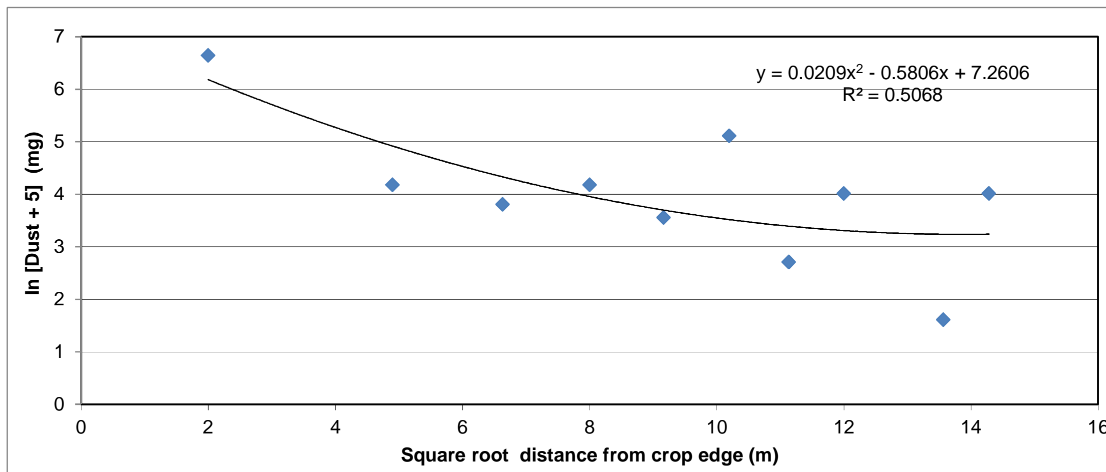

Figure 6-11 Amount of dust (log-transformed) collected on plastic sheets (1m x 1m) during harvest of a commercial pea crop at Kingsford in December 2009

Leaves of pea seedlings inoculated with dust all developed lesions of appearance similar to blackspot lesions immediately below the inoculated area (Figure 6-12). Leaves of control pea seedlings to which only RO water with Tween 20 had been applied did not develop lesions. No fruiting bodies developed on any leaf lesions.

Figure 6-12 Lesions on leaves of pea seedlings 7 days after inoculation with a slurry of dust, collected during harvest of a field pea crop affected by blackspot, in RO water with 0.1% Tween 20. Lesions formed where inoculum had been applied, but did not expand beyond that area.

6.5 Discussion

6.5.1 Release of L. maculans ascospores after drought

Pseudothecia released ascospores in large numbers, without preconditioning, until 16 weeks after placement in the field in drought conditions, and were still releasing ascospores after 23 weeks. The decline in ascospore release from Week 23 (16 December 2009) coincided with a change in the condition of the pseudothecia as observed under the microscope. Asci containing mature ascospores were ejected upon application of gentle pressure to the cover slip, but few or no ascospores were released. This condition of the pseudothecia had not been described before and was not anticipated at the start of the experiment. The term "Stage D2" was introduced into the rating system to describe it. Stage D2 was defined as "pseudothecium, asci and ascospores are mature, and the pseudothecium discharges asci upon gentle pressure but asci release few or no ascopores" (Section 2.2.4). Pseudothecia on stubble from Weeks 23 and 28 were at an interim stage, in which some, but not all, asci had ceased to release ascospores, and some, but not all were ejected from the pseudothecium. It was not always possible to discriminate between Stages D2 and C, in both of which few or no ascospores were released. Furthermore, as previously noted, it was difficult to discriminate between Stages B and C, which are differentiated from one another by the number of ascospores per ascus, and the number of septa in the ascospores, both which proved difficult to identify due to overlap of the asci within the pseudothecium. These two factors may explain the increase in the proportion of pseudothecia rated Stages B and C at Weeks 23 and 28 compared with earlier weeks (Figure 6-7). Many of these may have been more appropriately considered to be at Stage D2.

The cessation of release of ascospores from asci, and consequent ejection of entire asci containing mature ascospores from the pseudothecium, were assumed to be caused by a hardening of the ascus wall, possibly due to ageing of the asci. However, as this condition was first observed in mid-December (early summer), the presumed hardening of the ascus wall may have occurred as a result of either higher temperatures, lower humidity, or a combination of the two. Further investigation is required to determine the cause of this effect and whether it would still occur if stubble was exposed to prolonged dry conditions (i.e. no rain) combined with cool temperatures and high relative humidity, such as experienced at Kingsford in winter.

Pseudothecia on stubble collected from the shelter in January, which had stopped releasing ascospores, did not regain the ability to release ascospores when placed in conditions suitable for pseudothecial maturation for 24 hours. This was in keeping with the findings of (McGee, 1977), who reported that batches of blackleg-affected canola stubble, brought in from a shelter in January, did not release ascospores after preconditioning. That author found, however, that canola stubble brought in from the shelter during April and May (16 and 17 months after harvest), did release ascospores after preconditioning. In the experiment reported here, there was no resumption of ascospore release from stubble removed from the shelter following the advent of cooler, moister weather in March and April (without preconditioning). Ascospore release after preconditioning was not tested, but, given the findings of (McGee, 1977), it is likely that exposure to conditions suitable for pseudothecial maturation would have resulted in a resumption of ascospore release from the stubble.

The results of this experiment suggest that ascospores from the season before the last are unlikely to constitute a major source of inoculum at the start of the growing season. However, stubble had been kept completely dry throughout the course of the experiment. In practice, even in severe drought conditions, stubble on the ground in the field would most likely be subject to occasional wetting from rain or dew. It is possible that the presumed hardening of the ascus wall would have either been delayed, or not occurred at all, under such conditions. Furthermore, the fact that ascospores were released in considerable numbers even after being kept dry in the field for 23 weeks, suggests it may be possible for stubble to constitute a source of inoculum at the start of the season if dry weather commenced in spring rather than in winter as was simulated here. This would depend on whether the presumed hardening of the ascus wall was caused by high temperature and low humidity, or due to the passage of time, which, as noted above, requires further investigation. Further research is also required to determine the effect of intermittent moisture during winter followed by dry conditions in spring and summer, on ascospore release from 18-month-old stubble in autumn. The number of pseudothecia on canola stems did not decline over the course of the experiment, and, while there was a decline in ascospore viability, after 23 weeks more than 80% of ascospores were still viable. Therefore, should drought conditions commencing in spring not lead to failure of the ascus to release ascospores, there may

be plenty of viable ascospores remaining to infect crops more than 5 months later, in autumn.

6.5.2 Dispersal of D. pinodes in dust experiment

The exponential decline in the amount of dust collected onto the arms of rotorod samplers, and onto the plastic sheets, with distance from the crop edge, indicated that the dust originated from the harvested crop and not elsewhere.

At the furthest point (500 m from the crop edge) the amount of DNA detected in the rotorod samples was approximately 47,000 times the detection limit of the PCR test. In this study the disease had been readily detectable in the crop by visual inspection prior to harvest. In a biosecurity situation, it is desirable to detect a pathogen before it has become widely established, in which case the incidence of disease would most likely be much less. Furthermore, since the location of infected crops may be unknown, it would be useful to be able to detect pathogen DNA in air samples taken at some distance from an infested crop. Both of these factors would lead to a lower concentration of pathogen material in the air than detection close to a moderately-diseased crop as was reported here. Whether DNA would be detectable in these circumstances would depend on the magnitude of these factors. As an example, if the incidence of disease in the crop and the concentration of dust at the sampling point had each been 1% of what was the case in this study, then DNA would have been readily detectable (4.7 times the detection limit of the PCR test), providing pathogen material was well-mixed throughout the air. However, if either of those factors, or both combined, had led to a dilution of more than 4.7 times that, then it would not have been detectable. In order to increase the chance of detection of pathogen material in dust in the air, it would be necessary to sample larger volumes of air than were sampled in this study.

If the incidence of a pathogen in a crop is low, then the distribution of pathogen material in airborne dust generated at harvest of that crop is likely to be uneven, particularly in air near to the crop. However, as airborne dust moves away from the crop, mixing is likely to occur. In this study, dust from the harvester could be seen moving upward in air currents to high altitudes, as well as moving horizontally, and it was clear from these observations that dust was being dispersed over distances considerably greater than 500 m, and was subject to some turbulence as it moved. Movement of a cloud of dust in the atmosphere is similar to that of a plume of smoke from a chimney. As it moves

horizontally and vertically it becomes dispersed, and its movement can be explained by the theories of eddy diffusion (Lacey, 1988). This is a complex and incompletely understood subject, which is beyond the scope of the present study, but further information and references can be found in Lacey (1988). Suffice it to note that various equations have been developed to describe the dilution of particles in the air with distance from their source, in relation to diffusion and air turbulence. As distance from the source increases, airborne particles become less concentrated but better mixed. If a means were available to sample large volumes of air in the atmosphere during periods of harvest of field crops, the detection of pathogens present at low incidence may be feasible.

Although lesions developed on inoculated leaves of pea seedlings, the fact that no fruiting bodies were produced, in conditions optimal for sporulation, suggests that the seedlings had not become infected. The reason that lesions formed is unclear, but may have been due to a physical or other reaction of the plant to the presence of dust on the leaves, perhaps due to interference with gas exchange or some other physiological process. The apparent lack of infection suggests that dust did not contain viable inoculum, although it is possible that physical or other factors interfered with the infection process. Although the amount of dust collected onto plastic sheets was small, resulting in only small amounts of inoculum applied to seedlings in the second inoculation experiment, the lack of sporulation in the initial experiment, where up to 200 mg of dust was applied, indicates that insufficient dust was not the reason for the apparent lack of infection. Buchwaldt et al. (1996) used approximately 200 mg of dust per seedling to successfully inoculate lentil stems with dust collected during harvest of anthracnose-affected lentil crops in Canada. *D. pinodes* material was present in the dust, as evidenced by the detection of DNA in the rotorod samples, but it is not clear whether this material contained any infectious structures such as ascospores or chlamydospores. Microsclerotia, formed on the infected plant tissue, were considered to have been the most likely source of inoculum of *C. truncatum* in the study by Buchwaldt et al. (1996). Microsclerotia, being survival structures, may be better adapted to survival in dust than ascospores. *D. pinodes* forms both sclerotia and chlamydospores, but only when the pathogen is in contact with soil, and not directly on the plant. Therefore, the concentrations of *D. pinodes* chlamydospores and sclerotia in

dust generated at harvest, if present at all, are likely to be much lower than that of *C. truncatum* microsclerotia.

6.6 Conclusions

The decline in the numbers of *L. maculans* ascospores released from December onwards suggested that canola stubble from the season before the last is unlikely to release significant numbers of ascospores without a prior period of moist conditions. Further research is required to determine whether the presumed hardening of the ascus wall, which coincided with the decline in ascospore release, was due to warmer, drier conditions over summer, or to ageing of the pseudothecia. If the latter, then the fact that significant numbers of ascospores were released from stubble after 23 weeks of dry conditions suggests it may possible for release of viable ascospores to occur if dry conditions commenced in the spring, providing that winter rainfall had been low enough that not all ascospores had been released. Further research is required to clarify this.

The feasibility of detecting pathogen DNA by conducting PCR tests on samples of dust collected from the air during harvest of an infested crop was demonstrated. This technique may be useful for detecting pathogens of biosecurity significance, particularly if it were possible to sample and test large volumes of air. Further research would be needed to determine the logistics of such an approach.

There was no evidence from this study that *D. pinodes* was spread through the movement of inoculum in dust generated at harvest time, possibly because the pathogen does not produce resting structures such as sclerotia or chlamydospores on infected plant material before it is in contact with the soil.

The results of the experiments reported in this chapter provided insight into aspects of the epidemiology of the model pathogens to enable improvements in the comparison of field data with epidemiological models, and offered further indications of potential directions for research into the detection of plant pathogens of biosecurity significance.

7 GENERAL DISCUSSION

Methodology for detection and quantification of airborne spores using spore traps and quantitative PCR assays was developed and improved, and then tested on three model pathogens endemic on pulse and oilseed crops in South Australia. The aims of the research were, firstly, to investigate the suitability of the methodology for biosecurity surveillance, and secondly, to use the system to test predictions of spore release generated by epidemiological models.

The aims were met as follows:

1. The PCR tests were both specific and sensitive when applied to spore trap samples, and results of the PCR tests applied to field samples were borne out by close correlation with microscopic counts of ascospores, and with results of spore monitoring using trap plants;
2. The potential for reduced yield of DNA from spore trap samples collected during periods of extremely hot weather, and the possibility that dust may affect yields, were identified as factors to be considered in assessing qPCR results;
3. Storage of spore trap samples at -20°C was suitable for ascospores of the model pathogen *Leptosphaeria maculans*, with no reduction in the yield of DNA after 6 months;
4. The data from 2 years of monitoring for model pathogens were used to validate the spore trapping methodology, to calibrate epidemiological models to the field site and to refine these models to improve their performance as tools for disease management.

7.1 Development and improvement of the methodology

The PCR tests were sufficiently sensitive when applied to spore trap samples to detect single spores on some occasions, and results were validated by counts of ascospores on tapes bearing trapped spores. Poor reproducibility of results was largely addressed by replication of PCR assays and use of an exogenous control to allow variation to be taken into account. However, some imprecision remained in the system. As reported in Chapter 5, the assays were able to detect as little as one ascospore for some pathogens in some seasons e.g. for *L. maculans* in 2008 and for *M. pinodes* in 2009. However, the

limit of detection for *L. maculans* was higher in 2009 than in 2008. Furthermore, the yields of DNA were considerably greater from field samples than from samples spiked with spores during laboratory experiments (Chapter 3). This remaining imprecision was attributed to variations between batches or 'runs' of DNA extractions and of PCR assays, and difficulties in enumerating spores microscopically. This variability made it impossible to determine with complete accuracy the limit of detection. Other authors have also reported imprecision in quantification of spores in trap samples using qPCR, and a number of factors have been recognised as contributing to this imprecision. In reviewing the use of PCR-based assays in aerosol science, Peccia & Hernandez (2006) identified reagent and operator variability as factors, along with inconsistency in data analysis, and noted that all these factors need to be controlled or accounted for. Hospodsky et al. (2010) found qPCR instrument repeatability, which decreased (i.e. variability increased) as the amount of DNA approached the limit of detection by qPCR, to be the greatest source of variability, and suggested that detection limits of the method be factored into analysis of concentrations of biological material in air samples. Other sources of variability identified by Hospodsky et al. (2010) were efficiency of collecting samples, and extracting spores from the sampling medium and DNA from spores. Inhibition of the PCR is a further potential source of variation. McDevitt et al. (2007) suggested the inclusion of an internal standard control, such as exogenous DNA or reference organisms, when assaying air samples using qPCR, to check for inhibition of the PCR. In the studies reported in this thesis, yeast was used as an exogenous control to check for impaired PCR, whether caused by the presence of inhibitors or because of operator, machine or reagent error. Imprecision from other sources, such as inaccuracies in spore counts, were not measured, due to time constraints but, as noted in Chapter 5, were likely to be greatest when ascospores were few. This imprecision at low spore numbers was also reported by Van de Wouw et al. (2010) in relation to determining the frequency of avirulence alleles in airborne inoculum of *L. maculans* using qPCR. They advised that only samples with more than 50 ascospores be assayed to determine avirulence allele frequencies. Likewise, Karlen et al. (2007) reported that, when using qPCR, cycle thresholds (Section 1.4.3.1) of up to 30 were reproducible, but precision began to be compromised at higher cycle thresholds, i.e. lower DNA concentrations. In the studies reported in this thesis, the PCR was stopped at cycle threshold 40 to avoid generating false negative results. However, it is important to be

aware that quantification of yields of DNA from samples with small numbers of ascospores (less than approximately 20) was likely to be less accurate than from those with larger numbers.

Because of this variability it is suggested that, where limits of detection are important, such as in biosecurity surveillance, quality assurance measures and measures of the variability of the calculated DNA yields be included in the methodology. Use of an internal standard control and duplication of the assays are recommended, and consideration should be given to other measures such as inclusion of spiked samples with known numbers of spores to act as positive controls (Magarey et al., 2008), as well as processing spore trap samples in batches to minimise the error associated with different 'runs' of DNA extractions and of qPCR assays. If samples are to be processed in batches, it is important to determine whether storage of samples will affect the amount of DNA in the spores.

The need to develop protocols for storage of spores collected during surveillance for pathogens of biosecurity significance was noted by Jackson & Bayliss (2011). Few other researchers have investigated the effect of storage of spore trap samples on DNA, as noted in Chapter 4, and so the finding that storage of spore trap samples at -20°C was a suitable system for ascospores of *L. maculans* (Chapter 4) was an important one. It was also encouraging to find that the amount of DNA did not decline during storage of tape samples at -20°C, when comparison of ascospore counts and yields of DNA of samples collected during field studies was made (Chapter 5). Nevertheless, as noted in Chapter 4, this will need to be confirmed for other species and spore types.

7.2 Reliability of the spore trapping system in hot, dry environments

Jackson & Bayliss (2011) identified reliability of the spore trapping system as another key requirement to make it suitable for plant biosecurity. In these studies, the possible impact of weather factors on spore trapping results was investigated. The potential for reduced yield of DNA from spore trap samples collected during periods of extremely hot weather, and the possibility that dust may affect yields, were identified in laboratory studies (Chapter 4) as factors to be considered in assessing qPCR results from the field. In practice, examination of weather data during 2 years of field studies at Kingsford, South Australia (Chapter 5), indicated that the temperature inside the spore trap was unlikely to have risen to levels at which a reduction in DNA in *L. maculans* ascospores

might be expected, as found in the laboratory studies (45°C). Furthermore, there was no indication from the field studies that overall higher temperatures in summer than winter resulted in reduced yield of DNA from *M. pinodes* ascospores. South Australia has a Mediterranean climate, in which high summer temperatures coincide with very low humidity. The impact of high temperature on yield of DNA from spore traps in warm, moist conditions (in which spore germination may occur) requires further investigation, as does the impact of high temperature on other spore types and fungal species. Nevertheless the findings presented here suggest that high temperature is unlikely to be a major complicating factor in analysing results from spore trapping coupled with qPCR assays.

In investigations in which spore trap tapes were spiked with known numbers of conidia (Chapter 4), the presence of dust on the tapes resulted in an increased yield of DNA from spores, which could not be explained by presence of target DNA in the dust. It was speculated that the dust may have assisted in spore disruption and/or removal of conidia from the tapes. This effect may have been a peculiarity of the experimental method used, in which spores were applied to the tapes in the laboratory after the dust had been deposited during operation of the spore traps in the field. The lack of any major increase in yield of DNA from ascospores collected in dusty conditions in the field (Chapter 5), other than small amounts which could be attributed to presence of pathogen material in that dust, suggests that this was indeed the case, and that dust in field samples is unlikely to cause unexpectedly large increases in DNA yield from spore trap samples. On the contrary, dust in field samples can inhibit PCR assays (Driessen, 2005; Magarey et al., 2009), and there is also potential for it to cause a reduction in the number of spores captured onto adhesive surfaces during sampling. There was no indication of either of these effects in field studies reported here, possibly because ascospores of the model pathogens are only released in moist conditions.

7.3 Field comparison with other spore quantification methods

The validation of the DNA results from spore trapping in the field by results from trap plants (Chapter 5) lent further credibility to this methodology as a means of detecting and quantifying aerial inoculum, and of studying disease epidemiology. The methodology has been adopted by a number of researchers worldwide to study various aspects of epidemiology of a range of plant pathogens, including *L. maculans*, as

evidenced by the growing body of literature reporting its use. However, this is the first report of its use to study epidemiology of *D. pinodes*. The findings from this study enabled improvements to both the G1 Blackspot Manager model (Salam et al., 2011a) and the Blackleg Sporacle model (Salam et al., 2003), potentially enabling better prediction of ascospore release for these pathogens.

It is possible that similar epidemiological models can be used in biosecurity to identify the best time of year to detect airborne spores of exotic pathogens. Jackson & Bayliss (2011) noted the importance of knowing when and where to sample for organisms of biosecurity concern, which will vary depending on the organism. In combination with spore traps and PCR assays, predictive models potentially have application in monitoring for pre-determined pathogens in pre-entry quarantine, at high-risk sites such as ports of entry or near borders with other countries (Indonesia and Papua New Guinea in the case of Australia) and during responses to incursions of emergency plant pathogens. Spore trapping coupled with end-point PCR has recently been demonstrated as a useful methodology for delimiting surveys following an emergency plant pathogen incursion (Magarey et al., 2008; Magarey et al., 2009). The further refinement of this methodology by using qPCR offers the potential for gathering additional information on disease epidemiology during such surveys. As demonstrated in this study, the methodology is reliable at identifying changes in aerial inoculum density over time and space, and therefore lends itself well to determining the dynamics of pathogen spread and the timing of key life cycle events. This is important for determining the potential impact of a pathogen in a new environment and for pinpointing where and when control measures are likely to have the greatest effect.

When field data were compared with Blackleg Sporacle model predictions of *L. maculans* ascospore release (Chapter 5), it was assumed that ascospores released in autumn and early winter originated on canola stubble from two seasons before. However, the finding that 7-month-old canola stubble which had been exposed to prolonged dry conditions (and so could be expected still to have ascospores) did not release ascospores immediately upon wetting in late summer and early autumn (Chapter 6), suggested that canola stubble from two seasons before is unlikely to release significant numbers of ascospores without a prior period of moist conditions. Therefore it was just as likely that the ascospores released early in the season, during field studies reported in Chapter 5, originated on stubble from the previous season. When the early

ascospore release data were included in the comparisons (data not shown), the value of root mean squared deviation (RMSD) was reduced (from 0.534 to 0.349), reflecting the smaller overall deviations of field data from predictions early in the season compared with later in the season. However, the set of parameter values which gave the lowest RMSD remained unchanged, indicating that the model calibration still held true.

7.4 Potential modifications of the system to facilitate biosecurity surveillance

The small volume of air that is sampled by a Hirst-type volumetric spore trap (10 l/minute) is a limitation of the methods reported here when considering biosecurity surveillance (Jackson & Bayliss, 2011). Increasing the volume of air sampled would increase the likelihood of detection of target pathogens in air samples, whether present as airborne spores or, as discussed in Chapter 6, in dust generated from an infested crop at harvest time. This issue could potentially be addressed through application of qPCR assays to samples collected using higher volume samplers, such as the ionic spore trap (DS Scientific, Louisiana), or the Coriolis δ cyclonic air sampler (Bertin Technologies, France), both of which are able to sample more than 600 l/minute of air. There may also be potential to use unmanned aerial vehicles to sample air over greater distances, and at altitudes at which the samples may be more representative of a larger area, than is feasible with a ground-based trap (Jackson & Bayliss, 2011). Spore samplers mounted on unmanned aerial vehicles have been used to sample spores of plant pathogens using simple devices consisting of Petri dishes with selective media, from which microorganisms were subsequently cultured (Techy et al., 2010; Schmale et al., 2008). Mounting four such devices on an unmanned aerial vehicle enabled the sampling of 32 m^3 of air per minute (32,000 l/minute) (Schmale et al., 2008). The logistics of applying qPCR assays to samples collected from such large volumes of air would require further investigation. In particular, the potential for inhibition of the PCR assay would need to be considered as there are likely to be more extraneous particles. This could potentially be investigated using an exogenous control, such as was used in the studies reported in this thesis.

If the logistics of sampling larger volumes of air, and of detecting DNA in such air samples were resolved, then the opportunity to detect pathogen DNA in dust generated at harvest of an infested crop could further increase the scope of biosecurity surveillance, discussed in Chapter 6.

Another limitation of the use of PCR in conjunction with spore trapping, which is likely to be important if applying the methodology for biosecurity purposes, is that it does not normally enable determination of spore viability. It may be possible to overcome this by using reverse-transcriptase PCR (RT-PCR), to detect RNA, which is shorter-lived than DNA, and is therefore more indicative of cell viability (West et al., 2008). However, significant improvements in stabilising RNA will be needed to make this a practical field technique for the detection of pathogen RNA from spore trap samples. Another means of determining viability of the inoculum is to culture spores prior to performance of PCR assays. Providing suitable culture media are available, and quantification is not important, this can be used as a means of both increasing the amount of inoculum and determining its viability (Schaad et al., 1999; Ward et al., 2004).

The need to transport samples to the laboratory is also be an important consideration in biosecurity, as movement of potentially viable inoculum from within a quarantine zone may be prohibited or strictly controlled. Portable thermocyclers (PCR machines) have been developed, which could potentially enable the rapid establishment of a PCR diagnostic facility within a quarantine zone, and the cost of such PCR technology is diminishing rapidly (Schaad et al., 2003; West et al., 2008). Depending on the type of spores in question, DNA extraction equipment may need to include a centrifuge (15,000 x g) and, potentially, a homogeniser such as a FastPrep® machine. In some circumstances, it may be more practical to use secure systems to transport samples to an existing PCR diagnostic facility (see www.crcplantbiosecurity.com.au/sites/all/files/packagingbrochure.pdf).

In the studies reported here, qPCR assays were applied for the simultaneous detection of three model pathogens but there is potential for increasing that number through the use of multiplex assays, which use multiple primer sets within a single PCR mixture. Spore traps could therefore be used to monitor for a large number of exotic pathogens within a single crop or cropping system. In Australia such a system might be used to monitor for important cereal pathogens of biosecurity concern, such as those causing karnal bunt (*Tilletia indica*) and cereal rusts (*Puccinia* spp.; exotic strains or species), a number of which, e.g. *Puccinia graminis* f. sp. *tritici* pathotype Ug99, have been identified as high risk biosecurity threats (www.planthealthaustralia.com.au/go/phau/biosecurity/grains). While the advantages of multiplexing are clear, it must be borne in mind that less DNA

template would be available for each target, which would reduce the sensitivity of the qPCR assays. The timing of spore release may also limit the application of multiplex PCR assays. Nevertheless, this is an area of research which may hold promise for biosecurity surveillance.

7.5 Conclusion

The good match between results of spore trapping coupled with qPCR assays, and those obtained using trap plants, as well as predictions of ascospore release generated by epidemiological models, indicated that this methodology is a useful tool for epidemiological studies. The improved methodology was shown to be sensitive, specific and robust in a range of environmental conditions, and was used to improve epidemiological models for use in disease management. Such models also have potential for use in determining where and when to survey for pathogens of biosecurity concern. Spore trapping coupled with qPCR proved to be a useful tool for epidemiological studies, with good prospects for use in biosecurity surveillance.

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9 APPENDICES

Appendix 1 Media for culturing fungal isolates

Water agar

Bacto-agar (Difco)	20 g
RO (reverse osmosis) water	1,000 ml

Potato dextrose agar (PDA)

Potato dextrose agar (Oxoid)	39 g
RO water	1,000 ml

Quarter strength potato dextrose agar (¼ PDA)

Potato dextrose agar (Oxoid)	10 g
Bacto-agar (Difco)	10 g
RO water	1,000 ml

Coon's agar (Ali et al., 1978)

Maltose (Sigma)	4 g
Potassium dihydrogen phosphate (KH_2PO_4)	2.68 g
Potassium nitrate (KNO_3)	2 g
Magnesium sulphate ($MgSO_4$).$7H_2O$	1.2 g
Distilled water	1,000 ml
Agar (Oxoid no. 3)	20 g

Autoclave culture media at 121°C for 20 minutes. Dispense agar media into 9-cm diameter Petri dishes, approx. 15 ml per plate, and allow to cool and solidify.

Appendix 2 Spore tape preparation

Dissolve Tanglefoot® adhesive (product number E95113, Australian Entomology Supplies, NSW, Australia) in hexane in a fume hood at a rate of 1.2 g Tanglefoot® per ml hexane. Place the spore trap drum on a Burkard roller frame, and fix a length of Melinex tape to the drum using a short section of double-sided adhesive tape to hold it in place. Apply the adhesive mixture using a number 20 S&S Flat Taklon paint brush (510FT), as follows: Apply six to 12 evenly-spaced dabs of adhesive, then rest the brush flat on the tape surface while rotating the drum once, thus ensuring all of the tape is evenly covered. Allow the tape to dry in the fume hood for 20 minutes before replacing in the drum case.

Appendix 3 Recipes for stains and slide mountants

Aniline blue (0.1%) in lactoglycerol

RO water	24 ml
Lactic acid	20 ml
Glycerol	20 ml
Aniline blue in ethanol (0.5%)	16 ml

Mix RO water with lactic acid and glycerol. Add aniline blue in ethanol. Shake well. Store at room temperature.

Aniline blue (0.1%) in Mowiol (Lacey & West, 2006)

Mowiol	35 g
RO water	100 ml
Glycerol	50 ml
Phenol (optional)	2 g
Aniline blue	0.16 g

Put Mowiol and phenol in 80 ml RO water and leave to stand overnight. Add glycerol to the mixture and heat in water bath or warm gradually in microwave and stir to mix. Dissolve aniline blue in 20 ml RO water, add to the Mowiol mixture during the heating process.

Aniline blue (0.2%) lactoglycerol in Mowiol

Mowiol	35 g
RO water	80 ml
Glycerol	40 ml
Lactic acid	30 ml
Aniline blue	0.32 g

Put Mowiol in 70 ml RO water and leave to stand overnight. Add glycerol and lactic acid to the mixture and heat in a water bath or warm gradually in microwave and stir to mix. Dissolve aniline blue in 10 ml of RO water, add to the Mowiol mixture during the heating process.

Trypan blue (0.1%) in Mowiol with phenol (Lacey & West, 2006)

Mowiol	35 g
RO water	100 ml
Glycerol	50 ml
Phenol	2 g
Trypan blue	0.16 g (0.32 g for 0.2%)

Put Mowiol and phenol in 80 ml RO water and leave to stand overnight. Add glycerol to the Mowiol mixture and heat in water bath or warm gradually in microwave and stir to mix. Dissolve trypan blue in 20 ml RO water, add to the Mowiol mixture during the heating process.

Appendix 4 Relative advantages and disadvantages of stains and mountants

No.	Stain/mountant	Advantages	Disadvantages
1	0.1% aniline blue in lactoglycerol (Driessen, 2005)	Stained spores bright blue.	Did not set. Stain ran off the tape segment. Difficult to get coverage of whole tape. Required sealing with nail polish to prevent stain leaking during storage.
2	0.1% aniline blue in Mowiol without phenol	Set overnight.	Did not stain spores.
3	0.1% aniline blue in Mowiol with phenol	Set overnight.	Did not stain spores well. Phenol is toxic (requires use of fume hood when preparing stain and slides).
4	0.2% aniline blue in Mowiol with phenol	Set overnight.	Did not stain spores well. Phenol is toxic.
5	0.2% aniline blue lactoglycerol in Mowiol	Set after several days.	Stain precipitated, leaving particles which partially obscured spores.
6	0.1% trypan blue in Mowiol with phenol (as per Mowiol recipe)	Set overnight. Spores expected to be preserved by phenol. Spores are visible.	Did not stain spores well. Phenol is toxic.
7	0.2% trypan blue in Mowiol with phenol	Set overnight Spores expected to be preserved by phenol. Spores were visible (stained pale blue)	Did not stain spores brightly. Phenol is toxic.

Appendix 5 Experiments which did not lead to improvement in DNA assays

Experiment 15

The aim of this experiment was to determine if the difference in yield of DNA from spores on tape and spores in suspension could be eliminated by cutting the tapes in half. It was found that there was no advantage in cutting spore trap tapes prior to insertion into bead tubes for DNA extraction, compared with leaving tape segments intact.

Experiment 16

This was a pilot experiment to compare yield of DNA and consistency of results between two DNA extraction procedures; Method 1 (Appendix 6) and a method based on MicroLYSIS® (Microzone) (Rogers et al., 2009). The two DNA extraction methods were applied to ascospores of *L. maculans* which had been deposited directly onto segments of Melinex tape and counted before DNA was extracted and quantified. Using Method 1 (Appendix 6) the yield of DNA was variable, ranging from 128 to 998 fg/ascospore, and there was no significant positive-slope linear relationship between yield of DNA and number of ascospores. Using the MicoLYSIS® method the results were more consistent (range 22-96 fg/ascospore), with a significant linear relationship between yield of DNA and number of ascospores ($P < 0.01$; $R^2 = 0.97$), but the yield of DNA (mean 38 fg/ascospore) was considerably less than that obtained using Method 1 (mean 375 fg/ascospore). The low yield obtained using the MicoLYSIS® method was thought to be related to poor recovery of lysate (20–30 µl out of 60 µl of MicroLYSIS) following the homogenisation (bead-beating) step. The experiment was repeated to determine if higher yields were attainable by recovering a larger volume of lysate after the homogenisation step.

Experiment 17

This was a follow-up to Experiment 16 to compare DNA yield and consistency of results between two DNA extraction procedures; Method 1 (Appendix 6) and a method based on MicroLYSIS® (Microzone) (Rogers et al., 2009). The methods were the same as for the previous experiment. Several of the samples from which DNA was extracted using Method 1 (Appendix 6) yielded no DNA; the mean yield of DNA from the remaining three was 31 fg/ascospore. Samples from which DNA was extracted using

the MicroLYSIS® method yielded between 24 and 174 (mean 63) fg/ascospore. There was no significant linear relationship between ascospore numbers and DNA yield ($P < 0.05$) for either of the treatments. Thus neither of the methods resulted in consistent DNA yields. For the MicroLYSIS® method this may have been partly due to variability in the amount of lysate recovered after the bead beating step (30–50 µl). For Method 1 (Appendix 6) it may have been due to inconsistent disruption of ascospores during the homegenisation step. Yeast was not included in this experiment because the MicroLYSIS® method did not lend itself to addition of yeast. Therefore it was not possible to adjust results to account for variations in DNA extraction efficiency between samples. The MicroLYSIS® method gave only slightly higher DNA yields in this experiment than from the previous experiment, although a larger amount of lysate was removed following the bead beating step (30–50 µl out of 60 µl of MicroLYSIS compared with 20–30 µl in the previous experiment).

These results indicated that there was no advantage in using the MicroLYSIS® method over the modified PowerPlant® method.

Appendix 6 Protocols for DNA extraction from spore trap samples

Method 1. PowerPlant® DNA Isolation Kit (MoBio Laboratories) with modifications

Equipment required

1. Centrifuge for 2 ml tubes (15,000 x *g*)
2. Pipettor (50 µl – 200 µl, 100µl – 1000µl)
3. Homogeniser such as FastPrep® machine (FP120 Savant Instruments, Holbrook, NY, USA) or Precellys® 24 Bead Mill Homogeniser (0.3 ml – 1.8 ml; Part Number: 13-RD000, Omni International, GA 30144, USA).
4. 2 ml bead tube suitable for use in the homogeniser, such as the PowerPlant® bead tube, with ball bearings removed, or 2 ml screw-cap free standing tube (Catalogue No. I2340-00, Scientific Specialities Inc., CA, USA)
5. Ballotini glass beads, 0.85 mm diameter
6. PowerPlant® DNA Isolation Kit (Catalog No. 13200)

Instructions

1. Insert Ballotini glass beads into bead tubes (0.2 g per tube)
2. Insert spore trap tape sample, consisting of a segment of Melinex tape (48 mm x 9.5 mm) with Tanglefoot® adhesive, into the tube in such a manner as to ensure the tape is wound around the inside of the tube with the adhesive facing inwards
3. Pipette 600 µl of B1 extraction buffer (0.2 M sodium phosphate, pH 8.0), with 1 µl/ml Nonidet P40 and 0.05 mg/ml dry yeast (Defiance, NSW, Australia) into the tubes
4. Homogenise samples using either a FastPrep® machine or Precellys® Homogeniser at 6,000 rpm for two periods of 40 seconds with 2 minutes cooling on ice in between
5. Follow the standard protocol for the PowerPlant® DNA Isolation Kit from Step 7 onwards, as follows:

MoBio Laboratories, Inc.
PowerPlant™ DNA Isolation Kit
Experienced User Protocol

"Please wear gloves at all times

…

7. Make sure the PowerPlant™ Bead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.
8. Transfer the supernatant to a clean 2 ml Collection Tube (provided). **Note**: Expect between 400 to 500µl of supernatant. Supernatant may still contain some plant tissue particles.
9. Add 250 µl of Solution PB2 and invert the tubes to mix the contents. Incubate at 4°C for 5 minutes.
10. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
11. Avoiding the pellet, transfer the entire volume of supernatant to a clean 2.2 ml Collection Tube (provided).
12. Add 1 ml of Solution PB3 and invert the tubes at least 5 times to mix the contents. Incubate at room temperature for 10 minutes.
13. Centrifuge the tubes at room temperature for 15 minutes at 13,000 x *g*.
14. Discard the supernatant and resuspend the pellet in 100 µl of Solution PB6. Note: The tubes do NOT have to be air dried as residual isopropanol will not affect the process.
15. Add 500µl of Solution PB4 and vortex briefly to mix.
16. Load the entire volume (600 µl) onto a Spin Filter and centrifuge at 10,000 x *g* for 1 minute.
17. Remove the Spin Filter basket, discard the flow through, and replace the Spin Filter basket back in the tube.
18. Add 500 µl of Solution PB5 and centrifuge at room temperature for 30 seconds at 10,000 x *g*.
19. Discard the flow through from the 2 ml Collection Tube.
20. Centrifuge again at room temperature for 1 minute at 10,000 x *g*.
21. Carefully place Spin Filter in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution PB5 onto the Spin Filter.
22. Add 50µl of Solution PB6 to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog No. 17000-10).
23. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.
24. Discard the Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20°C to -80°C). Solution PB6 does not contain EDTA."

Method 2. PowerPlant® DNA Isolation Kit with further modifications

As for Method 1 except that samples were frozen for at least 16 hours before commencement of DNA extraction, and 0.6 g Ballotini glass beads were used instead of 0.2 g.

Equipment required

1. Freezer (-20°C)
2. Equipment listed for Method 1

Instructions

1. Freeze samples at -20°C for at least 16 hours
2. Insert Ballotini glass beads into bead tubes (0.6 g per tube)
3. Steps 2-5 of instructions outlined for Method 1