Curtailing and managing exotic fungal spores incursions into Australia

Papori Barua

Master of Biotechnology; Bachelor of Botany, Chemistry & Zoology

This thesis is presented for the degree of Doctor of Philosophy of The University of Western Australia

School of Agriculture and Environment

Plant Pathology/ Biosecurity

2018
Thesis Declaration

I, Papori Barua, hereby declare that this thesis is my own account of my research. This thesis has been substantially accomplished during enrolment in the degree. The content of this research has not previously been submitted for a degree at any tertiary educational institution.

No part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of The University of Western Australia and where applicable, any partner institution responsible for the joint-award of this degree.

This thesis does not contain any material previously published or written by another person, except where due reference has been made in the text.

The work(s) are not in any way a violation or infringement of any copyright, trademark, patent, or other rights whatsoever of any person.

This thesis contains published work and/or work prepared for publication, some of which has been co-authored.

Name

Date: 11/03/2019

Papori Barua
Dedication

I dedicate this thesis to my dearest husband the late Dr. Pranjal Bora who was and remains my constant source of motivation and support while I was undertaking this work and through my life.
Abstract

Fungal pathogens are a major cause of yield losses across agricultural crops and to the associated industries. Some fungi are additionally important as they can produce mycotoxins, secondary metabolites that can cause diseases in human and animals. Fungal spores are an integral part of the fungal life cycle and are mainly adapted for dispersal and need to remain dormant to survive unfavourable environmental conditions. Further, fungal spore dispersal plays a critical role in disease introduction, spread and establishment. While fungal spores may be primarily dispersed by air or rain splash, dispersal can also occur through human activities allowing pathogens to spread locally or to new geographic areas. In the era of increased personal and business travel, military deployments and exercises, sporting events and trade in commerce, the risk of inadvertent introduction of exotic fungal pathogens particularly by spores via introduction of contaminated equipment and commercial goods into a country or an area is increasing. While pathogens may get introduced through flower, seed, plant product imports, machinery movement and natural phenomena, these are not the only pathways. The rise in global movement of people and commodities, particularly from regions or countries posing a high biosecurity risk, increases the likelihood of incursion(s) of new fungal pathogens into new areas via spore contamination of biological and/or inert ‘carrier materials’.

Long-lasting viable fungal spores are one of the critical aspects in the spread of pathogenic fungi. While there are several detection methods available for the detection of fungal spores, their detection on potential ‘carrier materials’ had remained a challenge due to lack of a rapid reproducible method for assessing spore viability. In response to this, I first developed a rapid and miniaturized system using Alamar Blue (resazurin dye; 7-hydroxy-3H-
phenoxazin-3-one 10-oxide) for assessing fungal spore viability, using the ascomycete *Leptosphaeria maculans* (cause of blackleg disease on canola) as a ‘model pathogen’. This assay is dependent on the metabolic activity of viable fungal spores to convert the dark blue of resazurin (maximum absorbance 605nm) into the pink colour of resorufin (maximum absorbance 573nm). This assay is more rapid and reproducible, with detection of viable spores within two hours. The Alamar Blue assay uses an optimised microtitre based format that was far superior for determining fungal spore viability in comparison with current conventional techniques such as trypan blue staining, the TC10 cellometer cell counter, or by assessing germination of the spores under a microscope. The successful application of the Alamar Blue assay to measure fungal spore viability in my studies has important benefits for biosecurity operations, by enabling faster and reliable confirmation of viability of potential invasive exotic fungal pathogens, enhancing the ability to impede potential consequent disease outbreaks. This assay was further validated by assessing the viability of spores from three other diverse fungal genera, viz. plant pathogens of rice (*Magnaporthe oryzae*), wheat (*Puccinia striiformis* f. sp. *tritici*), and subterranean clover (*Kabatiella caulivora*) and also a yeast (*Saccharomyces cerevisiae*). The effectiveness of the Alamar Blue assay was confirmed by successfully determining the relative retention times of viable *L. maculans* ascospores, conidia/resting hyphae of *K. caulivora* and urediniospores of *P. graminis* f. sp. *tritici* across a range of different potential spore-carrier materials, including steel, fabric, wood, paper, rubber and leather, over a time period of 365 days.

Viability of three different fungal pathogen spores on a range of materials, including metals, fabrics, woods and plastics under different temperature conditions of 23°C/4°C, 36°C/14°C, and 45°C/15°C day/night was assessed. All
tested materials were not only effective carriers that maintained long-term viability of ascospores of *L. maculans*, urediniospores of *P. graminis* f. sp. *tritici* and conidia/resting hyphae of *K. caulivora*, but at higher temperatures than previously known. There were significant (*P*<0.001) differences between carrier materials and between temperature regimes in terms of fungal spore and resting hyphae survival. Further, viability of spores on these materials was also dependent on the type of fungal spore.

At the lowest temperature regime (23°C/8°C day/night) urediniospores of *P. graminis* f. sp. *tritici* remained viable on these materials for up to 365 days, while both ascospores of *L. maculans* and conidia and resting hyphae of *K. caulivora* remained viable for up to 240 days. *K. caulivora* conidia and hyphae became thick walled and melanised with time. At 23°C/4°C day/night, these melanised conidia maintained viability on steel, corrugated steel, galvanised steel and all tested fabrics, wood and random mixed materials for up to 240 days. Similarly, *L. maculans* ascospores remained viable for up to 240 days, but only on Tasmanian oak (*Eucalyptus regnans*) and pine wood (*Pinus radiata*). In contrast, urediniospores of *P. graminis* f. sp. *tritici* were viable on aluminium, paper, rubber, all fabric and all woods for up to 365 days. At 36°C/14°C day/night, *K. caulivora* conidia and resting hyphae remained viable for up to 240 days on leather, paper, plastic and all the fabric and wood materials. While at 36°C/14°C day/night *L. maculans* ascospores remained viable on pine wood for up to 180 days and *P. graminis* f. sp. *tritici* urediniospore for a maximum of 300 days on denim and jute. At 45°C/15°C day/night, *K. caulivora* conidia and resting hyphae remained viable up to 240 days on fleece wool, *Eucalyptus marginata* (jarrah wood) and on paper; *L. maculans* ascospores remained viable up to 60 days but
only on jute; while *P. graminis*. f. sp. *tritici* urediniospores remained viable to a maximum of 180 days on cotton and on jute.

Viability of these spores were assessed by using the Alamar blue assay. Both melanised conidia and resting hyphae of *K. caulivora* recovered even after 8 months were still able to germinate and *K. caulivora* colonies successfully re-established on potato dextrose agar. At least 30% of intact *L. maculans* ascospores recovered after 30 day from inert carrier materials were also able to germinate on artificial growth media. Similarly, *P. graminis*. f. sp. *tritici* urediniospores recovered even after 300 or 365 days from the lower two temperature regimes were able to germinate and successfully initiated infections of wheat seedlings.

Studies were also undertaken into the effect of commonly used commercially available disinfectant and fungicides to inhibit fungal spore germination and to assess their potential use for decontamination of infested carrier materials. *In vitro* studies were undertaken to determine the effects of five chemical fungicide/disinfectant treatments [propiconazole (Tilt 250EC), azoxystrobin (Amistar 250EC), didecyldimethyl ammonium chloride (Sporekill), alkali metal salts of alkylbenzene sulfonic acid and coconut diethanolamide (Farmcleanse), and potassium peroxymonosulfate (Virkon)] in preventing spore germination of *P. graminis* f. sp. *tritici*, *K. caulivora*, *L. maculans* and *Magnaporthe oryzae*. My results showed azoxystrobin and propiconazole to be the most effective with zero spore germination following decontamination treatment of carrier materials. In contrast, didecyldimethyl ammonium chloride had least ability to inhibit spore germination. All chemicals at least partially inhibited germination of spores on the different infested carrier materials although the extent varied with the pathogen and the type of carrier material. Overall, maximum reductions in spore
germination occurred at the manufacturer’s recommended concentration and increasing the fungicide/disinfectant concentration above this rate generally did not significantly reduce spore germination further. My studies highlight the potential for increased use of fungicides, particularly demethylation inhibitor and QoI fungicides, and/or to a lesser extent other disinfectants, to disinfest carrier materials known or likely to be contaminated with fungal spores.

In summary my findings confirmed the critical importance of materials as long-term carriers of viable conidia and resting hyphae, ascospores and urediniospores. My findings not only highlight the potential for spread of the current highly virulent *K. caulivora* Race 2, and the many *L. maculans* or *P. graminis* f. sp. *tritici* races and/or their new pathotypes, across their respective cropping regions inside and outside of Australia via farming equipment, clothing and other carrier materials. My results also highlight the wider biosecurity implications from the transportation of fungal-infested carrier materials previously considered as low-risk. There is clearly an urgent need to re-evaluate potential carrier materials such as metals, plastics, fabrics and woods in regards to the movement of fungal plant pathogens. My research also highlights the potential for using chemical treatments for effective decontamination and disinfestation of transported fungal plant pathogen spores, on or inside of carrier materials, as a means to reduce the biosecurity risk in regards to spore movement on such materials.
## Contents

Thesis Declaration ........................................................................................................... i  
Dedication ......................................................................................................................... iii  
Abstract ............................................................................................................................. v  
Acknowledgements ........................................................................................................... xiii  
Authorship declaration: Co-authored publications ........................................................ xvii  

### CHAPTER 1  
Introduction, Literature Review, Research hypothesis, originality and thesis structure ..... 1  
1.1 Introduction ................................................................................................................ 1  
1.2 Exotic Incursions ....................................................................................................... 2  
1.3 Fungal pathogen invasion and importance ............................................................... 3  
1.4 Survival of fungal Spores ........................................................................................ 5  
1.5 Entry pathways and mode of transportation to the host/hosts ............................... 6  
1.5.1 Aerial dispersal ...................................................................................................... 6  
1.5.2 Rain Dispersal ....................................................................................................... 7  
1.5.3 Carrier materials and dispersal of fungal spores ............................................ 7  
1.6 Detection and viability of fungal spores ................................................................... 9  
1.7 Mitigation and decontamination ............................................................................. 11  
1.8 Historical control, decontamination and management methods ....................... 12  
1.8.1 Historical control methods ................................................................................. 12  
1.8.2 Fungicides and fumigants .................................................................................... 13  
1.9 Selection of pathogens for proposed studies ........................................................... 14  
1.9.1 *Puccinia graminis* f. sp. *tritici* ......................................................................... 16  
1.9.2 *Leptosphaeria maculans* .................................................................................... 18  
1.9.3 *Kabatiella caulivora* .......................................................................................... 19  
1.9.4 *Magnaporthe oryzae* ........................................................................................ 21  
1.10. Studies Described In This Thesis ......................................................................... 22  
1.11. Hypothesis ............................................................................................................. 23  
1.12. Research Aims ....................................................................................................... 23  
1.13. Originality and significance of the project ......................................................... 23  
Bibliography .................................................................................................................... 27  

### CHAPTER 2  
A rapid and miniaturized system using Alamar blue to assess fungal spore viability: implications for biosecurity ................................................................. 41
CHAPTER 3
Inert Materials as Long-term Carriers and Disseminators of Viable Leptosphaeria maculans Ascospores and Wider Implications for Ascomycete Pathogens ........................................................................................................55

CHAPTER 4
Long-term viability of the northern anthracnose pathogen, Kabatiella caulivora, facilitates its transportation and spread ...........................................................................63

CHAPTER 5
Extended survival of Puccinia graminis f. sp. tritici urediniospores: implications for biosecurity and on-farm management .................................................................75

CHAPTER 6
Disinfestation of diverse fungal pathogen spores on inert contaminated materials: biosecurity implications ...........................................................................................................85

CHAPTER 7
7.1. General Discussion ..........................................................................................126
7.1.1. Rapid Fungal spore Viability Assay ..............................................................126
7.1.2. The role of inert materials as way to spread natural spore inoculum to new areas ..................................................................................................................129
7.1.3. Challenges in removal of fungal spore contamination ..............................132
7.1.4. Effect of temperature on spore viability over time .................................133
7.1.5. Relevance of extended spore survival .....................................................135
7.1.6. Relevance of subsequent germination and host infection from surviving spores .........................................................................................................................135
7.1.7. Chemical decontaminants ..............................................................136
7.2. Conclusion and the future ..............................................................................138

Bibliography .......................................................................................................141
Appendix ...........................................................................................................147
Presentations, Proceedings, Media Release ........................................................147
Acknowledgements

I wish to express my deepest gratitude to my supervisor, Professor Martin Barbetti for his excellent supervision and for providing me with a challenging and motivating project to develop my own research and analytical skills. It is a great experience to complete my study under the supervision of a great pathologist and respectful scientist, who treats his students respectfully to achieve their goals.

I would like to acknowledge my co-supervisors Dr Ming Pei You, Dr Kirsty L Bayliss and Dr Vincent Lanoiselet for their efforts, guidance and continuous support during my PhD journey. I am particularly grateful to Dr. Ming Pei You for her guidance in statistical analysis.

I am thankful to all my supervisors for their helpful discussions and constructive criticism and showing me how to analyze and compare my work, also for critically evaluating my writing and for improving my presentation skills.

My sincere thanks to Robert Creasy and Bill Piasini in the University of Western Australia Plant Growth Facilities for the technical support and help.

I would also like to thank the facilities, and scientific and technical assistance from the Centre for Microscopy, Characterization and Analysis, University of Western Australia, particularly the wonderful assistance of Lyn Kirilak and Dr Peta Clode.

I am grateful to the scholarship/financial support of the Plant Biosecurity Cooperative Research Centre (PBCRC) Bruce, ACT 2617, Australia, for project CRC62042 and operational funds from the School of Agriculture and Environment, The University of Western Australia.
I would also like to thank some people in the PBCRC, particularly Anthony Buckmaster, Jo Luck, John Austen, Michael Robinson and Tony Steeper, for their helpful discussions and suggestions during the course of my research.

I would also like to thank Professor Simon McKirdy for organizing my internship with Chevron at Barrow Island.

I would like to say a very big thank you to my colleagues and lab buddies particularly Akeel, Azam, Hebba, Dolar, Hieu, Margaret, Nireshini, Prodhan, Tamsal, Xiangling and Yupin for providing a fun, intellectual and a safe laboratory environment to play and work in!

My sincere thanks also goes to Dr. Wayne Reeve for his constant support and ideas, also for helping to cheer me up when needed!

A very big thank you to my friends Dr. Leila Eshraghi, Dr. Nader Aryamanesh and Dr. Margaret Gallagher for their constant motivation.

My heartiest thanks and appreciation to all the members of Assam Association of Western Australia for providing me all the support and looking after me as a family at the hardest time of my life away from my home here in Perth.

A very big thank you to Chandan, Farhan, Himankar, Mithi, Munmi, Nayan, Nivedita, Reetam, Sabiha, Sangeeta, Sanghamitra, Tanveer and Vivek for being my constant support.

A very big thank you to my friends Dr. Rebecca Swift, Dr. Rui Tian, Suraiya Bajwah and Sucharita Ravi for their constant motivation, being there for me and for giving me the much needed company.

A very big thank you to my friend Mr. Arun Gaur for all his encouragement and support.
A very big thank you to my parents in-law - Ma, Papa and brother in-law Preetam for giving me inspiration.

My sincerest gratitude to my sister Aparajita (Apu), my brother in-law Rantu and my nephew Progyan for your constant love and inspiration.

Finally I would like to acknowledge my dearest husband the late Pranjal Bora for his constant love and support. This day would not have arrived without the enormous support and constant inspiration from you. I do not have enough words to say how fortunate I feel and grateful to God that I had you as my life partner.

This research was supported by an Australian Government Research Training Program (RTP) Scholarship.
Authorship declaration: Co-authored publications

This thesis contains work that has been published.

Details of the work:

Location in thesis: Chapter 2

Student contribution to work:
Experimental design, work, data collection, record and analysis of the data and writing.

Co-author signatures and dates:

<table>
<thead>
<tr>
<th>Co-author</th>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Martin Barbetti</td>
<td></td>
</tr>
<tr>
<td>Ming Pei You</td>
<td></td>
</tr>
<tr>
<td>Kirsty L Bayliss</td>
<td></td>
</tr>
<tr>
<td>Vincent Lanoiselet</td>
<td></td>
</tr>
</tbody>
</table>
Details of the work:
Leptosphaeria maculans Ascospores and Wider Implications for
Ascomycete Pathogens Plant Disease 102, 720-726.

Location in thesis: Chapter 3

Student contribution to work:
Experimental design, work, data collection, record and analysis of the data and writing.

Co-author signatures and dates:

<table>
<thead>
<tr>
<th>Name</th>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Martin Barbetti</td>
<td></td>
</tr>
<tr>
<td>Ming Pei You</td>
<td></td>
</tr>
<tr>
<td>Kirsty L Bayliss</td>
<td></td>
</tr>
<tr>
<td>Vincent Lanoiselet</td>
<td></td>
</tr>
</tbody>
</table>
Details of the work:


Location in thesis: Chapter 4

Student contribution to work:
Experimental design, work, data collection, record and analysis of the data and writing.

**Co-author signatures and dates:**

<table>
<thead>
<tr>
<th>Name</th>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Martin Barbetti</td>
<td>![Signature]</td>
</tr>
<tr>
<td>Ming Pei You</td>
<td>![Signature]</td>
</tr>
<tr>
<td>Kirsty L Bayliss</td>
<td>![Signature]</td>
</tr>
<tr>
<td>Vincent Lanoiselet</td>
<td>![Signature]</td>
</tr>
</tbody>
</table>
Details of the work:


Location in thesis: Chapter 5

Student contribution to work:
Experimental design, work, data collection, record and analysis of the data and writing.

**Co-author signatures and dates:**

- Martin Barbetti
- Ming Pei You
- Kirsty L Bayliss
- Vincent Lanoiselet
Details of the work:

Location in thesis: Chapter 6

Student contribution to work:
Experimental design, work, data collection, record and analysis of the data and writing.

**Co-author signatures and dates:**

<table>
<thead>
<tr>
<th>Co-author</th>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Martin Barbetti</td>
<td></td>
</tr>
<tr>
<td>Ming Pei You</td>
<td></td>
</tr>
<tr>
<td>Kirsty L Bayliss</td>
<td></td>
</tr>
<tr>
<td>Vincent Lanoiselet</td>
<td></td>
</tr>
<tr>
<td>Student signature:</td>
<td>Coordinating supervisor signature:</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Date: 11/03/2019</td>
<td>Date: 11/03/2019</td>
</tr>
</tbody>
</table>

I, Professor Martin Barbetti certify that the student statements regarding their contribution to each of the works listed above are correct.
CHAPTER 1

Introduction, Literature Review, Research hypothesis, originality and thesis structure

1.1 Introduction

Australia being an island has a unique biodiversity which makes the local flora more vulnerable to exotic pests and diseases (Roger 2010). The agriculture and forestry industries contribute an important share to Australia’s economic and social prosperity. The increase in global movement of people and different materials, particularly from the areas with high biosecurity risks, increases the likelihood of incursion of new fungal pathogens via spore contamination into Australia. Such exotic introductions remain a threat to Australia’s agriculture, forest industries and biosecurity.

The term biosecurity is used widely and in different contexts. The Panel of experts chaired by Roger Beale (2008) defined biosecurity as “the protection of the economy, environment and human health from the negative impacts associated with entry, establishment or spread of exotic pests (including weeds) and diseases” (Roger 2010). Disease outbreaks from exotic introductions, including even deliberate introductions or “bioweapons”, could cause the extinction of endangered native flora species, the destruction of genetic diversity in farm plants and the productivity of major food crops, even to the extent where they could be a threat to traditional human livelihoods (Cariappa et al. 2002; Dudley and Woodford 2002). Of particular concern today is that plant pathogens could easily be deliberately introduced in
different locations by terrorists or criminals to initiate multiple and concurrent outbreaks of crop disease (Wheelis et al. 2002).

1.2 Exotic Incursions

An assessment of exotic incursions into Australia over the 25 years to 1996 revealed that rate of incursions of plant pests and diseases were 10 times greater than in animals (Nairn et al. 1996). This led to recommendations to increase monitoring, improve awareness, inspection of vessels, and review traveller’s statements i.e. whether going to a farm, camping, outdoor activities etc., and increased inspection of cargo. The probable issues of dangers by land-bridging, which involves movement of shipping and road containers between metropolitan areas before quarantine clearance, have also been raised by Nairn et al (1996) and they recommended that all containers should be subject to thorough external inspection at their port of entry as a minimum.

With rapid globalisation, the risks of introduction of fungal pathogens, particularly by means spore contamination, are increasing. While this vulnerability cannot be removed, effective response measures to minimize the degree of damage from naturally and/or intentionally introduced diseases (Wheelis et al. 2002) need to be improved. The Australian Quarantine Review Committee has made clear recommendations on preparedness and response in relation to incursions by establishment of critical plant diagnostic laboratories, secure post-entry quarantine facilities, and by highlighting funding options needed for a national secure containment facility for plant pests and diseases (Nairn et al. 1996). Development of new diagnostics, vaccines and sensing technologies for early identification of plant disease outbreaks has fostered improved preparedness for disease outbreaks and
their impact, both essential components of plant health surveillance and response systems (French et al. 2007; Wheelis et al. 2002). However, to identify and respond to new and unknown diseases, high level epidemiologic skills, surveillance methods and better resources are required (Cariappa et al. 2002).

1.3 Fungal pathogen invasion and importance

There have been well documented reports of exotic fungal pathogen invasions into Australia. One example was the wheat stripe rust pathogen (*Puccinia striiformis* f. sp. *tritici*) which was first detected in Australia in 1979 (O’Brien et al. 1980). A study of the initial pathotype of the stripe rust suggested that it was of European origin (Wellings 2007; Wellings et al. 1987). Another exotic pathogen invasion was *Phytophthora cinnamomi*, which has infested natural ecosystems, including the Jarrah forests (*Eucalyptus marginata*) of Western Australia, and continues to be a threat to many other Australian native plants and to the survival of some ecosystems (Anderson et al. 2010; Brandis 1983; Dell et al. 2007). Another is sugarcane smut caused by the fungus *Ustilago scitaminea*, which is primarily spread by air-borne spores and infected cuttings and was first reported in Australia in the Ord River Irrigation Area (ORIA) of Western Australia in July 1998 (Croft and Braithwaite 2006) and is now present in all major Australian sugarcane growing regions. More recently *Puccinia psidii* (myrtle rust) was reported in New South Wales in April 2010 (Carnegie and Cooper 2011) and has since then spread to Queensland and Victoria (Pegg et al. 2014). Most of these pathogenic fungi are the result of exotic pathogen invasion into Australia (Mireku and Simpson 2002).
Fungal pathogens are a major cause of yield losses across agricultural crops (Khangura et al. 2007; Wellings 2011) and their associated food industries (Bebber and Gurr 2015; Savary et al. 2012). Some fungal pathogens have the ability to completely destroy crops, for example, *Puccinia graminis* (wheat stem rust) (Fisher et al. 2012), and to severely disrupt natural ecosystems, for example *Ophiostoma ulmi* (Dutch elm blight) (Hubbes 1999) and *Phytophthora ramorum* (sudden oak death and ramorum blight) (Grünwald et al. 2008). Fungal pathogen can not only disturb the natural ecology but also can impact in the human welfare and the society. The classic example is the Irish potato famine caused by the pathogen *Phytophthora infestans*, which not only caused a loss of more than 1 million human lives but resulted in increased crime and led to social instability during 1843-1852 (Austin Bourke 1964; O’Grada 1995).

Exotic fungi are also important as some can produce mycotoxins, secondary metabolites that can cause diseases in human and animals (Tan et al. 2012; Zain 2011). Exposure to mycotoxins generally occurs through the intake of contaminated agricultural products or residues in foods such as meat, milk and eggs, but can also occur through dermal contact and inhalation (Frenich et al. 2011). Examples of effects on humans include Fusarium Head Blight of cereals (Tan et al. 2011a; Tan et al. 2012), exotic yellow rice poisoning (Udagawa and Tatsuno 2004) and ergotism (Wild et al. 1996). These can lead to various ailments such as alimentary toxic aleuria, hepatic diseases, various haemorrhagic syndromes, from associated mycotoxins (Bellemore et al. 1994; Capriotti et al. 2012). Ingestion of wheat contaminated by T-2 mycotoxin caused a severe loss of human lives during the plague of Athens (Bellemore et al. 1994). Examples of effects on grazing animals include
Fusarium contamination of annual medic pods as occurs in Western Australia where sheep refuse to graze toxic pastures (Tan et al. 2011b). To date approximately 500 fungal metabolites, have been reported, many of which are toxic to man and/or animals (Capriotti et al. 2012).

1.4 Survival of fungal Spores

Fungal spores are specialised resistant bodies important in the survival of fungi (Willetts 1971). Spore morphology, structure and chemical composition of the cell wall play an important role in the resistance to stress and hence also survival (Feofilova et al. 2012; Setlow 2006). Resistance of spores to stress is highly variable across different fungi and some fungal ascospores belong to the most stress-resistant eukaryotic cells described to date (Wyatt et al. 2013). Fungal spores are mainly seasonal in their release but many types can be found in the air all year round while others typically peak in the summer (Savary et al. 2012) or late autumn/early winter (Salam et al. 2007; Savage et al. 2012). Also some fungal spores have special rigid cell walls and can survive heat, drought, or exposure to other molecules (Wyatt et al. 2013). For example, thick walled oospores and chlamydospores can survive and can remain dormant for a long period of time under extreme climatic conditions until favourable conditions for infection occur (Browning et al. 2008; Crone et al. 2013). Spore survival is affected by different environmental factors. For example, survival of Peronospora destructor conidia was affected by the rate of dew deposited on the leaves (Hildebrand and Sutton 1984).

Dispersal processes play an important role in the long term survival, they facilitate flow of genetic structure of populations with spatial structure (Bohrer
et al. 2005; Johst et al. 2002; Trakhtenbrot et al. 2005) and help in colonisation of new areas (Isard et al. 2007). The survival of some wind dispersed fungal pathogens depends on the specific time of spore release (Savage et al. 2012). Also, the daily, seasonal and diurnal variation can affect the release and subsequent dispersal of spores by a particular fungal species (Savage et al. 2010; Savage et al. 2011b). The timing of release is determined by the wind conditions encountered by the spore or propagules and hence plays an important role in the survivability and spread of a population, particularly in a fragmented environment (Savage et al. 2011a).

1.5 Entry pathways and mode of transportation to the host/hosts

Fungal spores can be dispersed locally or trans-border by aerial, water (rain splash) or by human activities (McNeill et al. 2011; Savage et al. 2012). Fungal spore dispersal plays a critical role in disease introduction, spread and establishment. Long distance aerial dispersal of plant pathogenic fungal spores spreads diseases across and sometimes between continents (Brown and Hovmøller 2002). Even the release patterns of spores of fungal plant pathogens can be related to their relative abilities to survive during dispersal and to infect new hosts (Savage et al. 2013).

1.5.1 Aerial dispersal

Many fungal plant pathogens are dispersed by air (Nagarajan and Singh 1990; Savary 1986). For example, seasonal, long-distance aerial dispersal of rust spores has been documented for several rust pathogens throughout the world (Aylor 1982; Brown & Hovmøller 2002). Wheat rust spores are primarily wind-dispersed via fresh urediniospores and can be carried several hundred kilometres away (Soubeyrand et al. 2007). Many
fungal pathogens that occur endemically in rotation crops in Australia are also likely to be initiated mainly by airborne ascosporic inoculum (Bathgate and Loughman 2001). Long-distance aerial transport of fungal spores offers an ideal and important avenue for the initial introduction of a pathogen to a new crop, new region or new continent, as well as in the seasonal re-introduction of host-obligate fungi that cannot survive the native season (Aylor 1986, 1999).

1.5.2 Rain Dispersal

Dispersal of spores of a plant pathogen by rain-splash is an efficient mechanism for spreading large numbers of inoculum propagules from a source (Fitt et al. 1989). For example, conidia of the blackleg pathogen Leptosphaeria maculans are readily dispersed via rain splash (Hall 1992). Even aerial dispersed spores can be dispersed by rainfall events, as demonstrated for both P. recondita f. sp. tritici and P. striiformis, even though spores of both of these rust species are primarily dispersed by wind.

1.5.3 Carrier materials and dispersal of fungal spores

The role of different materials in the entry and dispersal of fungal pathogens is extremely important and can largely determine the success or failure of pathogen introductions.

1.5.3.1 Human activities

While fungal spores may be primarily dispersed by wind or rain, dispersal can also occur through human activities allowing pathogens to spread to new geographic areas (Wellings et al. 1987). High possibilities for spread involve plant pathologists and farm workers becoming contaminated with fungal spores during routine farm work, visits to research plots, and surveys of disease where disease incidences are very high. For example, in 1982, an
estimated 70,000 viable rust urediniospores were brought into Australia with the travellers and the Australian Plant Quarantine Service recognised the particular risks of clothing as a means of transporting rusts (Sheridan 1989). The study of initial pathotype of *P. striiformis* f. sp. *tritici* (Pst) initially suggested it to be of European origin, and later studies provided evidence that it was most likely transmitted as spores trapped on traveller’s clothing (Wellings 2007; Wellings et al. 1987). However, the process of transportation of the fungal spores from clothes to the crop hosts still remains unclear.

In general, data regarding the role of different materials as fungal spore carriers remains very limited. The proposed study in this PhD of different materials as fungal spore carriers will increase the understanding of fungal spore entry pathways into Australia and will help in the development of mitigation programmes and decontamination processes for biosecurity. My PhD study is mainly focussed on the fungal spore dispersal consequent to human activities.

### 1.5.3.2 Paper and other fibres

A wide range of different fibres are used in day to day life, e.g., book, gifts, wrappers, packing etc. Different fibre materials can easily become contaminated by fungal spores. This is illustrated by fungal contamination as one of the major problems for libraries leading to irreversible damage of the paper (Rakotonirainy et al. 2003). Fungi can also contaminate packaged food and beverages, and their contaminating packing materials by producing TCA (2, 4, 6-trichloroanisole) is responsible for much of the musty/muddy off-flavour of contaminated food (Miki et al. 2005; Tindale 1987). Tindale et al. (1989) isolated 31 species of fungi from fibreboard cartons, paper sacks and jute
sacks. Further, 38 species of fungi have been isolated from the timber flooring of six general-purpose freight containers (Hill et al., 1995) and included a variety of genera such as *Alternaria* (1), *Aspergillus* (7) *Cladosporium* (2) *Eurotium* (4), *Mucor* (1), *Paecilomyces* (1), *Penicillium* (16), *Phoma* (1) *Trichoderma* (4) and *Ulocladium* spp.

### 1.5.3.3 Other inert metals

Metals also could be potential carriers of fungal spores. Much of the equipment used in day to day life is made of metal, including farming tools, vehicles, shipping containers and their cargos that could allow fungal spores from a disease infested part of the world to enter Australia. Although there has been some into carrier materials; most investigations were related to living materials such as plant germplasm, fruits, seeds and pollen (Vanneste et al. 2011) rather than inert materials. Current data regarding materials such as metal, plastics, and paper etc. as spore carriers is extremely limited. The relative risk of incursion of pathogenic fungal spores with different materials needs urgent assessment as such data is critical in enabling biosecurity and quarantine sectors to take appropriate precautions and action and this is a focus of this PhD study.

### 1.6 Detection and viability of fungal spores

To develop new and robust effective management processes for managing spore incursions into Australia, it is required to develop methods for rapid detection of the presence of fungal pathogen spores on different materials that can act as possible carriers. Detection of propagules of plant pathogenic fungi is an important component not only of quarantine efficiency but also of effective disease management in general (Miller 1996). Most of the
aerially transported spores dispersed from the source region remain within the mixed layer of the atmosphere before descending back to the ground (Clarke et al. 1983). The survivability of spores in the atmosphere depends on the temperature, relative humidity and significantly on the UV radiation (Aylor 1986). Spore viability is generally monitored by assessing germination of the spores. However, spore germination can be time consuming and not all fungal spores readily germinate, hence rapid viability tests are required (Yu et al. 1984). Moreover, conventional cell counting methods are challenging when there is a cell-clumping issue. There have been various assays developed to determine the viability of different cells in vitro, for example, tetrazolium salt to determine the number of viable organisms (Kairo et al. 1999), and an ATP assay (measurement of Adenosine tri phosphate by using bioluminescence) as the ATP status of the cells reflects their viability (Maehara et al. 1987). Zill et al. (1988) demonstrated that an ergosterol assay can be adapted to identify the viability and growth of fungi. Further, there are other biomarkers which could be used to viability test, including NADH, LDH, capases, live and dead cell proteases (www.promega.com). Cell or spore viability can also be monitored by using dye exclusion and dye uptake methods and fluorescent staining methods. However, dyes can sometime enter and destroy cells with broken cell walls and fluorescence interference from test compounds can occur (Strober 2001). DE Bolle et al. (2011) used a BacTiter-GloTM Assay to detect the viability of spores of three fungal species: Botrytis cinerea, Aspergillus niger and Mucor piriformis. The Alamar Blue bioassay has also been used to assess cell viability and cytotoxicity in an extensive number of cell types including bacteria, yeast, fungi (hyphae), protozoa and cultured mammalian and piscine cells (Rampersad 2012). Flow cytometry is also often
used to check viability in bacteria and yeasts, but little work has been done with fungal spores (Mesquita et al. 2012). Real time PCR protocols have been also used for the detection of fungal spores (Zhao et al. 2007). For example, Barnes and Szabo (2007) developed four real time PCRs for independent detection of the cereal and grass rust pathogens *Puccinia graminis*, *P. recondita* f. sp. *secalis*, *P. striiformis*, and *P. triticina* using Taqman probes. Although there are many potential methodologies as illustrated above, all have limitations, particularly in terms of rapid detection methods for viable fungal spores.

From reviewing this literature it is evident that there is not only a gap of information regarding the variable means of transportation of viable fungal spores to the host or hosts from different infested carrier materials to cause an infection, but that the methods *per se* for detecting spore viability require further investigation and to be made with a more fungal pathogen-specific focus. These areas need to be investigated and new methodologies to be developed if we are to better apply methodologies to better assess and understand the risks in the field in relation to biosecurity for different individual fungal pathogens. Addressing these gaps are also a key focus of my PhD studies.

### 1.7 Mitigation and decontamination

“Successful integrated pest management strategies depend on an accurate evaluation of ‘immigrant’ inoculum coming into a managed area” (Aylor 1999). For successful control and eradication of a crop pathogen, the processes of identification and decontamination need to commence relatively soon after introduction, when the concentration of infection is restricted to only
a few infected individuals, and the dispersal distance of spores is short (Wheelis et al. 2002). Eradication of fungal pathogens with multiple hosts and with wind-assisted spore dispersal will be the most challenging, as they can rapidly spread over a large area (Savage et al. 2012). For example, while *P. striiformis* f. sp. *tritici* has a predominant host range of wheat, it also includes certain barley, rye, weedy grasses and triticale genotypes (Holmes and Dennis 1985; Wellings 2007).

### 1.8 Historical control, decontamination and management methods

#### 1.8.1 Historical control methods

In-crop rotation aids in separation of the infested stubble from subsequent crops. Spore production and the extent of aerial dispersal of spores can be reduced temporarily by ploughing infested fields. However, while conventional methods of rotation and destruction of the plant residues can be helpful, infection still can occur due to wind dispersal of spores and from survival of spores in a dormant or resting state. For this reason, use of resistant cultivars over susceptible cultivars is more effective long term (Croft and Braithwaite 2006). Options for management of fungal diseases may include the use of resistant varieties, crop rotation, removal and destruction of infected plant residues and use of fungicides (Khangura and Barbetti 1999). Screening of cultivars for resistance towards a pathogen, importing potential new resistant varieties, and breeding for resistant genotypes within Australia could all be helpful in preparing for any exotic disease outbreaks that eventuate (Croft and Braithwaite 2006).
1.8.2 Fungicides and fumigants

Fungicides are extensively used, particularly in vegetable crops, and can be effective in disease control (Hewitt 1998). However, they are expensive, not environmentally friendly and/or not good for human health, and the timing of application is critical for controlling the disease effectively (Hrelia et al. 1996; Khangura and Barbetti 2005). Many contaminated materials are either fumigated or sprayed with fungicides. Chemicals such as fluquinconazole, flutriafol, and gluphosinate-ammonium were able to effectively delay the development of pseudothecia and decrease in ascospores discharge of *L. maculans* by 100%, 99%, and 96%, respectively, from the canola residues (Wherrett et al. 2003). Also, fungicides benomyl, carbachin + thiram, iprodione, prochloraz, thiabendazole, and tolclofos-methyl were effective against seed borne *L. maculans* on canola under controlled treatments (Kharbanda 1992). However, in the field, foliar-applied fungicides can be much less effective because conidia and ascospores are released throughout the growing season (Barbetti 1975; Kharbanda 1992). Fumigants like methyl bromide have been used in many quarantine situations to disinfest plant and other commodities. While fumigants and some fungicide residuals can be harmful to humans and the environment (Weisskopf et al. 2013), and sometimes may also induce biosynthesis of mycotoxins or other harmful metabolites (Schmidt-Heydt et al. 2013), they are likely highly effective in decontaminating materials contaminated with fungal spores (Liu et al. 2011). Apart from fungicides, many disinfectants are sporicidal and are found to be effective against a wide range of microorganisms. Most of them have chlorine, aldehyde, alkali salts or peroxxygen as their active compounds. Peroxygen compounds have been used as disinfectants and found to be effective against a wide range of micro-
organisms (Hernández et al. 2000). For example, Virkon, a peroxygen formulation, is claimed to provide safe and effective control of all types of known bacteria, fungi, moulds, yeasts and viruses (Broadley et al. 1993). However, while disinfectants are used in surface sterilisation, cleaning vehicles, farm equipment, machines etc., they are predominantly used in hospitals and in laboratories for bacterial spore decontamination (Garcia-de-Lomas et al. 2008; Sagripanti and Bonifacino 1996).

A successful fungal spore control program must include bio-safe management practices, likely along with the use of fungicides and other treatment methods. An effective Integrated Pest Management (IPM) programme of fungal diseases may include the use of resistant varieties, crop rotation, removal and destruction of infected plant residues and use of fungicides (Khangura and Barbetti 1999; Khangura and Barbetti 2002). In my PhD studies I evaluated the effectiveness of five different commonly used commercially available disinfectants and fungicides to inhibit fungal spore germination and assessed their potential for use for effective decontamination of infested carrier materials.

1.9 Selection of pathogens for proposed studies

Many fungal pathogens currently present in Australia cause severe losses of agricultural yield and result in increased expense as a consequence of control measures (Murray and Brennan 2009, 2010; West et al. 2001). Furthermore, many fungi species are serious pathogens to agriculture across multiple hosts (Loope and Uchida 2012), and if introduced into Australia could have a significant adverse impact on several different crop industries. Some such pathogens produce airborne (e.g., rusts and L. maculans) spores that
are wind dispersed over long distances or are very ‘sticky’ water splashed spores (e.g., the clover scorch pathogen *Kabatiella caulivora*), and in all cases can remain dormant for varying periods during adverse weather conditions (Bayliss et al. 2001; Savage et al. 2013). The study of the effectiveness and role of different materials as carriers of these known pathogens above (i.e., *P. graminis* f. sp. *tritici*, *P. striiformis* f. sp. *tritici* *L. maculans*, *K. caulivora*) will provide new understanding and the potential to predict additional likely pathways of entry of the different pathogenic spores. A brief summary of these four important plant pathogenic fungi and the reasons for selecting them as models for use in my PhD studies, is as follows.

Rusts are one of the most destructive plant pathogens. Rusts, classified under Basidiomycetes, are obligate bio-trophic fungi. They are of major economic importance worldwide, causing huge losses of many important crops and also in natural ecosystems (Viljanen-Rollinson and Cromey 2002). The three most important rusts affecting wheat are: stem rust (*Puccinia graminis* f. sp. *Tritici*; *Pgt*), stripe rust (*P. striiformis* f. sp. *tritici*; *Pst*) and leaf rust (*P. triticina*). All three continue to pose the major threat to wheat production across Australia, with major epidemics approximately once per decade; and even in years of smaller epidemics losses suffered by many wheat growers are not sustainable (Brennan and Murray 1988; McIntosh 2007) with an estimated annual loss there of $147 m (Murray and Brennan 2009).

The fungicide application for stripe rust control in Australia alone cost AU$8 million in 1983 and between AU$ 40 to 50 million/year during 2003-2005 (Chen 2005; Wellings 2007). The total loss of yield of wheat due to rusts was
estimated to be 1.6% of the average annual value of production of wheat for Australia over the period 1998–99 to 2007–08 (Murray and Brennan 2009). The cost of this yield loss caused by rusts was estimated to be, on average, $68 million/year for Australia (Murray and Brennan 2010). However, the combined cost for controlling (including fungicidal and cultural control, and breeding resistant cultivars) of these three wheat rusts is estimated at $\geq$1.5 billion/year, with fungicides contributing 41, 9 and 2% of the total costs for stripe, leaf and stem rusts, respectively (Murray and Brennan 2009).

1.9.1. *Puccinia graminis* f. sp. *tritici*

Stem rust of wheat, (*Pgt*) is of major economic importance worldwide, with severe epidemic outbreaks reported in different regions, including South Africa, China, East Africa, Europe, Canada and South America (Kolmer 2001; Kolmer et al. 2007; Morgounov et al. 2012; Roelfs and Bushnell 1985; Saari and Prescott 1985). In the United States of America *Pgt* caused major wheat stem rust epidemics from 1900-1954 (Kolmer et al. 2007; Roelfs 1978) with an average yield loss of 19.3% in South Dakota, 25.4% in Minnesota and 28.4% in North Dakota (Singh et al. 2015). Under suitable environmental conditions, yield losses to stem rust can be 70% or more reaching up to 100% under extreme disease conditions (Beard et al. 2004; Saari and Prescott 1985). In Australia, it has constituted a significant threat to cereal production since early 1900s (Brennan and Murray 1988; Loughman et al. 2005; McIntosh 2007; Park 2007) and is considered the most important disease threat to wheat production in Australia (Zwer et al. 1992). Severe rust epidemics in Australia in 1889, 1899, 1947 1950 and 1973 resulted not only in significant production losses (up to 100% for some crops) but also impacted
on farmer welfare (Park 2007; Rees 1972; Rees and Syme 1981). Stem rust poses the most serious threat of the three rust diseases most commonly found attacking wheat (Beard et al. 2004; Loughman et al. 2005; McIntosh 2007; Park 2007). Losses in WA for stem rust are commonly in the 10–50% range (Loughman et al. 2005), but this can increase to up to 90% when epidemics commence in early spring (Beard et al. 2004) and has the potential to destroy entire individual cereal crops (Beard et al. 2005). While Australia-wide losses from stem rust, currently average only $8 m pa, losses of up to $478 m pa are estimated if stem rust is not controlled (Murray and Brennan 2009). Stem rust continues to be major problem in wheat growing regions in Australia.

1.9.1.1 **Survival of *P. graminis* f. sp. tritici**

*Pgt* is a macrocyclic, heteroecious fungus that generally requires both primary (wheat or grasses) and alternate (*Berberis* or *Mahonia* spp.) host plants to complete its lifecycle via sexual teliospores (Schumann and Leonard. 2000 ). However in Australia, teliospores are non-functional in disease epidemics as no alternate hosts are found (Park 2007). The pathogen only survives through generation and dispersal of asexual urediniospores and/or parasitic survival of mycelium on remnant living susceptible cereal hosts (e.g., wheat and triticale in particular, but also barley and some grasses) (Park 1997). These may offer a ‘green bridge’ for stem rust carryover from one season to another, with subsequent wind dispersal of urediniospores over large distances. However it is challenging to explain the occurrence of some rust outbreaks in the Mediterranean-type climatic region of South Western Australia characterised by hot dry summers having minimal rainfall over summer, restricting growth of any alternative host plants and cool wet winter
growing seasons. *Pgt* urediniospores are relatively tolerant of a range of light and temperature conditions, especially when relative humidity is low (Hernandez Nopsa and Pfender 2014; Singh et al. 2002).

### 1.9.2 *Leptosphaeria maculans*

This chosen pathogen, *L. maculans*, causes blackleg, is one the most serious diseases of *Brassica* species (Barbetti and Khangura 1999; Fitt et al. 2006) and is a major threat to oilseed rape (canola) production worldwide, including Australia (Li et al. 2006; West et al. 2001). *L. maculans* belongs to the Ascomycetes and can infect most cruciferous crops (Johnson and Lewis 1994).

The primary source of the inoculum for this disease is ascospores that are released from pseudothecia on infested canola stubble and are wind dispersed. Throughout the growing season ascospores are discharged from mature pseudothecia with each rainfall and infect canola crops (Howlett et al. 2001; McGee and Petrie 1979). *L. maculans* initially colonizes the tissue as a biotroph and with internal spread of fungus into vascular tissue and becomes necrotrophic producing pycnidia in the dead tissue (Hammond and Lewis 1987).

Pycnidia are produced on infested stubble, cotyledons, leaves and stems of the infected plants throughout the growing season and produce large numbers of asexual spores called conidia. Conidia can be dispersed by rain or direct contact (Hall 1992). The seedling infection is caused by invasion of the cotyledons and young leaves via stomata in particular (Hammond and Lewis 1987; Li et al. 2004). While conidia were initially considered to be the secondary source of inoculum as they are required in higher concentration
than ascospores for infection under controlled conditions (Wood and Barbetti 1977), later studies demonstrated that conidia do have a major role in their own right in disease epidemics (Li et al. 2006; Travadon et al. 2007). The Mediterranean climate of Western Australia also encourages severe epidemics of blackleg (Barbetti and Khangura 1999) estimating economic losses in this region alone of up to AU$50 million per year (Khangura et al. 2007).

1.9.2.1 **Survival of L. maculans**

Survival of the fungus *L. maculans* on oilseed rape residues depends on the environmental and weather conditions (Huang et al. 2003; West and Fitt 2005). Blackleg pseudothecia can be carried over on residues of the host plant encouraging fungal survival on the stubble due to slow decomposition of the crop residues in dry summers of Western Australia and can survive up to 4 years (Khangura and Barbetti 1999). In some situations, *L. maculans* may even remain up to 7 years on oilseed rape residues (Petrie 1995), but residues can be decomposed within 2 years in mild, wet conditions in the UK (West et al. 1999). Also detectable amounts of *L. maculans* DNA had been derived from the organic matter of the soil from the rape fields after 1 year, establishing the survival of the spores in the soil (Sosnowski 2006). However, survivability of viable ascospores decreases slowly with the increased temperature and time exposure (Huang et al. 2003).

1.9.3 **Kabatiella caulivora**

The third pathogen chosen is *K. caulivora* causes the disease clover scorch. This disease is of major economic importance in the temperate regions of the world particularly in Asia, North America and Europe and Australia on
perennial and annual *Trifolium* spp. pastures (Beale 1972; Johnsson 1975). The disease progresses rapidly at temperatures 18°C to 25°C and, low light intensity and wet conditions (Beale 1972). The disease spreads to other regions as spores. The conidia are produced in sticky masses and transported by rain or by attaching to animals or birds and can be carried for long distances by infected hay or seed (Beale 1972).

Both perennial and annual *Trifolium* spp. pastures are affected by clover scorch. *K. caulivora* is the most important foliar pathogen of subterranean clover (*Trifolium subterraneum*) (Barbetti and Sivasithamparam 1986). Clover scorch remains a major limitation to pasture production, both in quality and quantity (Barbetti 2007; Barbetti 1996). Heavily infected swards produce little seed and with greatly reduced hay production (Johnsson 1975). For example, in highly susceptible varieties such as Woogenellup and Yarloop, a seed yield reduction of 90 percent or more has been measured (You et al. 2007). Reductions of about 30% in grazing capacity and up to 50% reduction in hay production of this annual pasture legume have been estimated due to clover scorch during favourable seasons (You et al. 2007).

The disease was initially controlled in Australia by sowing resistant subterranean clover varieties (Bayliss et al. 2002). However a new race of clover scorch (Race 2) was identified in 1991 and later emerged in Esperance in Western Australia where it caused severe damage to subterranean clover varieties previously resistant to *K. caulivora* (Barbetti 1996). This newer Race 2 is capable of infecting many of the previously highly resistant varieties to Race 1 throughout southern Australia (Barbetti 1989; You et al. 2007).
1.9.3.1 Survival of *K. caulivora*

*Kabatiella caulivora* spores are primarily spread by wind-driven rain-splash, as well as livestock and, also can get attached to the hay cutting equipment (Beale 1972; You et al. 2007). Conidia of *K. caulivora* can survive in dry seed storage for months; though the viability decreases over time. However investigations show that the conidia of the pathogen can survive a wide range of temperature conditions, including -10°C (Cole and Couch 1957; Johnson and Lewis 1994). The spores can survive during the winter on infested leaves, debris of the host plants (Sampson 1928). Previous studies have shown that conidial suspensions of *K. caulivora* can remain viable for long periods at -20°C (You et al. 2005).

1.9.4 *Magnaporthe oryzae*

The fourth pathogen chosen *Magnaporthe oryzae* which causes rice blast. Rice blast is one of the most destructive rice diseases across the world caused by the ascomycete *M. oryzae* (Dean et al. 2012; Raveloson et al. 2018). Other grasses species such as turf grass, crabgrass and wheat are infected by species closely related to *M. oryzae* (*M. grisea, M. poae, M. rhizophila* and *M. salvinii*), species that infect different grass species and cause other important plant diseases. *M. oryzae* can infect rice throughout its growth stages and all parts including the roots (Sesma and Osbourn 2004; Sharma et al. 2012). Rice is one of the main crops grown widely across the globe. Rice blast can cause 10- 100% of crop losses depending on the severity (Sivapalan 2016). For example in 1995, rice blast disease caused loss of 1090 tonnes of crops in Bhutan (Strange and Scott 2005) and in the Ord Valley in 2011 the disease caused total losses of up to $1.2 million (Sivapalan et al.
2012). Rice blast (*M. oryzae*) was first detected in Western Australia in 2011 (You et al. 2012).

There have been many strategies used to control the disease including the use of resistant rice varieties (Filippi and Prabhu 1997; Sharma et al. 2012). However, the fungus has been able to establish resistance to chemical treatments and is able to infect the resistant varieties of the rice over time.

1.9.4.1. *Survival of M. oryzae*

Rice blast is a polycyclic disease transmitted by asexual conidia (Raveloson et al. 2018). The pathogen survives winter as mycelium or conidia on diseased rice stubble, seed or in living plants (Faivre-Rampant et al. 2013; Guerber and TeBeest 2006). Raveloson et al. (2018) demonstrated that rice blast survived on stems and sporulation was observed up to 18 months later, which can be the primary inoculum to initiate an epidemic of blast. Conidia are transported by wind/water and infect rice plants after landing on them.

1.10. *Studies Described In This Thesis*

**Justification for these studies**

In the era of increased personal and business travel, military deployments and exercises, sporting events and trade in commerce, the risk of inadvertent introduction of exotic fungal pathogens particularly by spores via introduction of contaminated equipment and commercial goods into Australia is increasing. Many of these exotic fungal pathogens pose a threat to our agricultural, horticultural and natural ecosystems if introduced into Australia; e.g., the exotic stem rust strain Ug99. However, the potential for such introductions into Australia needs further investigation to define the risks,
especially via carrier materials historically not previously seriously considered to be of high risk.

1.11. Hypothesis

The overall hypothesis of the project is that economically important exotic fungal plant pathogens can arrive in Australia via a range of different contaminated carrier materials and that it is possible to develop and apply prototype tools to detect the contamination of different carrier materials with viable fungal spores, and that effective methods for decontamination of contaminated carrier materials can be developed.

1.12. Research Aims

1. Develop a rapid and reliable method relevant for detecting viable exotic fungal spores on, and within, carrier materials.

2. Determine the relative abilities and effectiveness of different materials to act as spore carriers, including assessing spore viability on/from different carrier materials

3. Develop reliable and effective procedures for decontamination of infested carrier materials.

1.13. Originality and significance of the project

The detailed literature review undertaken ensures that this work is original. Although similar experimental methods have been reported in other studies, this research project targets issues that have not been investigated in relation to exotic fungal spore incursions into Australia.

The work undertaken in this study:
I. Delivers new knowledge, skills, techniques and capacity to detect, identify and manage exotic fungal spore incursions.

II. Provides the foundation data for formulating accurate risk assessments for the threats posed by different exotic pathogen-carrier material combinations.

III. Offers new insights into how future biosecurity responses can be matched with the biosecurity threat level posed by each studied pathogen-carrier material combination.

This work brings new understanding on managing fungal contaminations in relation to exotic fungal pathogen spores that could be introduced into Australia; and will be beneficial to the Commonwealth Department of Agriculture and Water Resources, Plant Health Australia, state government biosecurity agencies, other government agencies including the Department of Defence, and private industries.

Past research has been conducted on the impacts of exotic pathogenic fungal species on the plants, agriculture and/or on natural ecosystems. However, work on their entry pathways into a country or in a specific area or region is very limited (Desprez-Loustau et al. 2007). While earlier preliminary research conducted by the Cooperative Research Centre for National Plant Biosecurity (CRCNPB) demonstrated that it is possible to identify fungal spores on clothing materials, viability of spores were not determined in those studies (Wright et al. 2011). Apart from work of Holliday et al. (2013), there has been very little research into the fungal pathogen spore entry pathways into Australia; to date confined mainly to a limited study on the pathogen *Puccinia psidii* (causal agent of guava rust).
This PhD project defines the relative likelihood of, and means by which, viable exotic fungal spore incursions on or in different carrier materials can occur by assessing common pathogen species in Australia and likely entry pathways and deliver new knowledge, skills, techniques and capacity to better detect and manage future exotic fungal spore incursions into Australia.

1.14. Thesis structure

This PhD thesis is presented as a series of scientific research papers from the results of the studies undertaken, in accordance with postgraduate and research scholarships regulation 31(1) of the University of Western Australia. There are in total six chapters. The first chapter is the introduction and literature review of the project. Chapters 2 to 5, each presents the relevant scientific papers addressing the chapter topics and contains an independent introduction, materials and methods, results, discussions and acknowledgements. Additionally, each chapter has an independent reference section which acknowledges the contribution of previous related published works in the area. Hence, these five chapters can be read as a part of the whole thesis or as separate articles.

Chapter 1 covers introduction, a general literature review, background, the research hypothesis.

Chapter 2 A rapid and miniaturized system using Alamar blue to assess fungal spore viability: implications for biosecurity.

Chapter 3 Inert Materials as Long-term Carriers and Disseminators of Viable *Leptosphaeria maculans* Ascospores and Wider Implications for Ascomycete Pathogens.
Chapter 4 Long-term viability of the northern anthracnose pathogen, *Kabatiella caulivora*, facilitates its transportation and spread.

Chapter 5 Extended survival of *Puccinia graminis* f. sp. *tritici* urediniospores: implications for biosecurity and on-farm management

Chapter 6 Disinfestation of diverse fungal pathogen spores on inert contaminated materials: biosecurity implications

Chapter 7 discusses the final overall outcomes of this PhD research and suggests areas of further studies to follow on from the research of this thesis.
Bibliography


Brandis, A. J. 1983. Introduction to the detection and interpretation of the symptoms of jarrah dieback disease in Western Australia. Forests Department of W.A.


Cole, H., and Couch, H. 1957. Etiology and epiphytology of northern anthracnose of red clover. Pages 244-244 in: Phytopathology Amer. phytopathological Soc. 3340 Pilot Knob road, St Paul, MN 55121.


Dell, B., Hardy, G., O’Gara, E., and Shearer, B. 2007. The impact of *Phytophthora cinnamomi* on ecosystem function and health of Mediterranean forests, woodlands and heath lands in Western Australia.


Sivapalan, S. 2016. Agronomic Options for Profitable Rice-based Farming System in Northern Australia. Barton, ACT.


CHAPTER 2

A rapid and miniaturized system using Alamar blue to assess fungal spore viability: implications for biosecurity
A rapid and miniaturized system using Alamar blue to assess fungal spore viability: implications for biosecurity

Papori Barua · Ming Pei You · Kirsty Bayliss · Vincent Lanoiselet · Martin J. Barbetti

Accepted: 19 October 2016 / Published online: 26 October 2016 © Koninklijke Nederlandse Plantenziektenkundige Vereniging 2016

Abstract Long-lasting viable fungal spores are one of the important aspects in emergence, spread and disease development of pathogenic fungi. We developed a rapid and miniaturized system using Alamar Blue (resazurin dye; 7-hydroxy-3H-phenoxazin-3-one 10-oxide) for assessing fungal spore viability, using the ascomycete *Leptosphaeria maculans* (causing blackleg disease on canola) as a ‘model pathogen’. The assay is dependent on the metabolic activity of viable fungal spores to convert the dark blue of resazurin (maximum absorbance 605 nm) into the pink colour of resorufin (maximum absorbance 573 nm). The Alamar Blue assay uses an optimised micro-titre based format that was far superior for determining fungal spore viability in comparison with current conventional techniques including trypan blue staining, a TC10 cellometer cell counter, or by assessing germination of the spores under the microscope. This new assay was also more rapid and reproducible than current conventional tests to detect viable spores. Viable spores could be reliably detected within two hours. The successful application of the Alamar Blue assay to measure fungal spore viability in the current study has important benefits for biosecurity operations relating to faster and more reliable confirmation of viability of potential invasive exotic fungal pathogens and in minimising any consequent disease outbreaks. The effectiveness of the Alamar Blue assay was confirmed by successfully determining the relative retention times of viable *L. maculans* ascospores across a range of different potential spore-carrier materials, including steel, fabric, wood, paper, rubber and leather, over a time period of eight months. To further confirm the wide applicability of the Alamar Blue assay, it was successfully applied to detect viable spores of fungal pathogens of diverse taxonomic groups, including *Kabatiella caulivora*, *Magnaporthe oryzae* and *Puccinia striiformis* f.sp. *tritici*, and also of the yeast *Saccharomyces cerevisiae*.

Keywords Alamar blue · Resazurin · Spore viability · *Leptosphaeria maculans* · Blackleg · Plant biosecurity

Introduction

Fungal pathogens are a major threat to agricultural production (Khangura et al. 2007; Wellings 2011) and global food safety (Bebber and Gurr 2015; Savary et al. 2012). In Australia alone, the major pathogens of cereals and oilseeds are estimated to cause losses of SAUS1.3 billion/year (Murray and Brennan 2009, 2010, 2012). Furthermore, some fungi are additionally important as
they produce mycotoxins that can cause disease and even death in animals or humans (Tan et al. 2012; Zain 2011).

Fungal spores are an integral part of the fungal life cycle and are mainly adapted for dispersal and need to remain dormant to survive unfavourable environmental conditions (Wyatt et al. 2013). To retain viability until infection occurs, some fungal spores behave as specialised resistant bodies with high survival ability (Willett 1971). Long-lasting fungal spores are a critical aspect in the emergence, spread and consequent disease development of pathogenic fungi, as surviving spores constitute persistent fungal inoculum (Fisher et al. 2012; Strange and Scott 2005).

Fungal spore dispersal plays a critical role in disease introduction, spread and establishment. Fungal spores can be dispersed locally or over large distances by wind, water (e.g., rain splash) or by animal and human activities (McNeill et al. 2011; Savage et al. 2012). Long distance aerial dispersal of plant pathogenic fungal spores spreads diseases across and sometimes between continents (Brown and Hovmøller 2002). Even the release patterns of spores of fungal plant pathogens can be related to their relative abilities to survive during dispersal and to infect new hosts (Savage et al. 2013).

The rise in global movement of people and commodities, particularly from regions or countries posing a high biosecurity risk, increases the likelihood of introduction(s) of new fungal pathogens into new areas via spore contamination of biological and/or inert materials (Holliday et al. 2013; Hovmøller et al. 2008). Such potential exotic introductions pose a major threat to the agricultural, horticultural and forest industries (Grgurinovic et al. 2006). There are a wide range of potential carrier materials that could enable such entry and movement of exotic fungal spores, including inert materials widely used in everyday life such as paper, metal, fabrics, leather, timber and rubber tyres (Sterflinger 2010). For example, fungal contamination is a major problem on library books (Rakotonirainy et al. 2003). Similarly, Miki et al. (2005) and Tindale (1989) noted the importance of fungal contamination of food packing materials; and there have been examples of fungal contamination of tetra-packs and packets leading to food spoilage and health problems (Sturaro et al. 2006). Further, Tindale et al. (1989) isolated 31 species of fungi from fibreboard cartons, paper sacks and jute sacks; Hill et al. (1995) isolated 38 species of fungi from the samples of timber from the flooring of six general-purpose freight containers including plant pathogen species *Alternaria*, *Aspergillus*, *Phoma*, *Mucor*, *Eurotium*, *Cladosporium*, *Penicillium*, *Trichoderma* and *Ulocladium*; some of which are also capable of producing mycotoxins (da Cruz Cabral et al. 2013).

For plant biosecurity, accurate and rapid assessment of spore viability is critical (Jackson and Bayliss 2011). While there are several detection methods available for the detection of fungal spores, the detection of viable fungal spores on potential ‘carrier materials’ remains a challenge due to lack of a rapid reproducible method for assessing spore viability. Current methods for assessing spore viability mostly consist of visualization and spore germination counts, a laborious and time-consuming methodology (Gottlieb 1950). Further, not all spores germinate readily despite the fact that some can remain viable for long periods (Fries 1984) and subsequently germinate to become a potential risk when favourable conditions occur (Crone et al. 2013; Feofilova et al. 2012). Hence, in the context for biosecurity, there is critical need for development of a rapid, inexpensive and reliable quantification method to assess spore viability in order to mitigate potential exotic fungal infestations.

Alamar Blue (resazurin dye; 7-hydroxy-3H-phenoxazin-3-one 10-oxide) is a non-fluorescent and blue coloured dye in its oxidized state. However, viable cells have the ability to reduce resazurin to resorufin (7-hydroxyphenoxazin-3-one), the latter a highly fluorescent pink dye (Bueno et al. 2002). In living cells, oxidoreductases, the mitochondrial electron transport chain and/or cytosolic, microsomal and mitochondrial enzymes, reduce resazurin intracellularly to resorufin (Goegan et al. 1995). The two compounds are easily distinguished by different absorbance maxima, resazurin at 605 nm and resorufin at 573 nm. Resazurin was first used by Pesch and Simmert in 1929 to estimate the bacterial content of milk (Twigg 1945) and has been used for decades to monitor bacterial and yeast contamination of milk (O’Brien et al. 2000). Alamar blue or resazurin dye has been broadly used as a cell viability indicator in many cytotoxicity, cell growth proliferation assays (Borra et al. 2009; Riss et al. 2013; Sarker et al. 2007) as it is a rapid, non-radioactive, non-toxic, low cost and highly sensitive method (Castilho et al. 2015; Lim et al. 2016; Martin et al. 2003; Munshi et al. 2014). For example the Alamar Blue bioassay has been used to assess cell viability and cytotoxicity across a wide range of cell types including bacteria, yeast, protozoa, cultured mammalian cells (Fai and Grant 2009; Rampersad 2012; Tiballi et al. 1995), and also in medical research involving human or animal...
cells (Al-Nasiry et al. 2007; Bowling et al. 2012; Gloeckner et al. 2001; Khalifa et al. 2013; Nakayama et al. 1997; O’Brien et al. 2000). For fungal hyphae this assay has also recently been used to study the effects of stress and antifungal agents on the viability of hyphae of the rice blast pathogen Magnaporthe oryzae (Chadha and Kale 2015). However, this bioassay has not previously been applied to detect viability of plant pathogenic fungal spores. Hence we chose the plant pathogen Leptosphaeria maculans, belonging to the Ascomycetes, a class comprising numerous major pathogens of Brassica, legumes and cereal crops (Johnson and Lewis 1994), as a ‘model’ pathogen to develop an Alamar Blue bioassay specific for fungal spores. L. maculans was chosen as it is a serious and global disease of Brassicas and a pathogen readily available in Australia where it is considered the most important pathogen of canola (Barbetti and Khangura 1999; Fitt et al. 2006; Li et al. 2006; West et al. 2001). Further, the survival of pseudothecia of L. maculans on infested canola residues is known to depend on the environmental conditions (Huang et al. 2003; West and Fitt 2005); for example survival is up to four years where dry summers predominate as in Western Australia or less than two years in mild/wet conditions such as in the United Kingdom (West et al. 1999).

This study reports the development of a rapid, non-expensive, and portable bioassay using Alamar Blue in a miniaturized system adapted and optimised to measure the metabolic activity (i.e., viability) of spores using L. maculans as a ‘model pathogen’. This assay was compared with the traditional methods of spore viability assessments including cell counter, germination and staining methods. We further validated the assay by assessing the viability of spores from three other diverse fungal genera, viz. plant pathogens of rice (Magnaporthe oryzae), wheat (Puccinia striiformis sp. tritici), and subterranean clover (Kabatiella caulivora) and also a yeast (Saccharomyces cerevisiae). Here we report how this new bioassay transforms viability testing for fungal pathogen spores and discuss the implications from this study for plant biosecurity.

Materials and methods

L. maculans pathogen inoculum production

L. maculans ascospores were obtained by collecting infested canola stubble carrying pseudothecia in early winter (June) and then storing dry at 25 °C. Ascospores were produced following the protocol of Li et al. (2004). Briefly, infected stubble pieces were immersed in sterile deionized water (SDW) in covered plastic Petri dishes for 1 h at 25 °C under natural room fluorescent light and then maintained at 4 °C in the dark for 1 h. Ascospores were collected by washing them off the inside of the lid with SDW. The concentration of released ascospores was quantified using a haemocytometer (Superior®) counting chamber under a compound microscope.

Optimisation of L. maculans ascospore viability assay

Initially, the Alamar Blue bioassay was optimized as a spore viability test for L. maculans to determine the optimum time for maximum metabolic activity of spores. The assay was performed using 96 well cell plates in a spectrophotometer (Thermo Scientific Multiskan® Spectrum) with absorbance at 600 nm and 570 nm at 30 min intervals for a period of 3 h. Fresh L. maculans ascospore suspensions by tenfold serial dilution ranging in concentration from 10⁷ ml⁻¹ to 10 ml⁻¹ were set up in 96-well assay plates and used as the standards. Three replicate wells were used for each concentration and were set up using 100 μl of fresh ascospores for the standards with 20 μl of Alamar Blue reagent. Negative controls were similarly set up with no ascospores and only SDW to determine the extent of any background absorbance. The assay plates were covered with aluminium foil (to exclude light) and were placed on a rotary platform shaker (Innova™ 2100, New Brunswick Scientific) at 150 rpm for 1 min to mix the reagent and the spore suspensions. The plates were then incubated at 37 °C for each designated time period (0.5, 1, 1.5, 2, 2.5 and 3 h) and then the reaction was terminated by adding 50 μl of 3 % sodium dodecyl sulphate (SDS) before measuring the absorbance every 30 min for 3 h. Plates were then placed inside the spectrophotometer and shaken for 30 s using the linear shaking function in order to mix the samples. Then absorbance at 600 nm and 570 nm was measured using the spectrophotometer. The optimum time to obtain maximum absorbance (i.e., value directly proportional to the maximum metabolic activity of ascospores) was then calculated by subtracting the background absorbance value at 600 nm for control wells from the absorbance values of experimental wells at 570 nm.
Validation of methodology

Spores from three other diverse fungal genera, viz. plant pathogens of rice (Magnaporthe oryzae), wheat (Puccinia striiformis sp. tritici), and subterranean clover (Kabatiella caulivora) and also common yeast (Saccharomyces cerevisiae), were used to validate the methodology. The optimum incubation time to obtain maximum metabolic activity/viability for each species was recorded.

Other spore viability assessment methodologies tested

Six replicates each with 100 μl of fresh L. maculans ascospores of 10^6 ml^-1 concentration were used across a range of different methodologies, as outlined below, to determine the maximum time required to obtain maximum metabolic activity/viability (including sensitivity and rapidness of assessments). The number of viable ascospores was calculated and viability was expressed as a percentage of the total ascospores present.

Viability assessment - L. maculans ascospore germination

L. maculans ascospores were germinated on ½-strength potato dextrose agar (PDA) and the percentage germination was assessed using a compound microscope. Counts of germinated ascospores were conducted sequentially and regularly until no more germinated ascospores were found. The final germinated ascospore numbers were treated as the maximum number of ascospores that had germinated.

Viability assessment using trypan blue

To determine spore viability, the number of L. maculans ascospores was counted using a haemocytometer counting chamber under a compound microscope after staining with 0.4 % trypan blue in phosphate-buffered saline maintained at pH 7.2 (Strober 2001). Those that showed as trypan blue negative (i.e., unstained) represent viable spores while those that showed as trypan blue positive (i.e., stained) represent dead ascospores.

Viability assessment using a TC10 cellometer cell counter

A TC10 cellometer cell counter (Bio Rad) was used to count numbers of viable L. maculans ascospores. The protocol of one part of trypan blue dye mixed with 1 part of ascospore suspension (1:1) was followed. (Bio-Rad 2010). Application of Alamar Blue assay was used to determine viability of L. maculans ascospores on carrier materials over time.

Selection of carrier materials

Six different carrier materials, viz. metal (steel), fabric (cotton denim), wood (jarrah), leather, paper and rubber (tyre), were used in an experiment to determine the maximum period of viability for ascospores of L. maculans. These test materials were selected both to determine their effectiveness as potential spore carriers and also the effect of the materials per se on spore survival as they are common materials utilised in everyday life, found around farms, as used by travellers, and in the packaging and transport enterprises.

Inoculation of materials in a miniaturised system and the experimental design

To adapt and develop a new method that could cope with processing large sample numbers in a cost-effective way, firstly, a new technique to miniaturize samples was developed as illustrated in Fig. 1. The carrier materials were cut into 0.5 cm squares and placed into cells in a sterile 48-well cell culture plate (Greiner® sterile) along with the non-inoculated control treatments according to randomisation. Each of the six above mentioned test materials in each well was inoculated individually with 10 μl (10^6 ascospores ml^-1) of L. maculans and allowed to dry in a laminar flow for 3 h. The inoculated 48-well cell culture plates were sealed with Parafile® and then placed at 23 ± 1 °C day/8 ± 1 °C night under a light source consisting of both LED cool white and incandescent light bulbs (Quantum Flux MQ100, Apogee) at an intensity of 260 μmol m^-2 s^-1 or 320 μmol m^-2 s^-1, respectively. There were eight time points (factors). The experiment was run over an eight month period to assess spore viability commencing from month 1 (i.e., day 30) and repeated at 30 day intervals and the final assessment made at month 8 (i.e., day 240).

Recovery of L. maculans ascospores from inoculated test materials

L. maculans ascospores were recovered from the test materials by adding 800 μl of SDW containing 0.1 %
Tween® 20 directly into each treatment plate and placing plates on a rotary shaker for 40 min at 700 rpm (Fig. 1). Preliminary studies had showed that a concentration of 0.1 % Tween® 20 in SDW was optimal for removing ascospores from the materials without affecting their viability (data not shown).

Determination of viable number of *L. maculans* ascospores from inoculated test materials using Alamar blue assay

Initially, the optimized Alamar Blue assay was applied as a spore viability test for *L. maculans* ascospores recovered from the inoculated materials over time (Fig. 1). A standard curve of 570–600 nm absorbance versus a series of known ascospore concentrations was plotted to calculate the number of viable ascospores in residual solutions after washing. The total number of viable ascospores was determined using the regression equation from the standard curve. The viable ascospores were expressed as a percentage.

Experimental design and data analyses

All experiments were arranged in a completely randomized design, repeated twice, and with six replications for each of the three studies. For each study, the initial and repeat experiments were assessed by a paired t-test using GenStat (18th edition, GenStat Procedure Library Release PL23.2). As in each case there were no significant differences between each pair (*P* > 0.05), the data sets were pooled, re-analysed and presented as a single data set for each of the three studies. Single and multiple factor ANOVA were conducted using GenStat (18th edition, GenStat Procedure Library Release PL23.2) to compare the sensitivity of different methods on detecting viability of the *L. maculans* ascospores. Fisher’s least significant differences were used to compare differences, particularly the viable spore holding capacities between the materials. Duncan Multiple Range Tests were also performed across all data sets and confirmed all significant effects shown by ANOVA (Duncan Multiple Range data not shown).

Results

Optimisation of Alamar blue viability assay for *L. maculans* ascospores

The ability of fresh *L. maculans* ascospores to reduce resazurin to resorufin at 37 °C significantly (*P* < 0.001) increased with increasing incubation time from 0.5 to 2 h (Fig. 2). There was a linear positive correlation (*R*² = 0.99) between absorbance and the number of ascospores at the 2 h incubation time, while incubation times >2 h did not show any further significant increase in absorbance. Hence, the assay was optimised with an incubation time of 2 h at 37 °C for testing the viability of *L. maculans* ascospores.
Validation of methodology

The Alamar Blue assay was successful in measuring the spore viabilities of other diverse fungal pathogens including *Kabatiella caulivora*, *Magnaporthe oryzae*, *Puccinia striiformis* f. sp. *tritici*, and also for *Saccharomyces cerevisiae*, with optimised incubation times of 2 h, 2 h, 3 h and 1.5 h, respectively (Fig. 3).

Viability assessment - *L. maculans* ascospores germination

The percentage of the total germinated ascospores of *L. maculans* on ½ strength PDA was counted over a 120 h time period. Only 15 % of ascospores germinated within 2.5 to 12 h, 58 % ascospores germinated within 24 to 48 h. Up to 82.6 % of ascospores germinated within 72 h (Fig. 4). No changes were observed in the % germination after 72 h, although some of the remaining ascospores appeared visually to be intact yet did not show formation of any germ tube.

Viability assessment using haemocytometer and trypan blue

The mean number of viable ascospores of *L. maculans* detected using trypan blue was 82.2 % (Fig. 4). The time required was a minimum of 6 h in order to count the spores from all the replicates. Fewer viable spores were observed from the replicates analysed as time progressed past 4 h. With increasingly longer times of exposure increasingly greater numbers of spores became stained with the dye.

Viability assessment using a TC10 cellometer cell counter

The mean number of viable ascospores of *L. maculans* detected using a cellometer was 2.3 % (Fig. 4) after 1 h. The data recorded using a TC10 cellometer cell counter was very inconsistent across different replicates of each sample.

Comparison of the four different assessment methods

Analysis of variance of detected numbers of viable spores using the four different test methods, demonstrated significant differences between the different assessment methods \(P < 0.001\); l.s.d. = 1.863 (Fig. 4)]. The Alamar Blue assay was the most sensitive (i.e., detected highest viable spore number) and the most rapid, followed by trypan blue, then together the germination test on agar and the TC10 cellometer cell counter (Fig. 4). There was a significant difference between the times required to observe the maximum viability of the ascospores between the four different assessment methods (Fig. 4). However, as we did not try with fungal spores killed (e.g. by irradiation) to check the absence of false positive detection events in this study, this could be included for future studies.

Determination of viable number of *L. maculans* ascospores from inoculated test carrier materials over time using the Alamar blue assay

The percentage of viable *L. maculans* ascospores decreased gradually across one to eight months for all test carrier materials (Table 1). Across all test carrier materials, the highest percentage of viable ascospores recovered was from wood (31 %) after one month and this decreased to 29 % after two months, 22 % after three months, 16 % after four months, 11 % after five months, 11 % after six months, 9 % after seven months, and only 3 % after eight months. There was a significant effect \(P < 0.001\) of test carrier materials on viability of recovered ascospores over time. The lowest percentage of
Fig. 3  Validation of Alamar Blue assay using fungal spores of four different organism genera by assessing optimum incubation time for the maximum metabolic activity: the graphs show incubation times (n) for different spores of A = Magnaporthe oryzae (n = 6); B = Kabatiella caulivora (n = 6); C = Saccharomyces cerevisiae (n = 5) and D = Puccinia striiformis f. sp. tritici (n = 7), each at concentrations across 10 to 10⁷ ml⁻¹, for reducing resazurin (Alamar Blue) to resorufin. l.s.d. values at P < 0.05 were too small to show within Fig. 3 and are as follows: For ‘A’ l.s.d. values are 0.00734 (time), 0.00793 (concentration) and 0.01942 (time x concentration); for ‘B’ l.s.d. values are 0.00465 (time), 0.00502 (concentration) and 0.01231 (time x concentration); for ‘C’ l.s.d. values are 0.00484 (time), 0.00573 (concentration) and 0.01281 (time x concentration); and for ‘D’ l.s.d. values are 0.00506 (time), 0.00473 (concentration) and 0.01339 (time x concentration).

Fig. 4  Comparison between using the Alamar Blue viability assay, a TC 10 cellometer counter, spore germination counts, and trypan blue staining methods after using a haemocytometer counting chamber to determine the numbers of viable ascospores [expressed in percentage of total spores inoculated in the beginning (viz. 10⁶)] of Leptosphaeria maculans (P < 0.001, l.s.d = 1.863)
viable spores recovered were from fabric; 8 %, 4 %, 1 % and 1 % after 1, 2, 3 and 4 months, respectively. All the test carrier materials retained viable ascospores for a minimum for three months. Least retention of viable ascospores (1 %) was on steel for 3 months and no viable ascospores were recovered from steel after that time point. In contrast, a minimum of 1 % viable ascospores were retained on fabric for 4 months, leather for 5 months, rubber for 5 months, and paper for 6 months (Table 1).

**Discussion**

This is the first study to demonstrate utilization of an Alamar Blue reagent for determining the viability of fungal spores. Comparison with traditional methods for assessing spore viability, such as trypan blue staining, use of a cell counter, or assessing germination methods compared in this study, as well as in contrast with the MTT-assay and the MS and ATP assays (Page et al. 1993; Rampersad 2012) which cause cell death, Alamar Blue assay maintains cells alive for long-term monitoring (Al-Nasiry et al. 2007; Xiao et al. 2010). Not only is the Alamar Blue method a simple, rapid means to measure the viability of fungal spores, it is less expensive, non-radioactive and non-toxic to both pathogens and users and therefore does not require any special handling or disposal methods (Ahmed et al. 1994; Coban et al. 2014).

Alamar Blue offers a significant improvement as a novel technique for testing for viability of fungal spores as it is an excellent indicator of the metabolic capacity of cells (Freimoser et al. 1999). Further, in contrast to the trypan blue staining, use of a cell counter, or assessing germination methods compared in this study, as well as in contrast with the MTT-assay and the MS and ATP assays (Page et al. 1993; Rampersad 2012) which cause cell death, Alamar Blue assay maintains cells alive for long-term monitoring (Al-Nasiry et al. 2007; Xiao et al. 2010). Not only is the Alamar Blue method a simple, rapid means to measure the viability of fungal spores, it is less expensive, non-radioactive and non-toxic to both pathogens and users and therefore does not require any special handling or disposal methods (Ahmed et al. 1994; Coban et al. 2014).

The germination method is a conventional means for estimating spore viability. However not all spores may germinate readily. Further, the germination method is a laborious, space and time consuming process and sometimes requiring specific media for different species. In contrast, Alamar Blue can be added directly to the spore suspension sample and easily utilized in 96 to 384-well plates depending on the sample size; with spore viability data recorded using either fluorescence or absorbance. In addition, it took up to 72 h with the germination method to get maximum ascospore germination/viability (82.6 %) while it took only 2 h to measure the maximum viability of *L. maculans* ascospores using the Alamar Blue assay.
Although cell counters have been used to count viable cells both in animals and plants, use of the TC10 cellometer cell counter for fungal spores has been quite limited. Most cell counters work best with a cell concentration range of $5 \times 10^4$ to $1 \times 10^7$ cells ml$^{-1}$ and with cell diameters in the range of 6–50 $\mu$m (Bio-Rad 2010). In this study the TC10 cellometer cell counter was not really appropriate for use for ascospores of *L. maculans* as the spore size for this pathogen ranges from width of 5–8 $\mu$m and in length from 30 to 70 $\mu$m (Punithalingam and Holliday 1972). The TC10 cellometer cell counter also produces variable results depending upon homogeneity of spore concentration. In contrast the Alamar Blue assay which could be performed using a non-homogenous spore concentration and any sizes of spores (Table 2) also worked well with very low spore concentrations.

Use of trypan blue is a very common practice to observe the viability of cells (Strober 2001). However there are also challenges using trypan blue, as it is a cytolysis or membrane leakage assay where the dye crosses cell membranes of the dead cells (Strober 2001), and can be toxic to live cells with extended exposure time (Mascotti et al. 2000). It is also a time consuming assay and is inappropriate where large numbers of samples need to be processed. In contrast, the Alamar Blue assay was a much more time efficient and a non-toxic method compared with haemocytometer counting using trypan blue (Table 2). Also, compared to spore germination and trypan blue staining, Alamar Blue did not require microscopic investigation to observe or confirm the viability of spores.

The successful application of the Alamar Blue assay to measure fungal spore viability in the current study has important implications for biosecurity in relation to potential incursions of exotic fungal pathogens. The current study demonstrates that the Alamar Blue assay readily detects viable fungal spores, and can easily be adapted and optimised to determine the viability of a wide range of fungal/oomycete pathogens. To validate the assay we used spores of four other organisms and the assay was successful in measuring the spore viabilities of other three fungal pathogens including *Magnaporthe oryzae* that causes blast disease on rice (Wilson and Talbot 2009), *Puccinia striiformis* f.sp. *tritici* that causes stripe rust disease on wheat (Wellings 2011), *Kabatiella caulivora* that causes northern anthracnose disease on clover (Barbetti 1995), and also of brewer’s yeast cells (*Saccharomyces cerevisiae*) (Legras et al. 2007).

As confirmation of the wide application potential of the Alamar Blue assay for determining fungal spore viability, we successfully applied it to determine viability of *L. maculans* ascospores on a range of test materials including steel, fabric, wood, paper, rubber and leather in order to better understand the retention of viable fungal spores by these spore-carrier materials over a time period of eight months. Further, the sensitivity of this new assay was demonstrated in the current study, for example, by the greater potential of released *L. maculans* ascospores to survive much longer periods than previously considered possible from historical epidemiological studies with this pathogen (Huang et al. 2003; Li et al. 2007).

Despite the success of the Alamar Blue assay for fungal spore viability, it does need to be optimised for each application and cell model (Lim et al. 2016; Rampersad 2012). It is recognised that this assay is not a fungal diagnostic tool to identify fungi or differentiate spores of quarantine from non-quarantine fungal species. However, the Alamar Blue assay can easily be

---

**Table 2** Comparison of advantages and disadvantages between the four tested spore viability assessment methods (Alamar Blue assay, trypan blue stain, spore germination count and TC10 cellometer cell counter).

<table>
<thead>
<tr>
<th></th>
<th>Alamar blue assay</th>
<th>Trypan blue dye</th>
<th>Spore germination</th>
<th>Cell counter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (with maximum viability)</td>
<td>96 %</td>
<td>75 %</td>
<td>72 %</td>
<td>N/A</td>
</tr>
<tr>
<td>Time to observe maximum viability</td>
<td>2 h</td>
<td>6 h</td>
<td>120 h</td>
<td>1 h</td>
</tr>
<tr>
<td>Toxicity</td>
<td>Non-toxic and does not kill the spores</td>
<td>Toxic and kills the spores when the dye enters inside the spores following membrane leakage</td>
<td>Not applicable</td>
<td>Dye used is toxic to the spores</td>
</tr>
<tr>
<td>Cell size</td>
<td>Any</td>
<td>Any</td>
<td>Any</td>
<td>Any</td>
</tr>
<tr>
<td>No. samples analysed at a time</td>
<td>96 to 384</td>
<td>One sample</td>
<td>Not applicable</td>
<td>One sample</td>
</tr>
<tr>
<td>Microscopic examination</td>
<td>Not required</td>
<td>Required</td>
<td>Required</td>
<td>Not required</td>
</tr>
</tbody>
</table>

---
supplemented with other detection and diagnostic tools of pathogen identification such that it can quickly detect the presence of viable spores where pathogens of quarantine relevance are detected. For example this assay could be readily adapted to assess the viability of eucalyptus or guava rust (Puccinia psidii), or new strains of stem rust (Puccinia graminis) like Ug99 that pose serious environmental and economic biosecurity threats when introduced into regions where they do not yet occur (Carnegie and Cooper 2011; Wellings 2007). However, as the current experiments were performed in sterile microwells, spores were not exposed to degradation by microorganisms nor to any detrimental effects of, for example, sun UV light, as may occur for materials outside the laboratory. Furthermore, released spores may not land on a susceptible host, further reducing the threat. This being so, until additional studies clarify this relationship, the percentage of viable spores found in the current study should not be directly interpreted as an ‘absolute’ for the percentage of spores available to infect plants.

**Conclusion**

This study demonstrates the benefits of novel application of the Alamar Blue assay over traditional methods of spore viability assessment; highlighting it advantages of being a non-toxic, more reliable and faster method, where up to 96–384 samples in a micro-titre based format can be processed at a single time. Fungal spore metabolic activity conversion of dark blue resazurin into pink resorufin was a reliable indicator of the presence of viable spores of fungal pathogens and a yeast. Finally, novel application of this Alamar Blue assay methodology to easily and rapidly observe and assess fungal spore viability fosters future research studies to investigate patterns and changes in spore viability of spores across wide ranging exotic and non-exotic fungal and oomycete pathogens.

**Acknowledgments** We greatly appreciate the scholarship and operating funding provided by the Plant Biosecurity CRC for project CRC62042: ‘Curtailing and managing exotic fungal spore incursion into Australia’, and financial support by School of Plant Biology at the University of Western Australia. We thank Mr. Robert Creasy and Dr. Michael Considine from the University of Western Australia for the use of facilities and technical help during these studies.

**References**


symptomatic and asymptomatic annual and herbaceous perennial plant species. *Fungal Biology*, 117, 112–123.


CHAPTER 3

Inert Materials as Long-term Carriers and Disseminators of Viable *Leptosphaeria maculans* Ascospores and Wider Implications for Ascomycete Pathogens
Research

Inert Materials as Long-Term Carriers and Disseminators of Viable Leptosphaeria maculans Ascospores and Wider Implications for Ascomycete Pathogens

Papori Barua and Ming Pei You, School of Agriculture and Environment and the UWA Institute of Agriculture, Faculty of Science, The University of Western Australia, Crawley, WA 6009, Australia; Kirsty L. Bayliss, School of Veterinary and Life Sciences, Murdoch University, WA 6150, Australia; Vincent Lanoiselet, Department of Primary Industries and Regional Development Western Australia, South Perth, WA 6151, Australia; and Martin J. Barbetti, School of Agriculture and Environment and the UWA Institute of Agriculture, Faculty of Science, The University of Western Australia

Abstract

The viability of ascospores of the Phoma stem canker (blackleg) pathogen, Leptosphaeria maculans, was tested on a range of carrier materials, including metals, fabrics, woods, and plastics, and under different temperature conditions of 23 and 4, 36 and 14, and 45 and 15°C day and night, respectively. At 23 and 4°C (day and night, respectively), ascospores remained viable for up to 240 days on Tasmanian oak (Eucalyptus regnans) and pine wood (Pinus radiata). At 36 and 14°C (day and night, respectively), ascospores remained viable on pine wood for up to 180 days. At 45 and 15°C (day and night, respectively), ascospores remained viable up to 60 days on jute. There were also significant differences (P < 0.001) between carrier materials in their abilities to retain ascospores following washing. At least 30% of intact ascospores recovered from inert carrier materials were able to germinate on artificial growth media within 48 h of recovery and some ascospores were still viable after 240 days. These findings confirm that L. maculans ascospores remain viable for a much longer time in the absence of a host than previously considered. This demonstrates the importance of inert materials as long-term and long-distance carriers of viable L. maculans ascospores, and highlights their potential role for spread of L. maculans races to new regions and countries via farming equipment, clothing, and other associated materials. Local, national, and international biosecurity agencies need to be aware that the risks of spread of ascomycete plant, animal, and human pathogens via inert materials are significantly greater than currently assessed.

Leptosphaeria maculans (Phoma stem canker, blackleg) is the most important disease of oilseed rape and canola (Brassica napus) in many countries (Fitt et al. 2006; Johnson and Lewis 1994; West et al. 2001), including Australia (Barbetti and Khangura 1999; Li et al. 2006a; Sivasithamparam et al. 2005). Despite the availability of plant resistance in some varieties and widespread use of fungicides, Phoma stem canker causes significant production losses in Australia (Barbetti and Khangura 1999) and elsewhere (West et al. 2001). The estimated annual loss from Phoma stem canker to the Australian oilseed rape industry is approximately $77 million (Murray and Brennan 2012).

The primary source of L. maculans inoculum is from wind-dispersed, sexually produced ascospores released from pseudothecia on infected canola stubble (Li et al. 2004, 2006a; McGee and Petrie 1979; Wherrett et al. 2003, 2004). L. maculans ascospores primarily infest seedlings initially, and cause lesions on cotyledons, leaves, and stems (Barbetti 1976; McGee and Petrie 1979). Infections are primarily through stomata or wounds (Li et al. 2004). In Australia, although L. maculans ascospores are discharged throughout the growing season, there are distinct ascospore emission peaks in late autumn and early winter (Khangura et al. 2007; Salam et al. 2003, 2007; Savage et al. 2012), the timing of which are dependent on geographic region and time of the year (Savage et al. 2013), and also on seasonal rainfall and temperatures (Salam et al. 2003, 2007). In the Mediterranean-type environment of southwest and southern Australia (Barbetti et al. 2012), release of L. maculans ascospores can be diurnal (Savage et al. 2010, 2012).

Survival of L. maculans on canola residues depends on the environmental conditions (Huang et al. 2003; West and Fitt 2005). Pseudothecia can remain viable for up to 4 years where dry summers predominate such as in Western Australia but only up to approximately 2 years in mild or wet conditions such as those in the United Kingdom (West et al. 1999, 2001). Infested residues are the primary source of inoculum for successive spread of the disease from season to season and between different areas (Huang et al. 2003; West et al. 1999). Li et al. (2007) highlighted the high relative importance of ascospore survival in soil in the disease epidemiology in situations where the ascospore showers have been little or infrequent, particularly if restricted to a short period at the beginning of growing season. However, survivability of ascospores has been reported to decrease both with time (Li et al. 2007) and with increased temperature (Huang et al. 2003). L. maculans can be transmitted through the seed (Lloyd 1959), and Wood and Barbetti (1977) observed that both pods and seed can be infected by L. maculans. At least 36% infected seed gave rise to mature infected plants, indicating that seedborne infection of L. maculans can play an important role in spreading the pathogen into fields of susceptible hosts (Wood and Barbetti 1977).

Recently, we developed a rapid and miniaturized system using Alamar Blue (resazurin dye; 7-hydroxy-3H-phenoxazin-3-one 10-oxide) for assessing fungal spore viability and, for developing this methodology, we used L. maculans as the “model pathogen” across six different carrier materials and made comparisons with Kabatiella caulivora, Magnaporthe oryzae, Puccinia striiformis f. sp. tritici, and the yeast Saccharomyces cerevisiae to prove its wider applicability (Barua et al. 2017a,b). However, what has never been properly investigated in detail is the potential for wider dissemination of new pathogen races of L. maculans (Balesdent et al. 2005) via attachment

1Corresponding author: M. J. Barbetti; E-mail: martin.barbetti@uwa.edu.au

Funding: We greatly appreciate the scholarship provided by the Plant Biosecurity Cooperative Research Centre (PB CRC), Bruce. ACT 2617, Australia, for project CRC6204: “Curtailing and managing exotic fungal ascospores incursion into Australia”, and the financial support by the School of Plant Biology at the University of Western Australia.

Accepted for publication 7 November 2017.

© 2018 The American Phytopathological Society
to a comprehensive range of different inert carrier materials and under different temperature regimes, as would be associated with movement of humans, farming equipment, clothing, and commodities across regions and continents. In addition, although temperature is known to play an important role in formation and establishment of stem cankers and consequent disease severity (Barbetti 1975; Li et al. 2006b), its role in ascospore survival has not been investigated in terms of survival on inert carrier materials.

Although the movement of this pathogen through infected seed or infested crop debris is a well-recognized risk (Fitt et al. 2006), alternative means of movement and entry of *L. maculans* through dormant conidia have not been studied in the long-term viability of *L. maculans* ascospores on a range of 15 different inert carrier materials and under three different temperature regimes.

Materials and Methods

**Inoculum preparation.** *L. maculans* ascospores were produced from collected stubble carrying mature pseudothecia from previously infested fields in early winter and stored dry at 25°C for a few weeks until needed. Ascospores were produced following the protocol of Li et al. (2004). Briefly, infected stubble pieces were immersed in 10 ml of sterile deionized (DI) water in covered Petri plates for 1 h at 25°C under fluorescent light and then maintained at 4°C in the dark for 1 h. Ascospores were collected by washing them off the inside of the Petri plate lids with DI water. The concentration of released ascospores was measured using a hemocytometer (Superior Marienfeld).

**Selection of carrier materials.** In total, 15 different carrier materials were selected to determine their effectiveness as potential spore carriers and any direct effects of the materials upon spore survival. These were metals (aluminum, brass, corrugated iron sheet, galvanized steel, painted steel, and zinc) fabrics (denim, fleece, silk, and fiber polyester) woods (*Pinus radiata* [pine] and *Eucalyptus regnans* [Tasmanian oak]), and miscellaneous (glass, jute, and plastic). These were selected as test materials because they are common, everyday materials used around farms or used in everyday life, found around farms or associated in one way or another in the cargo and transport industry, or commonly used by travelers.

**Inoculations of carrier materials and measurement of survival of *L. maculans* ascospores over time at different temperatures.** The sterile test materials were organized in sterile Petri dishes and inoculated according to Barua et al. (2017a). Inoculations of carrier materials and measurement of survival of *L. maculans* ascospores are defined as the materials and determination of viability.

Recovery of *L. maculans* ascospores from inoculated carrier materials and determination of viability. The ascospores were recovered from the carrier materials and the numbers and their viability were assessed according to Barua et al. (2017a). Briefly, ascospores were recovered from the carrier materials by adding 800 ml of 0.1% Tween 20 directly to each well of the treatment plates and placing the plates on a rotary shaker for 40 min at 700 rpm. After washing, the carrier materials were removed from the residual ascospore suspension and prepared for microscopy. The Alamar Blue bioassay was optimized as a viability indicator for ascospores of *L. maculans* and the optimum time for maximum metabolic activity was 2 h. *L. maculans* ascospore suspensions, ranging in concentration from 10<sup>7</sup> ml<sup>-1</sup> to 10<sup>1</sup> ml<sup>-1</sup> by 10-fold serial dilution, were used as comparison standards. Three replicate wells were used for each concentration and were set up using 100 μl of fresh ascospores for the standards, with 20 μl of Alamar Blue reagent. In the same way, residual ascospore solutions after washing were set up with 100 μl of residual solution or wash suspension containing ascospores with 20 μl of Alamar Blue reagent. Negative controls contained 100 μl of DI water and 20 μl of Alamar Blue reagent to determine the extent of any background absorbance. The absorbance at 600 nm (A600) and 570 nm (A570) was measured using a spectrophotometer (Thermo Scientific Multiskan Spectrum). The average of the background A600 for control wells was subtracted from all A570 of experimental wells. A standard curve of A570 nm minus A600 nm versus concentration of ascospores was plotted and used to calculate the percentage viability of *L. maculans* ascospores in residual solutions after washing. The percentage of viable ascospores was determined for each replicate using the regression equation from the standard curve, and the mean was calculated from the six replicates. The correlation between the germinated ascospores and the viability assay was also determined.

**Observation of germination of ascospores on test materials.** Fresh ascospores as controls were germinated on half-strength potato dextrose agar (1/2 PDA) to observe the percent viability under a compound microscope at regular time intervals of 6, 12, 24, 48, and 72 h. Similarly, using the same time intervals, the number of ascospores germinated on the test carrier materials was also calculated. Ascospore suspensions recovered after washing the test materials until no further germinated spores were recovered from the inoculated materials. Any nongerminated intact or broken ascospores recovered from materials after washing were also plated on 1/2 PDA to assess their ability to germinate. The germination of the treated ascospores on the materials or on 1/2 PDA was compared with germination of the fresh ascospores used as a control, with maximum germination of the control ascospores at 90% by 48 h and no further increase by 72 h.

**Experimental design and data analyses.** All experiments were arranged using a completely randomized design, repeated twice, with six replications for each of the three temperatures. The relationship between the initial and repeat experiments was assessed using a paired *t* test using GenStat and for homogeneity of variances across the original and repeat experiments using Bartlett’s test (Snedecor and Cochran 1989). In each case, there were no significant differences between each pair of experiments (P > 0.05) using *t* test and variances were similar using Bartlett’s test; therefore, the data sets were pooled, reanalyzed, and presented as a single data set.

**Results**

At 23 and 8°C (day and night, respectively), viable ascospores were recovered for up to 240 days from Tasmanian oak and pine; 180 days from jute and from painted steel; 150 days on silk; 120 days on corrugated iron sheet, denim, and glass; 90 days on plastic, 60 days from fiber polyester and brass; 30 days from fleece; and up to 21 days from galvanized steel. At 36 and 14°C (day and night, respectively), viable
ascospores were recovered for up to 180 days from pine; 90 days from painted steel; 60 days on aluminum, fiber polyester, jute, and zinc; 30 days on brass, fleece, and Tasmanian oak; 21 days on denim and silk; 14 days on corrugated iron sheet; up to 7 days on plastic; and up to 6 days on galvanized steel and glass. At 45 and 15°C (day and night, respectively), viable ascospores were recovered for up to 60 days on jute; 21 days on denim and silk; 14 days on fiber polyester; 7 days on pine; 6 days on plastic, painted steel, and Tasmanian oak; 5 days on corrugated iron sheet, glass, and zinc; 4 days on aluminum and fleece; and up to 3 days on brass and 2 days on galvanized steel.

Specific differences in decrease in ascospore viability for each carrier material are provided as follows.

**Metals.** At 23 and 8°C (day and night, respectively), after 1 day, the viability of the ascospores decreased by 70% on aluminum, galvanized sheet, and zinc; 75% on painted steel; 80% on corrugated iron sheet; and 90% on brass. Ascospore viability for each type of metal carrier material decreased by 99% within a time period of 180 days. By day 7, there was a decline in viability of the ascospores up to 85 to 90% on all the metals at 23 and 8°C (day and night, respectively). At 36 and 14°C (day and night, respectively), ascospore viability declined by 75% on aluminum and zinc; 90% on brass, corrugated iron sheet, and painted steel; and 98% on galvanized sheet after day 1. A 99% decrease in spore viability was observed by day 90 on painted steel, day 60 on zinc, day 30 on aluminum and brass, day 14 on corrugated iron sheet, and day 6 on galvanized sheets, with no further viability observed subsequently. The percentage of viable spores at 45 and 15°C (day and night, respectively) decreased by 81% on zinc; 90% on aluminum, corrugated iron sheet, and painted steel; and 99% on brass and galvanized sheet after day 1. Viability of spores decreased by 98% at
6 days on painted steel and at 4 days on aluminum and 99% at day 5 on zinc and day 1 on brass and galvanized sheet (remained 99% till day 3 and 2, respectively), with no further viability observed subsequently (Fig. 1).

**Fabrics.** At 23 and 8°C (day and night, respectively), the viability of spores from silk decreased by 45% on silk, 62% on denim and fiber polyester, and 65% on fleece material after day 1. The viability of the ascospores decreased by 99% on day 150 on silk, day 120 on denim, day 60 on fiber polyester, and day 30 on fleece, with just 1 week with no viable spores recovered subsequently. At 36 and 14°C (day and night, respectively), viability of ascospores after day 1 decreased by 62% on silk, 70% viability on denim and fiber polyester, and 85% on fleece. The viability of the ascospores decreased to 99% by day 60 on fiber polyester, day 30 on fleece, and day 21 on denim and silk, with no viable spores recovered subsequently. At 45 and 15°C (day and night, respectively), the viability of the ascospores decreased by 86% on silk, 90% on denim and fiber polyester, and 96% on fleece after day 1, with a further decrease to 99% by day 21 on denim and silk, day 14 fiber polyester, and day 4 on fleece, with no viability recorded subsequently after these time periods (Fig. 2).

**Woods.** At 23 and 8°C (day and night, respectively), the viability of the ascospores decreased by 39% on pine wood and by 49% on Tasmanian oak wood after day 1, with a further decrease in viability by 97% by day 240 on both pine and Tasmanian oak, with no further viability observed subsequently. At 36 and 14°C (day and night, respectively), viability of ascospores after day 1 decreased by 68% on pine wood and by 70% on Tasmanian oak wood, with 99% decrease in viability on day 60 on pine and day 30 on Tasmanian oak. At 45 and 15°C (day and night, respectively), the viability of the ascospores decreased by 81% on pine and 83% after day 1, with a further decrease in viability by 99% on day 7 on pine and 97% on Tasmanian oak on day 6, with no viability recorded subsequently (Fig. 3).

**Miscellaneous.** At 23 and 8°C (day and night, respectively), the viability of the ascospores decreased by 57% on jute, 69% on glass, and 71% on plastic. A 99% decline in viability was observed by day 180 on jute, day 120 on glass, and day 90 on plastic, with no viability recorded after that. At 36 and 14°C (day and night, respectively), viability of ascospores after day 1 decreased by 75% on jute, 93% on glass, and 94% on plastic, with 99% decrease in viability by day 60 on jute, day 7 on plastic, and day 6 on glass, with no further viability recorded. At 45 and 15°C (day and night, respectively), the viability of the ascospores decreased by 89% on jute and 97% on glass and plastic after day 1, with further decrease in viability by 97% on day 7 on jute and 99% on glass and plastic on day 5 and 6, respectively, with no further viability detected (Fig. 4).

**Observation of germination of ascospores on test materials.** The mean maximum germination of the control ascospores was 90% and obtained within a time period of 24 to 48 h on 1/2 PDA (Table 1). There was no change in the germination of control ascospores after that time point. In comparison with control ascospore germination, ascospore germination was 1% on metals, 5% on wood, 5% on fabric, and 2% on miscellaneous materials, up to 48 h. Maximum germination of the nongerminated intact or broken ascospore suspension recovered after washing or recovery from the different material groups onto 1/2 PDA was 30% from metals, 50% from woods, 51% from fabrics, and 41% from the miscellaneous materials for the 48-h time period (Table 1). There was strong positive

**Fig. 2.** Maximum percentage of viable *Leptosphaeria maculans* ascospores recovered from fabrics (denim, fleece, silk, and fiber polyester) at three different temperature regimes (Temp 1 = 23 and 8, Temp 2 = 36 and 14, and Temp 3 = 45 and 15°C, day and night, respectively) and the ability of the materials to retain or release viable ascospores over time. Regression analysis was undertaken with a logarithmic model \(\log_2\) to define trend lines for each test material at each temperature regime. Parameters that define slope, y-intercept, asymptotes, and \(R^2\) are presented for each test material for each temperature regime.
correlation between the germinated ascospores and the viability assay \(y = 0.9901x + 1.2694, P < 0.001, R^2 = 0.99\).

**Discussion**

This is the first study to confirm the long-term viability of *L. maculans* ascospores on such a wide range of different carrier materials and under different temperature conditions. These results highlight the potential for dissemination of *L. maculans* on inert carrier materials and, in particular, the role of these materials as an alternative way to spread natural ascospore inoculum to new areas, in the absence of any host plant tissues. At least 30% of intact ascospores recovered from inert carrier materials were able to germinate on artificial growth media within 48 h of recovery and some ascospores were still viable after 240 days. Even broken ascospores, following their natural fragmentation at septae, still germinated, particularly from the nonterminal end cells of ascospores. These findings are

![Fig. 3.](image1.png)

Fig. 3. Maximum percentage of viable *Leptosphaeria maculans* ascospores recovered from woods (*Pinus radiata* [pine] and *Eucalyptus regnans* [Tasmanian oak]) at three different temperature regimes (Temp 1 = 23 and 8, Temp 2 = 36 and 14, and Temp 3 = 45 and 15°C, day and night, respectively) and the ability of the materials to retain or release viable ascospores over time. Regression analysis was undertaken with a logarithmic model (log2) to define trend lines for each test material at each temperature regime. Parameters that define slope, y-intercept, asymptotes, and \(R^2\) are presented for each test material for each temperature regime.

![Fig. 4.](image2.png)

Fig. 4. Maximum percentage of viable *Leptosphaeria maculans* ascospores recovered from miscellaneous materials (glass, jute, and plastic) at three different temperature regimes (Temp 1 = 23 and 8, Temp 2 = 36 and 14, and Temp 3 = 45 and 15°C, day and night, respectively) and the ability of the materials to retain or release viable ascospores over time. Regression analysis was undertaken with a logarithmic model (log2) to define trend lines for each test material at each temperature regime. Parameters that define slope, y-intercept, asymptotes, and \(R^2\) are presented for each test material for each temperature regime.
should exacerbated the disease epidemiology.

Intact ascospores were previously known to have some resilience to unfavorable conditions. For example, Huang et al. (2003) showed that, ascospores of L. maculans could survive longer than 1 month when exposed to dry air at 20°C and Barua et al. (2017a) showed that viability declined rapidly even at favorable conditions of 23 and 8°C. Furthermore, Huang et al. (2003) found that ascospore viability and infective capabilities were degraded over several weeks by moderately high temperatures (up to 20°C) or lower relative humidity. Savage et al. (2012) were the first to demonstrate that robustness of ascospores provides a distinct advantage and enables L. maculans to have a distinct diurnal pattern, with a mid- to late-afternoon peak in ascospore release in Australia coinciding with meteorological conditions of warmer temperatures and lower humidity, because these are more conducive to their greater dispersal over larger distances than for analogous release at other times of the day. Current studies highlight the significant differences between temperature regimes in terms of survival of L. maculans ascospores and the combined effects of type of carrier material on the length of prolonged survival. For example, in the current study, at 23 and 8°C (day and night, respectively), L. maculans ascospores remained viable on pine wood and Tasmanian oak for up to 240 days. In contrast, at 36 and 14°C, ascospores could remain viable for up to 180 days but only on pine wood whereas, at 45 and 15°C, ascospores remained viable for only up to 60 days and only on jute.

The current study has not only demonstrated far greater resilience of L. maculans ascospores than previously reported but also has highlighted the “more favorable” inert materials that support this resilience. Clearly, there is a greater risk than previously anticipated for more widespread dissemination of existing as well as new or different L. maculans races into new areas, as a consequence of ascospores remaining intact and viable for long periods in the absence of a susceptible host. Minimizing development and spread of new or different L. maculans races is of critical importance to the long-term viability of the oilseed rape industries around the world (Balesdent et al. 2005), because their spread and build-up drives boom-and-bust cycles of effective and failed host resistance, respectively, both in Australia (Balesdent et al. 2005; Li et al. 2003, 2005, 2008) and elsewhere, such as in France (Roussel and Balesdent 2017).

The current studies also highlight the threat of dispersion not only of L. maculans as an important plant pathogen but also, potentially, other important ascomycete plant and animal or human pathogens. Destructive ascomycete plant pathogen examples include wheat scab and Dutch elm disease, while human ascomycete pathogens include the etiological agent of thrush, the yeast Candida albicans, and the dermatophytes causing athlete’s foot (Berbee 2001). Hence, the current study also emphasizes the broader biosecurity implications in relation to the overall transport and movement of ascospores via these carrier materials, highlighting significant potential for ascomycete pathogens in general being spread widely in association with movement of humans, farming equipment, clothing, and commodities across regions and continents. The relative risks will be commensurate with the relative abilities of the different carrier materials to retain ascospores, the relative survival capabilities of different ascomycete pathogens, and the relative comparative risks of other pathways of entry such as imported infected seed or seed infested with crop residues. That not all L. maculans ascospores could be removed from the carrier materials even after washing the materials highlights the challenges in eliminating ascomycete ascospore contamination from equipment, clothes, travelers, wood, crafts, and toys. Furthermore, it highlights to local, national, and international biosecurity agencies that the risk of spread of ascomycete plant, animal, and human pathogens via inert materials are significantly greater than current assessments suggest.

Acknowledgments

We thank R. Creasy and B. Piasini from the University of Western Australia for support and help with these controlled environment studies.

Literature Cited


Table 1. Germination (%) of ascospores of Leptosphaeria maculans on different test materials and on 1/2 strength potato dextrose agar (1/2 PDA) over 48 h.

<table>
<thead>
<tr>
<th>Germination (%) per time period</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germination of ascospores on materials</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metals</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Wood</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Fabric</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Germination of ascospores recovered from materials on 1/2 PDA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metals</td>
<td>10</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Wood</td>
<td>15</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Fabric</td>
<td>15</td>
<td>50</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>10</td>
<td>38</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>Germination of control ascospores on 1/2 PDA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>87</td>
<td>90</td>
<td>90</td>
</tr>
</tbody>
</table>

Data pooled across three repeated experiments with least significant difference (LSD) at P < 0.05 = 0.54 (germination), 0.36 (time), and 1.07 (germination-time). Significance: P < 0.001 for germination, time, and germination-time.


CHAPTER 4

Long-term viability of the northern anthracnose pathogen, *Kabatiella caulivora*, facilitates its transportation and spread.
Long-term viability of the northern anthracnose pathogen, *Kabatiella caulivora*, facilitates its transportation and spread

P. Barua<sup>ab</sup>, M. P. You<sup>a</sup>, K. L. Bayliss<sup>c</sup>, V. Lanoiselet<sup>d</sup> and M. J. Barbetti<sup>ab</sup>*

<sup>a</sup>School of Agriculture and Environment and the UWA Institute of Agriculture, Faculty of Science, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009; <sup>b</sup>Plant Biosecurity Cooperative Research Centre (PBCRC), Bruce, ACT 2617; <sup>c</sup>School of Veterinary and Life Sciences, Murdoch University, South Street, Murdoch, WA 6105, and <sup>d</sup>Department of Agriculture and Food Western Australia, 3 Baron-Hay Court, South Perth, WA 6151, Australia

The conidia and resting hyphae of the northern anthracnose pathogen of *Trifolium* species, *Kabatiella caulivora*, were effectively carried by, and maintained long-term viability on, a range of materials, including metals, fabrics, woods and plastics. Conidia and hyphae became thick-walled and melanized with time. There were significant (*P* < 0.001) differences in conidia/resting hyphae survival between carrier materials and between temperature regimes. At 23 °C/8 °C day/night, conidia and resting hyphae remained viable on steel, corrugated iron, galvanized steel, all tested fabrics, wood and random mixed materials for up to 8 months. At 36 °C/14 °C day/night, conidia and resting hyphae remained viable for up to 8 months, but only on cotton, denim, fleece, silk, leather, paper, plastic and all wood materials. At 45 °C/15 °C day/night, conidia and resting hyphae remained viable up to 8 months only on fleece wool, *Eucalyptus marginata* (jarrah wood) and paper. There were significant differences between carrier materials in their abilities to retain conidia and resting hyphae after washing (*P* < 0.001). Metabolic activity was confirmed for conidia and resting hyphae recovered after 8 months and *K. caulivora* colonies successfully re-established on potato dextrose agar. Findings confirmed the critical importance of materials as long-term carriers of viable *K. caulivora* conidia and resting hyphae, highlighting the potential for spread of a highly virulent *K. caulivora* race within and outside Australia via farming equipment, clothing and other associated materials. Results also have wider biosecurity implications for the transportation of fungal-infested carrier materials previously considered as low risk.

**Keywords:** carrier materials, conidial survival, *Kabatiella caulivora*, northern anthracnose

Introduction

In the era of increased personal and business travel, military deployments and exercises, sporting events and trade in commerce, the risk of inadvertent introduction of exotic fungal pathogens, particularly by spores, via introduction of contaminated equipment and commercial goods into a country or an area is increasing (Hovmøller et al., 2008; McKirdy et al., 2012). While pathogens may be introduced through flower, seed and plant product imports, and also by machinery movement and natural phenomena, their entry is not limited to these pathways only (McKirdy et al., 2012). Further, while fungal spores may be primarily dispersed by aerial or rain splash, dispersal can also occur through human activities allowing pathogens to spread locally or to new geographic areas (Fitt et al., 1989; Roper et al., 2010; Savage et al., 2012), playing a critical role in disease spread and development.

There is a wide range of potential ‘carrier materials’, widely used in many different situations, that could enable entry and spread of exotic fungal spores; these include fabrics, metals, paper, rubber tyre, leather, wood and building materials (Whinam et al., 2005; Sterflinger, 2010). For example, Oszczka et al. (2012) isolated four species of perithecia-forming Ascomycota, five species of corticioid and polyporoid Basidiomycota, and four unidentified sterile mycelia from basidiomycete taxa from timber, with some ascocarps containing vital spores. Furthermore, Hill et al. (1995) isolated 38 species of fungi from timber flooring of freight containers, including species such as Alternaria, Aspergillus, Eurotium, Mucor, Penicilium, Phoma and Trichoderma. Fungal contamination is also a major problem on library books, paper sacks and fibreboard cartons and also in food packing materials (Tindale et al., 1989; Zyska, 1997; Rakotoni-rainy et al., 2003). Although there has been research into carrier materials, most of these investigations were related to live materials such as plant germplasm, fruits, seeds and pollens (Vanneste et al., 2011) rather than inert materials. Current data regarding materials such as metal, plastics, fabrics and paper etc. as spore carriers is extremely limited. The relative risk of incursion of pathogenic fungal spores with different materials needs urgent assessment as resulting data is needed to guide the biosecurity and quarantine sectors to take appropriate precautions and action.

*E-mail: martin.barbetti@uwa.edu.au*
Northern anthracnose, also known as ‘clover scorch’ in Australia, caused by the fungus Kabatiella caulivora, is of major economic importance in the temperate regions of the world, particularly in Asia, North America, Europe and Australia (Beale, 1972; Johnsson, 1975; Barbetti & Sivasithamparam, 1986; Barbetti, 1996). The disease remains a major limitation to Trifolium spp. forages (Johnstone & Barbetti, 1987; Barbetti, 1989), particularly reducing herbage yield, hay and seed production of annual subterranean clover, Trifolium subterraneum (Johnsson, 1975; Bokor et al., 1978; Anderson et al., 1982). In highly susceptible varieties such as Wooramullup and Yarloop, seed yield reductions of 90% or more have been measured (Barbetti, 1989; You et al., 2007). Reductions of about 30% in grazing capacity and up to 50% in whole-farm hay production have also been attributed to clover scorch during seasons favourable for disease development (Barbetti, 1989; You et al., 2007). High rainfall areas of southwestern and southern Australia remain severely affected despite the widespread uptake by farmers of varieties with improved resistance (Nichols et al., 2014).

The disease progresses rapidly at temperatures of 18 to 25 °C, with low light intensity and wet conditions (Leach, 1962; Beale, 1972). Kabatiella caulivora produces conidia by budding and forms yeast-like colonies (Berkenkamp, 1969) with the conidia primarily spread by wind-driven rain splash, livestock and also by attachment to hay cutting equipment (Beale, 1972; You et al., 2007; Barbetti & You, 2014) and in infested hay or seed (Beale, 1972; Bokor et al., 1978; You et al., 2007). Conidia can change form and colour over time, producing several unusual features, such as thick melanized conidia and thick-walled hyphae (Colotelo & Grinchenko, 1962). Past investigations have demonstrated that the conidia of K. caulivora can survive a wide range of temperature conditions, from 3 to 24 °C, for at least 150 days, and that they can be stored for more than 3 months at −20 °C (You et al., 2005) and for 20 months at −10 °C (Cole, 1957). Kabatiella caulivora also forms thick-walled resting hyphae, presumably for long-term survival (Bayliss et al., 2003), allowing it to survive in stored infested seed and in dead infested plant residues for at least 18 months (Kellock, 1971; Beale, 1972), and enabling it to persist from one annual growing season to the next (Johnstone & Barbetti, 1987).

The pathogen invades, predominantly through the peti- olules, causing ‘turning’ of the leaflets to expose the under surface (Bokor, 1972; Bayliss et al., 2001; Barbetti & You, 2014), the characteristic symptom of K. caulivora infection on subterranean clover (Barbetti, 1989) and red clover (Sampson, 1928). Brown to black lesions become visible on the petiolules, petioles, stems, runners and peduncles, destroying the vascular tissues and causing leaves to wilt and collapse (Barbetti & You, 2014).

The disease was initially controlled in Australia by sowing resistant subterranean clover varieties (Bayliss et al., 2002; Nichols et al., 2014). While a single race of K. caulivora, race 1, was predominant in the clover-growing regions of Australia for decades (Bayliss et al., 2001), a new race, race 2, was identified in 1990 in Western Australia that has devastated fields of subterranean clover varieties such as Junee and Esperance, previously resistant to race 1 (Barbetti, 1995; Barbetti & You, 2014). Race 2 continues to spread within Western Australia and will probably in future spread across southern Australia, and potentially outside of Australia (Barbetti & You, 2014). However, although K. caulivora is known to survive in infested seed and dead infested plant residues, there is no information about its survival and/or dispersal via inert carrier materials and under different environmental conditions, despite such information being particularly relevant for curtailing the spread of race 2 both inside and outside of Australia. Hence, the aim of this study was to investigate the viability of K. caulivora conidia and resting hyphae on a range of different carrier materials and under different temperature conditions to determine their potential to foster or impede future spread of new races of K. caulivora inside and outside of Australia.

Materials and methods

Kabatiella caulivora inoculum

A single isolate of K. caulivora race 2, WAC5757 (Barbetti, 1995), was used. Lyophilized cultures in glass ampoules were rehydrated using sterile deionized water and plated onto potato dextrose agar containing 100 mg L⁻¹ of aureomycin hydrochloride. Cultures were maintained at 15 °C and subcultured every 5–8 days to maintain the budding, yeast-type form required for inoculation (Cole & Couch, 1959; Barbetti, 1995). A platinum wire loop of conidia was scraped from each culture and used to inoculate 100 mL of sterile malt extract broth in 250 mL Erlenmeyer flasks that were incubated at 20 °C on a rotary shaker at 150 rpm for 72 h. The conidial suspensions were filtered through four layers of muslin cloth to remove any hyphae. The concentration of conidia was determined using a haemocytometer counter (Superior Marienfeld) and the suspensions stored at −20 °C until needed. Prior to inoculation, conidia were resuspended in 0.01% Tween 20 and adjusted to a concentration of 10⁶ conidia mL⁻¹ using a haemocytometer counting chamber.

Selection of carrier materials

A total of 20 different carrier materials were selected to determine both their effectiveness as potential conidia carriers per se and also any direct effects of the materials upon conidial survival. These were: metals – aluminium, brass, corrugated iron sheet, galvanized steel, steel, zinc; fabrics – cotton, denim, fleece, silk, fibre polyester; woods – Eucalyptus marginata (jarrah), Pinus radiata (pine), Eucalyptus regnans (Tasmanian oak); and miscellaneous – glass, leather, jute, paper, plastic, rubber tyre. These were selected as test materials as they are materials commonly used on farms, and/or associated with commercial and farm transport and/or used by travellers.
Inoculations of carrier materials and measurement of survival of *K. caulivora* over time at different temperatures

The 20 materials for inoculation were cut into 0.5 cm squares and randomly placed into rows in a sterile 48-well cell culture plate (Greiner sterile) along with an equal number for non-inoculated control treatments. The material in each well was inoculated individually with 10 μL (10⁶ conidia mL⁻¹) of inoculum and allowed to dry in a laminar flow for 2–3 h. All sides of the inoculated culture plates were sealed with Parafilm and they were placed under one of the three controlled environmental conditions: 23 ± 1 °C day/8 ± 1 °C night, 36 ± 1 °C day/14 ± 1 °C night, or 45 ± 1 °C day/15 ± 1 °C night, with a photoperiod of 14 h from a light source consisting of cool white LEDs along with incandescent light bulbs with an overall intensity of 250 μmol m⁻² s⁻¹, 260 μmol m⁻² s⁻¹, 320 μmol m⁻² s⁻¹, respectively (Quantum Flux MQ100; Apogee). There were six replicates for each carrier material, for every sampling time and for each temperature treatment. A fully randomized design was used. Over a period of 8 months, samples were taken to assess the effect of test carrier materials and temperature on viability of conidia, and whether thick-walled melanized conidia and thick-walled hyphae developed over time. The ability of the test carrier materials to retain or release conidia, thick-walled melanized conidia and hyphae was assessed daily from day 1 until day 7, then weekly until day 30 (month 1), after which sampling was undertaken at regular 30 day intervals concluding at day 240 (month 8). The entire experiment was fully repeated twice.

Recovery of *K. caulivora* conidia and hyphae from inoculated carrier materials and determination of viability of conidia

The viability of conidia and hyphae was assessed using an Alamar Blue resazurin dye (7-hydroxy-3H-phenoxazin-3-one 10-oxide; Barua et al., 2017). Briefly, conidia and resting hyphae were recovered from the carrier materials by adding 800 μL of 0.01% Tween 20 directly to each well of the treatment plates and placing plates on a rotary shaker for 40 min at 700 rpm. After washing, the carrier materials were removed from the residual conidial suspension and prepared for microscopy. Initially, the Alamar Blue bioassay was optimized as a viability indicator for conidia of *K. caulivora* and the optimum time for maximum metabolic activity was determined to be 2 h. Kabatiella caulivora conidial suspensions, ranging in concentration from 10⁻⁷ mL⁻¹ to 10 mL⁻¹ by 10-fold serial dilution, were set up in 96-well assay plates and used as comparison standards. Three replicate wells were used for each standard concentration and contained 100 μL of fresh conidial suspension with 20 μL of Alamar Blue reagent. In the same way, 20 μL of Alamar Blue reagent was added to wells containing 100 μL of the conidial suspensions that remained after washing. Negative controls contained 100 μL of deionized water and 20 μL of Alamar Blue reagent to determine the extent of any background absorbance. The absorbance at 600 nm and 570 nm was measured using a spectrophotometer (Thermo Scientific Multiskan Spectrum). The average of the background absorbance value (A) at 600 nm for control wells was subtracted from all absorbance values of experimental wells at 570 nm. A standard curve of A₅₇⁰ nm minus A₆₀₀ nm versus concentration of conidia was plotted and used to calculate the percentage viability of *K. caulivora* conidia in residual solutions after washing. The conidial suspensions were filtered through muslin cloth to remove any hyphae. The percentage of viable conidia was determined for each replicate using the regression equation from the standard curve, and the mean was calculated from the six replicates.

**Observation of conidia germination, melanized conidia and hyphae**

The carrier materials removed from the residual conidial suspensions after washing at each time interval were prepared for scanning electron microscopy (SEM) studies to observe the attachment of conidia and hyphae on different carrier materials. Materials were fixed, dehydrated, dried and mounted on metal stubs following the standard SEM sample preparation technique from the Centre for Microscopy, Characterization and Analysis (CMCA) and Leser et al. (2009). The materials were coated with gold, platinum and carbon to increase conductivity.

Light microscopy studies were carried out to observe germination of conidia as well as formation of thick-walled melanized conidia and resting hyphae. A 50 μL-droplet of each residual solution after washing the carrier materials was placed on a glass microscope slide and covered with a glass cover slip. The percentage of hyaline conidia, melanized conidia and thick-walled melanized hyphae was determined using a BX51 (Olympus) microscope. There were three replications for each sample. The time period for maximum percentage germination of conidia was also assessed. The hyphae derived from the carrier materials were rehydrated in 500 μL of sterile deionized water for 6 h and plated on potato dextrose agar media, maintained at 20 °C, and observed for 7 days to monitor viability and growth of the hyphae.

**Experimental design and data analysis**

All the test carrier material treatments in the experiments were arranged in a completely randomized design with numbers of replications as indicated above. All experiments were fully repeated twice and the relationship between the initial and repeat experiments assessed by a paired t-test using GENSTAT (15th edition, GenStat Procedure Library Release PL23.2). Where there were no significant differences between experiments (P > 0.05), the datasets from the two most similar experiments were pooled, reanalysed and presented as a single dataset. Single and multiple factor ANOVA were conducted using GENSTAT to determine the effects of time and temperature on the viability of the *K. caulivora* conidia across a period of 8 months and to determine the rate of melanization of the conidia and hyphae over a period of 7 days.

**Results**

**Effect of temperature and time on recovery of viable conidia and hyphae of *K. caulivora* from inert materials**

The average percentage of viable conidia recovered from each carrier material varied with the temperature and the type of carrier material (P < 0.001). Viable conidia and hyphae were recovered for up to 8 months from all materials incubated at 23 °C/8 °C day/night except aluminium, zinc and brass, which remained viable for only 6 months, 5 months and 21 days, respectively (Table 1).
Table 1: Maximum period of viability of Kabatiella caulivora conidia observed on different carrier materials over 8 months, under three different controlled temperature regimes

<table>
<thead>
<tr>
<th>Material</th>
<th>Temperature</th>
<th>23 °C/4 °C day/night</th>
<th>36 °C/14 °C day/night</th>
<th>45 °C/15 °C day/night</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aluminium</td>
<td>6 months</td>
<td>3 months</td>
<td>3 months</td>
<td></td>
</tr>
<tr>
<td>Brass</td>
<td>21 days</td>
<td>14 days</td>
<td>2 days</td>
<td></td>
</tr>
<tr>
<td>Corrugated iron</td>
<td>&gt;8 months</td>
<td>2 months</td>
<td>21 days</td>
<td></td>
</tr>
<tr>
<td>Galvanized steel</td>
<td>&gt;8 months</td>
<td>14 days</td>
<td>6 days</td>
<td></td>
</tr>
<tr>
<td>Steel</td>
<td>&gt;8 months</td>
<td>2 months</td>
<td>2 months</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>5 months</td>
<td>2 months</td>
<td>2 months</td>
<td></td>
</tr>
<tr>
<td>Fabric</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotton</td>
<td>&gt;8 months</td>
<td>&gt;8 months</td>
<td>5 months</td>
<td></td>
</tr>
<tr>
<td>Denim</td>
<td>&gt;8 months</td>
<td>&gt;8 months</td>
<td>6 months</td>
<td></td>
</tr>
<tr>
<td>Fleece</td>
<td>&gt;8 months</td>
<td>&gt;8 months</td>
<td>&gt;8 months</td>
<td></td>
</tr>
<tr>
<td>Silk</td>
<td>&gt;8 months</td>
<td>&gt;8 months</td>
<td>6 months</td>
<td></td>
</tr>
<tr>
<td>Fibre polyester</td>
<td>&gt;8 months</td>
<td>7 months</td>
<td>4 months</td>
<td></td>
</tr>
<tr>
<td>Wood</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jarrah</td>
<td>&gt;8 months</td>
<td>&gt;8 months</td>
<td>&gt;8 months</td>
<td></td>
</tr>
<tr>
<td>Pine</td>
<td>&gt;8 months</td>
<td>&gt;8 months</td>
<td>7 months</td>
<td></td>
</tr>
<tr>
<td>Tasmanian oak</td>
<td>&gt;8 months</td>
<td>&gt;8 months</td>
<td>7 weeks</td>
<td></td>
</tr>
<tr>
<td>Miscellaneous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glass</td>
<td>&gt;8 months</td>
<td>7 months</td>
<td>6 months</td>
<td></td>
</tr>
<tr>
<td>Leather</td>
<td>&gt;8 months</td>
<td>&gt;8 months</td>
<td>7 months</td>
<td></td>
</tr>
<tr>
<td>Paper</td>
<td>&gt;8 months</td>
<td>&gt;8 months</td>
<td>&gt;8 months</td>
<td></td>
</tr>
<tr>
<td>Plastic</td>
<td>&gt;8 months</td>
<td>&gt;8 months</td>
<td>6 months</td>
<td></td>
</tr>
<tr>
<td>Rubber</td>
<td>&gt;8 months</td>
<td>6 months</td>
<td>6 months</td>
<td></td>
</tr>
<tr>
<td>Jute</td>
<td>&gt;8 months</td>
<td>7 months</td>
<td>7 months</td>
<td></td>
</tr>
</tbody>
</table>

At 36 °C/14 °C day/night, the viability of conidia and hyphae was more variable, with conidia on brass and galvanized steel remaining viable for only 14 days, but conidia on other materials remaining viable for up to 8 months (Table 1). At 45 °C/15 °C day/night, the maximum time period of viability of conidia and hyphae was markedly reduced for most materials, but viability persisted for 8 months on fleece (Table 1).

Viability of conidia and hyphae of *K. caulivora* recovered from carrier materials over time and temperatures

The different groups of materials varied in their capacity to retain viable conidia and hyphae and this also varied according to the temperature at which the materials were maintained. The percentage of viable conidia recovered from materials of a particular group varied with the temperature. There were significant (*P* < 0.001) differences between carrier materials, over time and between temperatures, in terms of viability of conidia and hyphae (Fig. 1).

Metals

At 23 °C/8 °C day/night, the maximum percentage of viable conidia and hyphae recovered at 8 months was 0.3% from both corrugated iron and from steel, at 1 month it was 9.8% from steel and at 1 week it was 19.1% from steel (Fig. 1). At 36 °C/14 °C day/night, the maximum percentage of viable conidia and hyphae recovered at 6 months was 0.2% from steel, at 1 month it was 3.4% from aluminium and at 1 week it was 5.4% from aluminium. At 45 °C/15 °C day/night, the maximum percentage of viable conidia and hyphae recovered at 5 months was 0.2% from zinc, at 1 month it was 1.1% from steel and at 1 week it was 6.1% from corrugated iron (Fig. 1).

Fabrics

At 23 °C/8 °C day/night, the maximum viable conidia and hyphae recovered at 8 months was 10.9% from cotton, at 1 month it was 29.2% for cotton, while at 1 week it was 39% from fibre polyester. At 36 °C/14 °C day/night, the maximum viable conidia and hyphae recovered at 8 months was 0.3% from cotton, while at 1 month it was 18.4% from denim and at 1 week it was 19.1% from cotton. At 45 °C/15 °C day/night, the maximum viable conidia and hyphae recovered at 8 months was 0.2% from denim, while at 1 month it was 0.5% from silk and at 1 week it was 7.7% from fleece (Fig. 1).

Woods

At 23 °C/8 °C day/night, the maximum viable conidia and hyphae recovered at 8 months was 8.3% from jarrah wood, at 1 month it was 12.1% from Tasmanian oak and at 1 week it was 15% from Tasmanian oak. At 36 °C/14 °C day/night, the maximum viable conidia and hyphae recovered at 8 months was 5.1% from pine wood, at 1 month it was 9.6% from jarrah wood and at 1 week it was 10.7% from Tasmanian oak. At 45 °C/15 °C day/night, the maximum viable conidia and hyphae recovered at 8 months was 0.3% from jarrah wood, at 1 month it was 4.8% from Tasmanian oak and at 1 week it was 8.9% from Tasmanian oak (Fig. 1).

Miscellaneous

At 23 °C/8 °C day/night, the maximum viable conidia and hyphae recovered at 8 months was 0.5% from plastic and rubber tyre, at 1 month it was 19.1% from jute and at 1 week it was 26.1% from jute. At 36 °C/14 °C day/night, the maximum viable conidia and hyphae recovered at 8 months was 0.2% from plastic, at 1 month it was 3.6% from plastic and at 1 week it was 8.9% from rubber tyre. At 45 °C/15 °C day/night, the maximum viable conidia and hyphae recovered at 8 months was 0.2% from leather and paper, at 1 month it was 2.3% from plastic and at 1 week it was 7.7% from rubber (Fig. 1).

Observation of melanized conidia and resting hyphae

Light microscopy studies revealed that the number of hyaline conidia observed at the time of inoculation decreased from day 1 (25%) to day 7 (0%) and some (12%) of conidia degenerated (Table 2). Within 2 days of inoculation, geminated and non-germinated conidia (>70%) changed their colour and shape, then elongated.
and formed thick cell walls that became melanized and septate in the majority (65%) of cases. Many of the conidia germinated and formed thick-walled resting hyphae over time. Maximum average percentage (up to 55%) of germination of conidia was observed within 1–5 days after inoculation (dai) across all carrier materials. All conidia that germinated were melanized, but approximately 15% of the melanized conidia did not germinate. Thick-walled melanized hyphae were observed from day 1 and the percentage of hyphae increased with the increase in germination (Table 2). At day 7, >50% of germinated conidia, 15% of non-germinated conidia and 35% of resting hyphae developed obvious melanization (Table 2). Conidia produced germ tubes and then formed mycelial colonies on the surface of the various materials (Fig. 2a–f). The colour of these mycelial colonies varied and changed over time (Fig. 3a–c). The mycelial colonies were pale white to light olive coloured or light brown when beginning to form, but changed to darker brown or black from 15 dai onwards (Fig. 3a–c). The mycelia eventually formed thick-walled resting hyphae within 30 dai (Fig. 3). After 5 months, although hyphae did not show any metabolic activity immediately, when soaked in water over a 4–6 h period, the subsequent swollen hyphae demonstrated viability by changing the colour of the resazurin dye. Resting hyphae that were then transferred to potato dextrose agar germinated and produced several germ tubes and then branching mycelia, further confirming their viability. Scanning electron microscopy revealed that K. caulivora conidia and resting hyphae remained attached to each of the carrier materials even after washing (Fig. 2a–f).

**Discussion**

This is the first study to demonstrate that K. caulivora can remain viable on inert carrier materials at

![Plant Pathology (2017)](image-url)
temperatures up to 45 °C for up to 8 months. In the absence of a plant host, *K. caulivora* survived on tested carrier materials by forming thick-walled melanized conidia and resting hyphae. While most conidia changed size and shape, developed thick walls and became melanized within 1–2 days of inoculation on the carrier materials, the 12% of the conidia that remained hyaline all degenerated. Where conidia had become elongated, melanized and septate, they produced multiple germ tubes arising randomly; these and resting hyphae formed mycelial colonies. The melanized thick-walled conidia and resting hyphae had a clear survival advantage, especially under higher temperatures and for a much longer period than did conidia that remained hyaline and degenerated. The colour of the mycelial colonies varied from initially pale white or light green to light brown and finally to darker brown, and were heavily melanized and black from 15 dai onwards. Cole & Couch (1959) reported mycelial colonies of *K. caulivora* to vary in colour among and within colonies, from white to pink to olive-green, brown and black in many shades, especially across different growth media. While such studies detailed changes in colour and shape of conidia on different growth media and the formation of melanized, thick-walled, dark green conidia and even anastomosis of mycelium under limited nutrient availability, the current study is the first report of this occurring on inert carrier materials. Such cell wall thickening and melanization changes have been assumed to assist with the survival of the pathogen under adverse environmental conditions (Sampson, 1928; Cole, 1957; Colotelo & Grinchenko, 1962). The current study also demonstrated that melanized conidia and mycelia can form a hyphal mass on carrier materials, with mycelia becoming anastomosed and forming round thick resting hyphal coils. Formation of such hyphal coils may be a thigmotropic response of the pathogen to the surface of the carrier materials, and adverse environmental conditions have also been suggested as a cause of such hyphal coils (Bayliss *et al.*, 2001; Piéard *et al.*, 2007). These fungal coils were also proposed as a possible mechanism to overcome host resistance (Bayliss *et al.*, 2003). Furthermore, and perhaps more importantly, the current study demonstrated, for the first time, that these hyphal coils not only remain viable for up to 8 months, but that they can, without producing any conidia, 'germinate' to produce multiple germ tubes and branching mycelium on potato dextrose agar.

There were significant differences between temperature regimes in terms of survival of conidia and resting hyphae, and upon which carrier materials they survived the best. For example, at 23 °C/4 °C day/night, conidia and resting hyphae remained viable on many test
materials for up to 8 months, including steel, corrugated iron, galvanized steel and all tested fabrics, wood and random mixed materials. However, at 36 °C/14 °C day/night, conidia and resting hyphae remained viable for up to 8 months only on cotton, denim, fleece, silk, leather, paper, plastic and all the wood materials. In contrast, at 45 °C/15 °C day/night, conidia and resting hyphae remained viable up to 8 months only on fleece wool, E. marginata (jarrah wood) and on paper. Although disease epidemics develop best at lower temperatures and in wet weather, conidia and hyphae of the pathogen are known to survive long periods at high temperatures in infested residues and with the ability to subsequently initiate disease when conditions are suitable (Sigrianski & Minyaeva, 1937; Leach, 1962; Helms, 1977). Berkenkamp (1969) reported that production of conidia and disease development was best at cool temperatures between 12 °C and 20 °C. Anderson et al. (1982) highlighted a strong relationship between environment and disease development in subterranean clover forages, with mean temperatures of 11–17 °C and frequent rainfall favouring disease development. In contrast, others have reported that more severe disease symptoms occur at temperatures between 20 °C and 22.5 °C (Guerret et al., 2016) or at temperatures between 20 °C and 24 °C (Cole & Couch, 1959; Darunday & Hanson, 1967). Prolonged warm to hot sunny weather stops disease development (Anderson et al., 1982) and K. caulivora is inactivated when the temperature reaches 28 °C (Berkenkamp, 1969; Johnsson, 1975). However, in the current study, conidia and resting hyphae of K. caulivora could survive at temperatures up to 45 °C on fleece wool, E. marginata (jarrah wood) and on paper for a period of at least 8 months; this highlights the significant potential for K. caulivora conidia and resting hyphae to survive for long periods at much higher temperatures than previously considered possible from other epidemiological studies with this pathogen. These findings have major implications both for the length of effective K. caulivora carryover across multiple forage growing seasons and for domestic and international movement of people and commodities that can effectively carry and retain conidia and resting hyphae. This poses an environmental and economic biosecurity threat in regions where northern anthracnose, or a specific race(s) of it, does not yet occur.

Although there were significant differences between carrier materials, it is of concern that thick-walled melanized conidia and hyphae of K. caulivora remained attached to and viable on a range of carrier materials for
up to 8 months. Importantly, there were also significant differences between carrier materials in their abilities to retain conidia and resting hyphae after washing. It is of even greater concern that metabolic activity was confirmed for conidia and resting hyphae recovered after 8 months and that \textit{K. caulivora} colonies could be successfully re-established on potato dextrose agar from such spores. Even after two to three washes, not all conidia or resting hyphae could be removed from the carrier materials, highlighting the challenges in eliminating contamination by fungal spores and hyphae from farm machinery, clothes of regional, national and international travellers, etc. Such contamination allows transportation and spread of \textit{K. caulivora} into areas where it, or a specific race of it, is not yet present. For example, \textit{K. caulivora} race 2 is predominantly found in the Esperance region of Western Australian (Barbetti, 1996, 2007) but could quickly spread much further afield on inert materials such as farm equipment and clothing. Many of the varieties of subterranean clover with resistance against \textit{K. caulivora} race 1 are very susceptible to race 2 (Barbetti, 1995, 1996; Bayliss et al., 2002), and the threat of spread of race 2 more widely across southern Australia threatens more than 1 million hectares of varieties that currently have effective resistance against race 1 (M. J. Barbetti, unpublished data). Furthermore, the increasingly variable and changing climatic conditions will probably encourage development of further new races of \textit{K. caulivora} (Guerret et al., 2016), as is already happening with other fungal pathogens in Western Australia, such as \textit{Sclerotinia sclerotiorum} on oilseed rape (Uloth et al., 2015). Any such \textit{K. caulivora} isolates that were better adapted to warmer conditions would pose a potential threat not only to currently affected regions, but also to regions where the disease is presently unimportant due to current unfavourable environmental conditions. As already noted, attachment of any new highly virulent races to one or more of these carrier materials opens the way for their spread into new regions internal and external to Australia.

The present study confirms the critical importance of materials as long-term carriers of viable \textit{K. caulivora} conidia and resting hyphae. It highlights the threat posed by the long-term maintenance of viability of \textit{K. caulivora} conidia and hyphae on inert materials associated with movement of humans, farming equipment, clothing and commodities across regions inside and outside of Australia where race 2 is not yet present. Finally, the results of the current study have wider biosecurity implications in relation to the transportation of fungal-infested carrier materials in general, as they highlight an urgent need to re-evaluate potential carrier materials that have historically long been considered of low biosecurity risk for movement of fungal plant pathogens.

\textbf{Acknowledgements}

The authors greatly appreciate the scholarship provided by the Plant Biosecurity Cooperative Research Centre (PBCRC), Bruce, ACT 2617, Australia, for project CRC62042: ‘Curtailing and managing exotic fungal conidia incursion into Australia’, and the financial support by the School of Plant Biology at the University of Western Australia. The authors also acknowledge the facilities, and scientific and technical assistance from the Centre for Microscopy, Characterization and Analysis, University of Western Australia, particularly the wonderful assistance of Lyn Kirilak and Dr Peta Clode. The authors would like to thank Robert Creasy and Bill Pia- sini from the University of Western Australia for support and help with these controlled environment studies. All authors state that they do not have any conflict of interest to declare in regards to this paper.

\textbf{References}


CHAPTER 5

Extended survival of *Puccinia graminis* f. sp. *tritici* urediniospores: implications for biosecurity and on-farm management
Extended survival of *Puccinia graminis* f. sp. *tritici* urediniospores: implications for biosecurity and on-farm management

P. Barua\textsuperscript{ab}, M. P. You\textsuperscript{a}, K. L. Bayliss\textsuperscript{c}, V. Lanoiselet\textsuperscript{ad} and M. J. Barbetti\textsuperscript{ab}\textsuperscript{*}

\textsuperscript{a}School of Agriculture and Environment and the UWA Institute of Agriculture, Faculty of Science, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009; \textsuperscript{b}Plant Biosecurity Cooperative Research Centre (PBCRC), Bruce, ACT 2617; \textsuperscript{c}School of Veterinary and Life Sciences, Murdoch University, South Street, Murdoch, WA 6105; and \textsuperscript{d}Department of Agriculture and Food Western Australia, 3 Baron-Hay Court, South Perth, WA, 6151, Australia

*Puccinia graminis* f. sp. *tritici* (Pgt), the causal organism of stem rust, is of global importance across wheat-growing countries. However, some epidemics commence without the obvious presence of ‘alternate’ or ‘green bridge’ hosts, suggesting urediniospores can survive in the absence of suitable host plants for many weeks. Testing a range of inert material types, including metals, plastics, fabrics and woods, highlighted a significant effect of material type and temperature on urediniospore viability ($P < 0.001$), with urediniospores remaining attached and viable on these materials (aluminium, paper, rubber, all fabric and all woods) for up to 365 days at 23/8 °C day/night. At 36/14 °C day/night, urediniospore viability was retained for a maximum of 300 days on denim and jute. Furthermore, at 45/15 °C day/night, urediniospores remained viable for a maximum of 180 days on cotton and jute. The frequency of recovery of attached urediniospores was also dependent upon the material type, with significant differences between materials in their abilities to retain urediniospores after washing ($P < 0.001$). Urediniospores recovered even after 300 or 365 days from the lower two temperature regimes successfully initiated infections of wheat seedlings. Results confirm the potential importance of inert materials as long-term carriers of viable Pgt urediniospores, highlighting risks of spread of new pathotypes and strains across wheat-growing regions, the significant biosecurity implications for contaminated carrier materials, and its likely survival across seasons without a host.

**Keywords:** biosecurity, *Puccinia graminis* f. sp. *tritici*, urediniospore survival, wheat stem rust

**Introduction**

Stem rust of wheat, caused by *Puccinia graminis* f. sp. *tritici* (Pgt) is of major economic importance worldwide, with severe epidemic outbreaks reported in South Africa, China, East Africa, Europe, Canada and South America (Roelfs & Bushnell, 1985; Saari & Prescott, 1985; Kolmer, 2001; Kolmer et al., 2007; Morgounov et al., 2012). In the United States of America Pgt caused major wheat stem rust epidemics from 1900 to 1954 (Roelfs, 1978; Kolmer et al., 2007) with an average yield loss of 19.3% in South Dakota, 25.4% in Minnesota and 28.4% in North Dakota (Singh et al., 2015). Under suitable environmental conditions, yield losses to stem rust can be 70% or more, reaching up to 100% under extreme disease conditions (Saari & Prescott, 1985; Beard et al., 2004). In Australia, it has constituted a significant threat to cereal production since the early 1900s (Brennan & Murray, 1988; Loughman et al., 2005; McIntosh, 2007; Park, 2007) and is considered the most important wheat disease in Australia (Zwer et al., 1992).

Severe rust epidemics of 1889, 1899, 1947, 1950 and 1973 in Australia resulted not only in significant production losses (up to 100%) but also impacted on farmer welfare (Rees, 1972; Rees & Syme, 1981; Park, 2007). The warm, moist conditions there promote the development of stem rust and increase its severity (Beard et al., 2004).

Control measures for stem rust are based on genetic resistance, eradication of alternate hosts, application of fungicides and preventive agricultural practices. An annual protection cost of A$124 million has been estimated for control measures against stem rust in Australia (Brennan & Murray, 1988). Stem rust has been controlled during the last century through resistant varieties using resistance genes (e.g. *Sr31*) and, outside of Australia, by eradication of the alternate barberry host (Morgounov et al., 2012). Although eradication of the alternate host eliminates sexual recombination in Pgt, urediniospores are still dispersed and transported long distances as a primary source of inoculum (Kolmer et al., 2007). While foliar fungicide sprays can be useful in the early stages of the infection, the extent of yield loss depends on the resistance of the...
wheat variety (Beard et al., 2004). Despite the widespread development and use of resistant varieties, fungicides still contribute 41% of the total costs for overall management of rust (Murray & Brennan, 2009a,b). This is particularly so in the wheat stem rust-prone summer rainfall areas of northern New South Wales and Queensland, where prior to the introduction of such varieties, the likelihood of significant crop losses was about one year in four (McIntosh & Brown, 1997).

In 1998 the identification of a new race of Pgt, Ug99, in Uganda, saw a renewed threat to wheat-growing countries (Pretorius et al., 2000). Ug99 or its variants are virulent on many currently resistant varieties and can cause 80–90% crop losses under favourable conditions (http://www.fao.org/agriculture/crops/rust/stem/rust-report/stem-ug99race/). Since then, Ug99 and related races have been detected in Kenya, Ethiopia, Sudan, Yemen, Iran, Zimbabwe, Mozambique, South Africa and Tanzania (http://www.fao.org/agriculture/crops/rust/stem/rust-re-report/stem-ug99race/). Similar, a new race, TKTTF, emerged in Turkey in 1990 and has subsequently been detected in Iran in 2010, Ethiopia and Lebanon in 2012 and Egypt in 2013 (Singh et al., 2015). Should these Pgt races become established in Australia, stem rust would probably re-emerge as the single most important disease of wheat.

Pgt is a macrocyclic, heterocercous fungus that generally requires both primary (wheat or grasses) and alternate (Berberis or Mahonia spp.) host plants to complete its life cycle via sexual teliospores (Schumann & Leonard, 2000). In Australia, teliospores are nonfunctional in disease epidemics as no alternate hosts are found (Watson & Luig, 1958; Park, 2007). Hence, the pathogen only survives through generation and dispersal of asexual urediniospores and/or parasitic survival of mycelium on remnant living susceptible cereal hosts (e.g. wheat and triticale in particular, but also barley and some grasses; Park, 1996, 1997). These can provide a ‘green bridge’ for stem rust carryover from one season to another, with subsequent wind spread of urediniospores over large distances (Anonymous, 2017). For this reason, rust epidemics in Australia are often worse following wet summers that have supported the widespread growth of volunteers (Hollaway, 2016). In contrast, it is challenging to explain the occurrence of some rust outbreaks in the Mediterranean-type climatic region of southwest Western Australia. This region is characterized by hot dry summers and cool wet winters and it has long been suspected by the authors that urediniospores of various cereal and other rusts can survive and remain infective over considerable periods, particularly under dry conditions without rainfall (M. J. Barbetti, unpublished data).

Although rust urediniospores in general are vulnerable to environmental factors, such as temperature, moisture and ultraviolet light (Zadoks, 1961), Pgt urediniospores are relatively tolerant of a range of light and temperature conditions, especially when relative humidity is low (Singh et al., 2002; Hernandez Nopsa & Pfender, 2014). It is widely claimed that rust urediniospores do not survive on seed, stubble or soil (Hollaway, 2016), only surviving in the field for several weeks, to germinate and infect susceptible hosts (Singh et al., 2002). However, urediniospores of bean rust (Uromyces phaseoli) survive over winter in residues in fields in North Dakota (Gross & Venette, 2001), potentially providing initial inoculum for the next season’s bean crops. Similarly, Twizeyimana & Hartman (2010) showed that viable soybean rust (Phakopsora pachyrhizi) urediniospores harvested from infected soybean leaves could be maintained at 23–24 °C at 55–60% relative humidity for up to 18 days, while freshly harvested urediniospores that were first desiccated remained viable for up to 30 days. Phakopsora pachyrhizi urediniospore survival is temperature sensitive, for example, urediniospores were killed after 4–6 h of exposure at 40–50 °C, but at 25 °C survival was 15 h (Twizeyimana & Hartman, 2010).

Another example is subterranean clover rust, Uromyces trifolii-repentis, which has been found to readily survive on infested dead subterranean clover residues from one growing season to the next in Western Australia (M. J. Barbetti, unpublished data). It is likely that the same occurs for Pgt and this has significant implications for ‘rolling’ stem rust epidemics from one year into the next in the absence of infected host materials.

There may be possible movement of Pgt pathotypes across regional, state and international borders via air and sea cargo or directly through other human activities (McNeill et al., 2011; Savage et al., 2012). While pathogens may be introduced with increasing international trade and tourism (Wellings, 2007; Holliday et al., 2013), there is a wide range of potential carrier materials that could facilitate entry and spread of exotic fungal spores (Barua et al., 2017b). Some extensively used materials such as fabrics, metals, paper, rubber tyres, leather and wood can be potential carriers of viable fungal spores (Hughes et al., 2010; Osyczka et al., 2012). While urediniospores are the main source of inoculum of Pgt and can be dispersed long-distance up to 2000 km by wind (Luig, 1985), they are also subject to human-mediated transport (Brown & Hovmöller, 2002; Aylor, 2003; Yamaoka, 2014) as occurs with a wide range of fungal species. At Honolulu International Airport, 65 fungal species from 39 genera were isolated from shoes of travellers arriving from San Francisco (Baker, 1966). Two significant instances of human-aired introduction of rust diseases are barley stripe rust into Columbia in 1975 (Roelfs & Bushnell, 1985) and wheat stripe rust (human, goods or machinery urediniospore contamination) into Australia in 1979 (Wellings et al., 1987; Wellings, 2007). Further, in the early 1980s, high numbers of plant pathogenic fungal spores, including Puccinia coronata, were collected from clothing and baggage of passengers arriving by air in New Zealand (Sheridan & Nendick, 1988; Sheridan, 1989). In 1982, these examples included an estimated 70 000 viable rust urediniospores brought into New Zealand on travellers’ clothing and baggage (Sheridan, 1989). Sheridan (1989) also monitored the movement of fungal spores attached to human bodies and clothing while conducting disease surveys of cereal
crops, and showed how human bodies and clothing carried many viable pathogenic fungi, including urediniospores of rusts such as *P. coronata*, Pgt and *Puccinia hordei*. Lana et al. (2012) reported low numbers of *Puccinia psidii* spores on wood products (timber and pulp) and suggested that these low numbers were due to adverse environmental conditions in the wood storage areas and during overseas transport that did not foster spore survival. However, in 2004, the Australian Plant Quarantine Service (AQIS) detected *P. psidii* urediniospores on kiln-dried *Eucalyptus* timber imports from Brazil, plastic wrapping and the external surfaces of shipping containers, and used molecular analysis to confirm maintenance of their viability throughout the 2-month sea journey (Grgrurinovic et al., 2006).

To address the biosecurity concerns outlined above, studies were undertaken to assess the long-term survival of Pgt urediniospores on various material surfaces. The present study highlights long-term survival of Pgt urediniospores on a range of different material types and across different temperatures, and discusses the implications.

**Materials and methods**

**Pgt inoculum**

Pgt urediniospores (pathotype as 34-1,2,7 rather than the more recent 34-1,2,7+Sr38 pathotype; Cuddy & Park, 2013) were provided by the Department of Agriculture and Food Western Australia. The urediniospores had been collected from infected wheat plants using a handheld vacuum collector and then dried over silica gel prior to storing at $-80\,^\circ\mathrm{C}$ until needed. Desiccated urediniospores were used for several reasons. First, fresh urediniospores were not consistently available across the time period of these repeated studies. Secondly, their use avoided any possible confounding effects of variation from successive urediniospore collections made across different wheat varieties, temperatures, ages of urediniospores, and their exposure to different relative humidities. In addition, urediniospores undergo natural desiccation from mid to late spring onwards under the rain-free Mediterranean period, a situation that may not change until autumn/early winter the following year. Viability of urediniospores was confirmed on potato dextrose agar (PDA) media (c. 95% relative humidity) and germination found to be $\geq80\%$.

Prior to inoculation, urediniospores were suspended in 0.001% Tween 20 and adjusted to a concentration of $10^6$ urediniospores mL$^{-1}$ measured using a haemocytometer counting chamber (Superior Marienfeld). Tween 20 was added directly to the treatment plates and then placed on a rotary shaker for 40 min at 700 rpm. Relative humidity (RH) in controlled environmental rooms was $25\pm1\,^\circ\mathrm{C}$ day/8±1 $^\circ\mathrm{C}$ night, 36±1 $^\circ\mathrm{C}$ day/14±1 $^\circ\mathrm{C}$ night, or 45±1 $^\circ\mathrm{C}$ day/15±1 $^\circ\mathrm{C}$ night, that were based on the 10-year climate data of 2005–2014 to represent the highest, lowest and intermediate average maximum/minimum temperature combinations for winter, summer and autumn/spring, respectively, in Western Australia. The three corresponding light intensities were 250, 260 or 320 mol m$^{-2}$ s$^{-1}$, respectively, using a photoperiod of 14/10 h for day/night from light sources of LED cool white and incandescent light bulbs (Quantum Flux MQ100; Apogee). Relative humidity (RH) in controlled environmental rooms was 25–37% during the day and 68–84% during the night and was comparable to regions where Pgt epidemics can commence, e.g. the Western Australian grain belt at Merredin (mean RH at 09:00 from October–March, 1911–2010, ranges from 53% to 63%, http://www.bom.gov.au/climate/averages/tables/cw_010093.shtml). There were six replicates in different 48-well cell culture plates for each inoculated carrier material and each uninoculated control material, with every sampling time and each temperature treatment arranged in a fully randomized design to measure the effect of temperature and time on urediniospore viability. The experiment was run over 365 days, sampling to assess the effect of temperature on viability of urediniospores and the ability of the materials to retain or release viable urediniospores, starting daily from day 1 until day 7, then weekly until day 30, after which sampling was undertaken at regular 30-day intervals concluding at day 365. The entire experiment was repeated twice (total of three identical experiments).

**Effect of temperature and time on viability of Pgt urediniospores**

The materials were cut into 0.5 cm squares, autoclaved and randomly placed into rows in sterile 48-well cell culture plates (Greiner sterile). The material in each well was inoculated individually with 10 $\mu$L ($10^6$ urediniospores mL$^{-1}$) of inoculum and allowed to dry in a laminar flow cabinet for 2–3 h. All sides of the inoculated culture plates were sealed with Parafilm wrap and placed under one of three controlled environmental conditions: $23\pm1\,^\circ\mathrm{C}$ day/8±1 $^\circ\mathrm{C}$ night, 36±1 $^\circ\mathrm{C}$ day/14±1 $^\circ\mathrm{C}$ night, or 45±1 $^\circ\mathrm{C}$ day/15±1 $^\circ\mathrm{C}$ night, that were based on the 10-year climate data of 2005–2014 to represent the highest, lowest and intermediate average maximum/minimum temperature combinations for winter, summer and autumn/spring, respectively, in Western Australia. The three corresponding light intensities were 250, 260 or 320 mol m$^{-2}$ s$^{-1}$, respectively, using a photoperiod of 14/10 h for day/night from light sources of LED cool white and incandescent light bulbs (Quantum Flux MQ100; Apogee). Relative humidity (RH) in controlled environmental rooms was 25–37% during the day and 68–84% during the night and was comparable to regions where Pgt epidemics can commence, e.g. the Western Australian grain belt at Merredin (mean RH at 09:00 from October–March, 1911–2010, ranges from 53% to 63%, http://www.bom.gov.au/climate/averages/tables/cw_010093.shtml). There were six replicates in different 48-well cell culture plates for each inoculated carrier material and each uninoculated control material, with every sampling time and each temperature treatment arranged in a fully randomized design to measure the effect of temperature and time on urediniospore viability. The experiment was run over 365 days, sampling to assess the effect of temperature on viability of urediniospores and the ability of the materials to retain or release viable urediniospores, starting daily from day 1 until day 7, then weekly until day 30, after which sampling was undertaken at regular 30-day intervals concluding at day 365. The entire experiment was repeated twice (total of three identical experiments).

**Recovery of Pgt urediniospores from inoculated carrier materials and determination of viable number of urediniospores**

Urediniospores were recovered from the carrier materials as described by Barua et al. (2017a,b). Briefly, 800 $\mu$L of 0.1% Tween 20 was added directly to the treatment plates and then plates were placed on a rotary shaker for 40 min at 700 rpm. Preliminary studies showed that a concentration of 0.1% Tween 20 in deionized water was optimal for washing urediniospores from the materials without damaging them (data not shown). After washing, the carrier materials were removed from the
residual spore suspension and prepared for microscopy studies. The urediniospore suspension was used to determine numbers of urediniospores recovered. Then, urediniospore viability was assessed using a recently developed, rapid and miniaturized system using alamarBlue (resazurin dye; 7-hydroxy-3H-phenoxa-

zine-3-one 10-oxide) where viable fungal spore metabolic activity converts dark blue resazurin into pink resorufin that provides a reliable indicator of the presence of viable spores (Barua et al., 2017a). In brief, the alamarBlue bioassay was initially optimized as a spore viability carrier for Pgt and optimum time for maxi-
mum metabolic activity was determined as 3 h. Pgt uredin-
iospore suspensions ranging in concentration from 10^7 to 10

urediniospores mL^-1 by tenfold serial dilution were set up in
96-well assay plates and used as comparison standards. Six
replicate wells were used for each concentration and were set up
using 100 μL of fresh urediniospore dilution for the standards
with 20 μL alamarBlue reagent. In the same way, residual ure-
diniospore solutions after washing were set with 100 μL of
residual solution/wash suspension containing urediniospores
with 20 μL alamarBlue reagent. Negative controls were also
similarly set up with no urediniospores, but only deionized
water, to determine the extent of any background absorbance.
The assay plates were covered with aluminium foil (to exclude
light) and then incubated on a rotary platform shaker (Innova
2100; New Brunswick Scientific) at 150 rpm at 22 °C for
1 min. Plates were then incubated at 37 °C for 3 h. The reac-
tion was terminated by adding 50 μL of 3% sodium dodecyl
sulphate (SDS) before measuring the absorbance. Plates were
then placed back on the rotary shaker at the same speed for
30 s to ensure a uniformly mixed end product before measuring
absorbance at 600 nm and 570 nm using a spectrophotometer
(Thermo Scientific Multiskan Spectrum). The average of the
background absorbance value at 600 nm for control wells was
subtracted from all absorbance values of experimental wells at
570 nm.

A standard curve of 570–600 nm absorbance versus uredin-
iospore concentration was plotted to calculate the viable number
of rust urediniospores in residual solutions after washing. The
absorbance readings were directly proportional to germination.
The total number of viable urediniospores was determined for
each replicate using the regression equation from the standard
curve, and the mean calculated from the six replicates.

**Determination of germination rate of the urediniospores recovered from carrier materials after time and temperature treatments**

After washing at each time interval, the residual spore suspen-
sions recovered from the carrier materials were examined. A 20 μL aliquot of the recovered residual spore suspension was
spread over PDA plates (≥95% RH) and incubated at 22 °C
(8 h photoperiod) to observe the germination of the uredin-
iospores. There were three single plate replications for each sam-
ples and 100 spores randomly selected to assess the percentage
of germinated urediniospores using a BX51 (Olympus) microscope.

**Ability of recovered Pgt urediniospores to infect wheat seedlings after temperature treatments**

Four-week-old wheat seedlings (cv. Wyalkatchem) were inocu-
lated with rust urediniospores recovered from materials after
12 months from 23/8 °C. Leaves of seedlings were inoculated
with 20 μL of recovered residual spore suspension at a concen-
tration of 10^7 urediniospores mL^-1. Three spot-inoculations
were made randomly on each leaf across six replicate pots of
wheat plants. Relative humidity was maintained at 85–90% for
24 h in a dew chamber and subsequently maintained in a glass-
house at approximately 18 ± 1°C. Six replicate pots of noninoc-
ulated wheat plants as controls were also similarly maintained.
Inoculated leaves from the test and noninoculated control plants
were collected from days 1 to 30 for microscopy studies. Simi-
larly, wheat seedlings were inoculated with urediniospores
recovered from 36/14 °C day/night after 10 months and from
45/15 °C night after 7 months.

To examine differences in the ability of urediniospores from
each treatment to cause infection, inoculated leaves were har-
vested at 12 days post-inoculation (dpi). Harvested leaves both
showing and not showing visible symptoms were subjected to
decolourization by immersing in a solution containing glacial
acetic acid and ethanol (1:1) for 1 day (Carrillo et al., 2013)
and then in acetic acid/ethanol/water (2:2:1) in plastic vials
maintained at 23 °C for 4–5 days (Uloth et al., 2015). Deco-
loured samples were washed with two changes of deionized
water and stained with 1% cotton blue in lactophenol for
3 min. Whole wet mounts of stained leaves on microscope slides
were then examined using a BX51 microscope mounted with an
Olympus DP71 camera system.

**Experimental design and data analysis**

Carrier material treatments were arranged in a complete ran-
domized design with numbers of replications as indicated above.
All experiments were carried out under controlled laboratory
and glasshouse environments. All experiments were repeated
twice (i.e. three identical experiments for each study) and the
relationship between the initial and repeat experiments assessed
using a paired t-test using GenStat 16th edition (GenStat Proce-
dure Library Release PL23.2). Where there were no significant
differences between experiments (P > 0.05), datasets from the
two most similar experiments were pooled, reanalysed and pre-
sented as a single dataset. All numbers of viable Pgt uredin-
iospores were expressed as a percentage of total number of
urediniospores inoculated. Single-factor ANOVAs were con-
ducted using GenStat to determine the effects of temperature on
the viability of the Pgt urediniospores at each assessment time
across the 365-day period and across test materials. Subse-
quently, multiple-factor ANOVAs including temperature, test
material and time were undertaken as there were no significant
changes to individual time point, temperature or test material
outcomes within the multiple-factor ANOVA compared with
single-factor ANOVAs. Fisher’s least significant differences were
used to show significant differences.

**Results**

**Effect of temperature and time on viability of Pgt urediniospores**

The extent of viability of urediniospores depended upon the
carrier material and the temperature over the 365-

day time period. The viability decreased over time. At 23/8 °C day/night, urediniospores remained viable for
365 days on materials except for brass, all different steels and plastic (Table 1). At 36/14 °C day/night, viabil-
ity was retained for up to 300 days on denim and jute.

At 45/15 °C day/night, urediniospores remained viable for a maximum of 180 days on cotton and jute.
Table 1 Mean number of viable urediniospores (%) of Puccinia graminis f. sp. tritici isolated from tested metals, fabrics, woods and other miscellaneous materials (‘other’) over a time period of 365 days’ incubation at each of the three different temperature regimes.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time (days)</th>
<th>Metals</th>
<th>Fabrics</th>
<th>Woods</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>23 ± 1 °C day/night</td>
<td>Aluminium</td>
<td>Brass</td>
<td>Corrugated steel</td>
<td>Galvanized Rusted steel</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
</tr>
<tr>
<td>6</td>
<td>14</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
</tr>
<tr>
<td>11</td>
<td>4</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
</tr>
</tbody>
</table>

Average | 21 | 18 | 11 | 19 | 12 | 12 | 12 | 37 | 26 | 18 | 36 | 38 | 27 | 44 | 32 | 28 | 17 | 36 | 24 | 17 | 26 |

36 ± 1 °C day/night | 1 | 11 | 6 | 11 | 1 | 10 | 20 | 9 | 32 | 52 | 29 | 20 | 19 | 15 | 23 | 11 | 20 | 1 | 14 | 26 | 11 | 20 |

14 ± 1 °C night | 2 | 9 | 4 | 10 | 1 | 1 | 20 | 5 | 29 | 46 | 25 | 19 | 18 | 11 | 15 | 8 | 20 | 1 | 14 | 20 | 10 | 20 |

(continued)
Table 1 (continued)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>240</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>270</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>300</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>330</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>365</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Average</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>14</td>
<td>18</td>
<td>8</td>
<td>7</td>
<td>8</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>16</td>
<td>18</td>
<td>18</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Material Temperature Time</th>
<th>Material</th>
<th>Temperature</th>
<th>Time</th>
<th>Material × Temperature</th>
<th>Material × Time</th>
<th>Temperature × Time</th>
<th>Material × Temperature × Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>23/8 °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Significance (P)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>l.s.d. (P &lt; 0.05)</td>
<td>0.47</td>
<td>0.47</td>
<td>2.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>36/14 °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Significance (P)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(continued)
Maximum time for retention of urediniospore viability on each material at three temperature regimes

**Metals**

At 23/8 °C day/night, the viability of urediniospores decreased by 50–70% on day 1 (after 24 h). The spore viability further decreased by 98–99%, with viable urediniospores recovered up to 365 days from aluminium (1%), 300 days from brass (1%), 120 days from corrugated steel (2%), 330 days from galvanized sheet (1%), 210 days from steel (1%) and rusted steel (1%), and 180 days from zinc (1%). At 36/14 °C day/night, viability of urediniospores decreased by 80–70% on day 1, with a further decrease in viability by 96–99%, with viable urediniospores recovered up to 5 days from aluminium (2%), 3 days from brass (1%), 4 days from corrugated steel (4%), 5 days from galvanized sheet (1%), 30 days from steel (1%), 4 days from rusted steel (1%) and 3 days from zinc (1%), with no further viability observed. At 45/15 °C day/night, viability of urediniospores decreased by 90–99% on day 1, with viable urediniospores recovered up to 4 days from aluminium (1%), 2 days from brass (4%), 3 days from corrugated steel (2%), galvanized sheet (1%) and rusted steel (1%), 6 days from steel (1%) and 3 days from zinc (1%), with no more viability (Table 1).

**Fabrics**

At 23/8 °C day/night, viability of urediniospores decreased by 26–36% on day 1, which further decreased to 90–99%, with viable urediniospores recovered from all the fabric materials up to 365 days; viz. cotton (10%), denim (4%), fleece (1%), silk (3%) and fibre polyester (1%). At 36/14 °C day/night, viability of urediniospores decreased by 48–81% after day 1, which further decreased to 99%, with viable urediniospores recovered up to 240 days from cotton (1%), 300 days from denim (1%), 150 days from fleece (1%), 180 days from silk (1%) and 180 days from fibre polyester (1%). At 45/15 °C day/night, viability decreased by 75–81% after day 1 with a further decrease to be 99%, with viable urediniospores recovered up to 180 days from cotton (1%), 150 days from denim (1%), 60 days from fleece (1%), 90 days from silk (1%) and up to 120 days from fibre polyester (1%) (Table 1).

**Woods**

At 23/8 °C day/night, viability of urediniospores decreased by 34–54% on day 1, which further decreased to 92–99%, with viable urediniospores recovered from all three wood materials for up to 365 days; viz. jarrah wood (1%), pine wood (8%) and Tasmanian oak (3%), with no further viability observed. At 36/14 °C day/night, viability of urediniospores decreased by 77–90% on day 1, with viable urediniospores recovered up to 60 days from jarrah wood (1%), 120 days from pine wood (1%) and 21 days from Tasmanian oak (1%). At 45/15 °C day/night, viability of urediniospores decreased by 82–93% on day 1, which further decreased by 99%,
with viable urediniospores recovered up to 7 days from jarrah wood (1%) and Tasmanian oak (1%) and up to 21 days from pine wood (1%) (Table 1).

**Others**

At 23/8 °C day/night, viability of urediniospores decreased by 33–60% on day 1, which further decreased to 90–99%, with viable urediniospores recovered up to 365 days from glass (1%), jute (8%), leather (10%), paper (8%) and rubber tyre (1%), and up to 300 days from plastic (1%). At 36/14 °C day/night, viability of urediniospores decreased by 64–83% after day 1, with a further decrease to be by 98–99%, with viable urediniospores recovered up to 3 days from glass (1%), 300 days from jute (1%), 60 days from leather (1%), 120 days from paper (1%), 5 days from plastic (2%) and 60 days from rubber tyre (1%). At 45/15 °C day/night, viability of the urediniospores decreased by 78–99% after day 1, with a further decrease to be by 99%, with viable urediniospores recovered up to 2 days from glass (1%), 180 days from jute (1%), 14 days from leather (1%), 60 days from paper (1%), 3 days from plastic (2%) and 30 days from rubber tyre (1%) (Table 1).

**Effect of material type on viability of Pgt urediniospores**

Different groups of materials, i.e. metals, woods, fabrics and ‘others’, had different capacity to retain viable urediniospores, and this was also dependent upon the temperature the materials were maintained at. The maximum and minimum average percentage of viable urediniospores recovered from the carrier materials varied with temperature and type of material. The average maximum viable urediniospores recovered at the three different day/night temperature regimes of 23/8 °C, 36/14 °C and 45/15 °C after 365 days were from pine wood (44%), denim (18%) and cotton (8%), respectively. There was a significant \( P < 0.001 \) negative correlation between time and temperature in terms of viability of urediniospores. At 23/8 °C, the maximum average percentages of viable urediniospores recovered after 365 days across each carrier material group were 21% from aluminium (metals), 44% from pine wood (woods), 37% from silk (fabrics) and 36% from leather (‘others’). At 36/14 °C, these were 6% from steel (metals), 4% from pine wood (woods), 18% from denim (fabrics) and 8% from jute (‘others’), and at 45/15 °C, were 2% from steel (metals), 2% from pine wood (woods), 8% from cotton (fabrics) and 6% from paper (‘others’).

The percentage of viable urediniospores recovered from materials within the same group type also varied with temperature. For example, while the maximum percentage of viable urediniospores recovered from metals at 23/8 °C was from aluminium (21%), the maximum viable urediniospores recovered from steel at 36/14 °C and at 45/15 °C were 6% and 2%, respectively. Among the woods, maximum viable urediniospores recovered from pinewood depended upon temperature regime; viz. 44%, 4% and 2% at 23/8 °C, 36/14 °C and 45/15 °C, respectively. The maximum viable percentage urediniospores obtained from fabrics varied with material and temperature, from silk (38%) at 23/8 °C, from denim (18%) at 36/14 °C and from cotton (8%) at 45/15 °C. Across the ‘others’ group, maximum viable urediniospores recovered was from leather (36%) at 23/8 °C, from jute (8%) at 36/14 °C, and from paper (6%) at 45/15 °C (Table 1).

**Validation of assay: determination of germination rate of urediniospores recovered from carrier materials after time and temperature treatments**

The germination of the spores varied over time and also with temperature. There was a significant \( P < 0.001 \) positive correlation between the percentage urediniospores germinated and percentage of viable urediniospores that reduced resazurin to resorufin in alamarBlue assay at 23/8 °C day/night over the time period of 365 days \( (R^2 = 0.97) \). Similar correlation was observed at 36/14 °C \( (R^2 = 0.99) \) and at 45/15 °C \( (R^2 = 0.95) \). At least 5% of the urediniospores recovered from 23/8 °C after 365 days, 1% from 36/14 °C after 300 days and 1% from 45/15 °C after 180 days successfully germinated on PDA.

**Confirmation of infection of wheat seedlings from recovered Pgt urediniospores and detection of Pgt from inoculated wheat plants**

Pgt urediniospores that had been recovered from 23/8 °C or 36/14 °C day/night caused infection symptoms on wheat following their inoculation. Early stages of infection were confirmed by the appearance of light brown discolouration of the leaf tissues at points of inoculation by 4 dpi. Subsequently, small brown rust lesions 0.5–2 mm developed at all inoculation sites within 7–10 dpi; and by 11–12 dpi, extension of lesions beyond the site of inoculation was evident. However, urediniospores recovered from 45/15 °C, despite being viable (1% germination), did not produce infection symptoms.

**Discussion**

The current study opens up new possible explanations of extended survival per se that can initiate subsequent rust epidemics. This study demonstrates a significant effect of material type and temperature on Pgt urediniospore viability, for the first time across a range of material types, including metals, plastics, fabrics and woods, with urediniospores remaining viable on tested carrier materials for up to 365 days (aluminium, cotton, denim, fleece, fibre polyester, silk, jarrah wood, Tasmanian oak, jute, leather, paper and rubber) at 23/8 °C, up to 300 days (denim and jute) at 36/14 °C, and up to 180 days (cotton and jute) at 45/15 °C. While the number of viable urediniospores decreased over time and with increasing

---

**Table 1:** The percentage of viable urediniospores recovered from carrier materials after different day/night temperature regimes of 23/8 °C, 36/14 °C and 45/15 °C. Notation: 'Others' includes metals, plastics, fabrics and woods. Positive correlation between the percentage urediniospores remaining viable on tested carrier materials and temperature was observed at 36/14 °C \( (R^2 = 0.99) \) and at 45/15 °C \( (R^2 = 0.95) \). At least 5% of the urediniospores recovered from 23/8 °C after 365 days, 1% from 36/14 °C after 300 days and 1% from 45/15 °C after 180 days successfully germinated on PDA.

<table>
<thead>
<tr>
<th>Material Type</th>
<th>Temp/Day/night</th>
<th>23/8°C</th>
<th>36/14°C</th>
<th>45/15°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metals</td>
<td>64%</td>
<td>90%</td>
<td>78%</td>
<td></td>
</tr>
<tr>
<td>Plastics</td>
<td>83%</td>
<td>99%</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td>Fabrics</td>
<td>5%</td>
<td>5%</td>
<td>5%</td>
<td></td>
</tr>
<tr>
<td>Woods</td>
<td>99%</td>
<td>99%</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>83%</td>
<td>99%</td>
<td>99%</td>
<td></td>
</tr>
</tbody>
</table>

---

temperature, there is clearly greater potential for Pgt urediniospores to survive for much longer periods in the absence of a host than the several weeks previously considered feasible. However, there were suggestions that stem rust urediniospores could be more durable, and clearly they have some resistance to atmospheric conditions providing their moisture content is moderate (20–30%), with long-distance transport and infection possible across the North American Great Plains (Roelfs, 1985), from Australia to New Zealand (Luig, 1985) and occasionally from East Africa to Australia (Watson & de Sousa, 1983). Importantly, in the current study, urediniospores recovered even after 12 months from the lower temperature regimes (23/8 °C and 36/14 °C) successfully infected wheat seedlings. However, the urediniospores recovered from the higher temperature (45/15 °C) were unable to infect the wheat seedlings, although a very small percentage of spores (1%) successfully germinated on PDA.

The current study shows the long period for which urediniospores can remain viable on a range of carrier materials and under a wide range of temperature conditions, and highlights the importance of deposition and ‘attachment’ of urediniospores on various carrier materials that further enhances dispersal potential, both locally and large-scale, as a consequence of a range of inadvertent human activities. Urediniospores of stem rust are relatively resistant to light and temperatures compared to other rusts at a relatively low humidity of 30% (Aydoğan & Boyraz, 2012). While nondesiccated and desiccated urediniospores of soybean rust (P. pachyrhizi) could remain viable up to 18 and 30 days at 23–24 °C and 55–60% RH, respectively, they only remained viable up to 4–6 h at 40–50 °C (Twizeyimana & Hartman, 2010). Urediniospores of P. psidii from Eucalyptus spp. maintained viability up to 10 days at 35 or 40 °C with 35–55% relative humidity (Lana et al., 2012). It is noteworthy that in the current study high temperatures up to 45 °C did not kill all urediniospores. This implies that some urediniospores could survive and remain viable from one season to the next, even under the relatively hot and dry environmental conditions that frequently occur between cropping seasons in Western Australia. Further, the frequency of recovery of urediniospores was dependent upon the carrier material type. These findings have major implications both for carryover across sequential cropping seasons to initiate stem rust epidemics, and for domestic and international movement of freight and commodities that clearly can effectively carry and retain viable urediniospores for up to 365 days.

The challenge in managing initiation and spread of Pgt epidemics is that urediniospores are primarily wind dispersed both locally and large scale (Sache, 2000). In particular, long-distance transport of urediniospores is important for distributing new genetic variants of rusts over larger areas (Rees, 1972), especially as the estimated mean rate of spread of Pgt approximates 35 km per day (Aylor, 2003). The dispersal patterns associated with the commencement and spread of rust epidemics is not only determined by the interaction between urediniospore availability and release timing, but also wind patterns (Savage et al., 2010). Wind plays an important role in urediniospore dispersal capacity, and it also dehydrates urediniospores, thereby promoting their long-term survival, as dried urediniospores survive longer than those retained in moist environments (Chen, 2005). In Israel, under a similar Mediterranean-type environment to southwestern Australia, it is observed that Puccinia dracaunculina does not complete its sexual cycle there, and urediniospores overwintering in dry leaves serve as the source of initial inoculum for the following season (Cohen et al., 2013). Similarly, Pgt epidemics can be generated from minute amounts of viable inoculum; for example, studies in the Netherlands have shown that even a single uredinium per hectare surviving the winter, a level of inoculum which is below detectable thresholds, was sufficient to generate a subsequent spring rust epidemic (Singh et al., 2002). A study by Dill-Macky et al. (1991) demonstrated that while initial inoculum pressure influences the rust development, a significant epidemic could develop in highly susceptible varieties of barley from a relatively small amount of inoculum. Hence, even just a few Pgt urediniospores attached to wheat residues and/or inert carrier materials could play an important role in the spread and subsequent initiation of infections and initiate subsequent epidemics from these apparently inconspicuous initial levels of inoculum.

The current study highlights a clear interaction between the type of carrier material and the quantity of urediniospores retained, with woods able to retain the most viable urediniospores, followed by fabrics and metals. Not only did retained urediniospores remain viable for up to a year on these materials, their subsequent ability to infect wheat plants under favourable conditions, even after 365 days, was confirmed. However, it is noteworthy that recovered urediniospores from carrier materials at 23/8 °C and 36/14 °C caused infection, while those recovered from 45/15 °C no longer retained the ability to successfully produce infections, the latter possibly for reasons outlined above. Although rusts are biotrophic pathogens (Duplessis et al., 2011), the urediniospores can easily be spread by wind or through infected plant material as well as contaminated clothing, footwear, baggage, plastic wrapping and the external surfaces of shipping containers etc. (Sheridan, 1989; Grgrurinovic et al., 2006).

In conclusion, the current study demonstrates that not only do Pgt urediniospores remain viable for a much longer period and at higher temperatures when attached to or embedded in nonliving materials than has been historically accepted, but also that at least some of these urediniospores still retain the ability to infect wheat. The outcomes of the current study demonstrate the potential importance of inert materials as long-term carriers of viable Pgt urediniospores, highlighting both the risks of spread of new pathotypes and strains into wheat-growing regions as well as significant biosecurity implications in general for contaminated carrier materials previously
considered as low risk. It is believed that these studies will not only prompt new evaluation of possible sources of carryover of stem and other rusts in Australia, particularly in relation to their survival and spread from infested stubbles, but a wider reconsideration of risks associated with transmission of fungal spores from one place to another involving human activities. Re-evaluation is especially needed for materials such as metals, plastics, fabrics and woods that have been historically considered of low biosecurity risk in regards to the movement of fungal plant pathogens.

Acknowledgements

The authors greatly appreciate the scholarship provided by the Plant Biosecurity CRC for project CRC62042: ‘Curtailing and managing exotic fungal spore incursion into Australia’, and financial support by the University of Western Australia. They would like to thank Robert Creasy and Bill Piasini from the University of Western Australia for support and help in the glasshouse during these studies. All authors declare that they have no conflict of interest.

References


CHAPTER 6

Disinfestation of diverse fungal pathogen spores on inert contaminated materials
Disinfestation of diverse fungal pathogen spores on inert contaminated materials

Papori Barua¹,² • Ming Pei You¹ • Kirsty L. Bayliss³ • Vincent Lanoiselet¹,⁴ • Martin J. Barbetti¹,²(*)

¹ School of Agriculture and Environment and the UWA Institute of Agriculture, Faculty of Science, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia;
² Plant Biosecurity Cooperative Research Centre (PBCRC), Bruce, ACT 2617, Australia;
³ School of Veterinary and Life Sciences, Murdoch University, South Street, WA 6105, Australia;
⁴ Department of Primary Industries and Rural Development Western Australia, 3 Baron-Hay Court, South Perth, WA 6151, Australia

*Corresponding author: M. J. Barbetti; E-mail: martin.barbetti@uwa.edu.au

Abstract In vitro studies were undertaken to determine the effects of five fungicide and disinfectant treatments [propiconazole (Tilt 250EC), azoxystrobin (Amistar 250EC), didecyldimethyl ammonium chloride (Sporekill), alkali metal salts of alkylbenzene sulfonic acid and coconut diethanolamide (Farmcleanse), and potassium peroxymonosulfate (Virkon)] in preventing the germination of spores of Puccinia graminis f. sp. tritici, Kabatiella caulivora, Leptosphaeria maculans and Magnaporthe oryzae. Germination was inhibited by all fungicides and disinfectants, with maximum reductions at the manufacturer’s recommended concentration. Overall, azoxystrobin was the most effective, reducing germination of M. oryzae by
89%, *L. maculans* by 78% and *P. graminis* f. sp. *tritici* by 77%. Propiconazole was the most effective in reducing germination of *K. caulivora* by 72%. The extent of inhibition of germination was dependent on the pathogen; for example, alkali metal salts of alkylbenzene sulfonic acid and coconut diethanolamide, and potassium peroxymonosulfate were more effective on *M. oryzae* and *P. graminis* f. sp. *tritici* compared with *L. maculans* or *K. caulivora*. Studies undertaken to define the effectiveness of the fungicides/disinfectants reducing germination of the pathogens on five inert carrier materials (steel, fabric, wood, paper, and rubber showed azoxystrobin and propiconazole to be the most effective, having 12% to 15% spore germination following decontamination treatment of carrier materials. The results demonstrate the potential for increased use of fungicides, particularly demethylation inhibitor and QoI fungicides, to decontaminate carrier materials to address the critical need to implement a practical commercial solution for dealing with threats posed by the long-term viability of these and other plant pathogens on inert materials associated with movement of humans, farming equipment, and commodities nationally and internationally.

**Keywords** *Puccinia graminis* f. sp. *tritici*, *Kabatiella caulivora*, *Leptosphaeria maculans*, *Magnaporthe oryzae*, fungicide, disinfectant, spore germination suppression

**Introduction**

Fungal plant pathogens are a major cause of yield losses across agricultural and horticultural crops world-wide and pose an on-going threat to the associated industries (Bebber and Gurr 2015; Khangura et al. 2007;
Savary et al. 2012; Wellings 2011). Fungal contamination of foods, household and other products and environments, is also associated with adverse health effects of animals and humans (Zain 2011), including via mycotoxins (Schoental 1994; Tan et al. 2011). Fungal spores can survive adverse environmental conditions and remain viable for years (Nagtzaam and Bollen 1994; Wyatt et al. 2013) and be dispersed locally or trans-border by air currents, water (rain splash) or by human activities to play a critical role in disease spread and development (McNeill et al. 2011; Savage et al. 2012).

With globalization, the risk of introduction of fungal plant pathogens to new regions, particularly by spore contamination, has increased, especially via movement of contaminated equipment and commercial goods (McKirdy et al. 2012). While this risk cannot be prevented, effective mitigation measures can minimize the degree of risk. An assessment of exotic incursions of plant pests into Australia during a 25 year period up to 1996 showed a 10 times greater incursion of plant pests than for animal pests (Nairn et al. 1996). This resulted in recommendations to increase monitoring, improve awareness and inspections, and review traveller statements (i.e., whether or not visiting a farm, camping, outdoor activities etc.), with the aim of reducing the incidence of both naturally and/or accidentally introduced plant pathogens (Wheelis et al. 2002). The threat of land-bridging via movement of shipping and road containers between metropolitan areas from ports before quarantine clearance was identified as an area of concern (Nairn et al. 1996).

In 1982 an estimated 70,000 viable rust urediniospores were collected from clothing and baggage of passengers arriving by air in New Zealand (Sheridan 1989), and in 1992, Phillips et al. (1992) stated that conidia of Magnaporthe grisea introduced to Australia on the clothes of international
travellers was a major risk. Further, the ready movement of *Phakopsora pachyrhizi* (soybean rust) spores by non-conventional means, such as on peoples trousers and shoes in the southern United States, was confirmed by Hartman and Haudenshield (2009). These and other examples highlight how plant pathogens could be introduced via international trade and tourism (Wellings 2007). More recently it has been demonstrated that fungal spores can remain viable for long periods when attached to various materials including fabrics, metals, paper, rubber, leather, and timber (Barua et al. 2017a,b; Osyczka et al. 2012). For example, urediniospores of *Puccinia graminis* f. sp. *tritici* could survive on inert materials without a host and successfully initiate infections of wheat seedlings after 300 days, increasing the likelihood of spread of the pathogen across wheat growing regions through contaminated carrier materials (Barua et al. 2018a). Similarly, spores of *Kabatiella caulivora* and *Leptosphaeria maculans* survived on inert materials for up to 240 days (Barua et al. 2017b, 2018b). Hence appropriate decontamination of carrier materials is one of the most important requirements for prevention of plant pathogen incursions.

For successful control and/or eradication of a plant pathogen, the processes of identification and decontamination need to commence as close as possible to the time of introduction, while numbers of spores and/or infections are restricted (Wheelis et al. 2002). However, containment and eradication of fungal plant pathogens that can infect multiple host plants, or those that have wind-driven spore dispersal, is challenging as they can rapidly spread over a large area (Roper et al. 2010; Savage et al. 2012). For example, while the host of *P. striiformis* f. sp. *tritici* is predominantly wheat, it also infects barley, rye, some weedy grasses, and triticale genotypes (Holmes and Dennis
and urediniospores are air-dispersed over long distances as a primary source of inoculum (Kolmer et al. 2007). Thus, decontamination of spores on introduced materials can be critical in preventing introduction of such an easily dispersed pathogen.

As a quarantine measure, many fungal-spore-contaminated materials are either fumigated or sprayed with fungicides or other more general-purpose chemical agents. Registered fungicides including propiconazole (Tilt 250EC) or azoxystrobin (Amistar 250EC) are used primarily in-field crop sprays, and are both systemic fungicides with curative and preventative action (Ivic 2010). These products would likely be suitable for decontaminating spores on carrier materials. As such, these fungicides should be considered as an option for spore decontamination, along with the many sporicidal disinfectants that are effective against a wide range of microorganisms including plant pathogens. Most of the sporicidal disinfectants contain chlorine, aldehyde, alkali salts or peroxygen as their active chemical group, with peroxygen widely accepted as an effective disinfectant against a broad range of microorganisms (Hernández et al. 2000). For example, potassium peroxymonosulfate has been claimed to provide effective control of bacteria, fungi, moulds, yeasts, and viruses (Broadley et al. 1993). While such disinfectants are widely used in surface sterilization, decontamination of vehicles, farm equipment and machines, their prime use has been for bacterial spore decontamination in hospitals, veterinary clinics and laboratories for human and other animal pathogen control (Garcia-de-Lomas et al. 2008; Sagripanti and Bonifacino 1996). For example, potassium peroxymonosulfate is used extensively for decontamination of high-risk animal and zoonotic pathogens, including foot and mouth disease (Fretzel et al. 2013).
There have been recent studies on survival of spores of the wheat stem rust pathogen, *P. graminis* f. sp. *tritici* (Barua et al. 2018a), the forage legume northern anthracnose pathogen, *K. caulivora* (Barua et al. 2017b), the rapeseed phoma stem canker pathogen, *L. maculans* (Barua et al. 2018b), and the rice blast pathogen, *M. oryzae* (Barua et al. 2017a). Subsequent *in vitro* studies were undertaken to determine the effect of five fungicide or disinfectant treatments [propiconazole (Tilt 250EC), azoxystrobin (Amistar 250EC), didecyldimethyl ammonium chloride (Sporekill), alkali metal salts of alkylbenzene sulfonic acid and coconut diethanolamide (Farmcleanse), and potassium peroxymonosulfate (Virkon)] in preventing spore germination of these plant pathogens. Studies were also undertaken to define the effectiveness of these chemicals for disinfestation of five common carrier materials: metal, fabric, wood, paper, and rubber. The biosecurity implications are discussed in relation to disinfestation of carrier materials that can transport fungal spores, particularly in relation to decontamination of carrier materials long mistakenly considered of low biosecurity risk in regards to the transport of fungal plant pathogens.

**Materials and Methods**

Fungicide and disinfectant selection

*In vitro* studies were undertaken to determine the effects of propiconazole (as Tilt 250EC, Syngenta Crop Protection, Sydney, New South Wales, Australia), azoxystrobin (Amistar 250EC, Syngenta Crop Protection), didecyldimethyl ammonium chloride (Sporekill, Nufarm Australia Ltd, Laverton
North, Victoria, Australia), alkali metal salts of alkylbenzene sulfonic acid +
coconut diethanolamide (Farmcleanse, Castrol Australia Pty, Guildford,
Australia) and potassium peroxymonosulfate (Virkon, DuPont Animal Health
Solutions, Sydney, New South Wales, Australia) on fungal spore germination.
These fungicide and disinfectants were chosen as they are registered for use
in Australia by the Australian Pesticides and Veterinary Medicines Authority
(APVMA). To determine the decrease in rate of spore germination in aqueous
suspensions amended with the fungicide or disinfectant, and to determine the
optimum concentration to reduce or eliminate germination, a total of five
concentrations of each was tested. The manufacturer recommended
concentration was used, plus two concentrations above and two
concentrations below that recommended concentration. All formulations were
prepared following manufacturer recommendations, mixing or diluting the
product with sterile deionized water as required (Table 1). For the in vitro agar
plate studies, each chemical was incorporated into the agar only at the
manufacturer’s recommended concentration. In all studies, there were six
replicates for each chemical treatment for each pathogen and for the control
and all experiments were repeated twice (i.e. conducted three times).

Preparation of pathogen inoculum

*Magnaporthe oryzae*: A single virulent isolate of *M. oryzae* (WAC13466;
race IA-1) collected from cultivated rice (*O. sativa*) in Kununurra, Western
Australia (Fang et al. 2017a), was chosen for this study. The isolate had been
stored on colonized filter paper discs and was revived by placing the discs
onto half strength potato dextrose agar (19.5 g l\(^{-1}\); PDA) and incubating in the
dark at 25°C for 7 days. To produce conidia, moist sterile wheat seed was prepared by soaking 100 g of seed in 100 ml deionized water (DI) water overnight in 250 ml Erlenmeyer flasks. The water was drained and the flasks containing the seeds were autoclaved at 120°C for 20 min, three times on each of three consecutive days. Agar plugs (0.5 mm²) from 7-day-old PDA cultures were added to the flask with sterile wheat and incubated in the dark at 23°C. The flasks were shaken every second day for 6 to 8 days to mix the materials until mycelia covered the wheat, when 100 ml DI water were added, the flask shaken for 3 min and water discarded. The neck of the flask was covered with Miracloth and incubated in the dark at 23°C until conidia were observed [using an Olympus (BX51) microscope, Olympus Australia Pty, Notting Hill, Victoria, Australia] and collected after 4 to 5 days by rinsing with DI water and filtering through double layers of sterilized muslin cloth to remove hyphae, wheat or agar pieces. Conidia were suspended in 0.001% Tween 20 in water and the concentration adjusted to 10⁶ spores ml⁻¹ using a haemocytometer (Superior Marienfeld, Lauda-Königshofen, Germany).

**Leptosphaeria maculans**: L. maculans ascospores were produced by collecting field crop residue with pseudothecia from rapeseed fields in early winter and storing the material dry in the laboratory at 25°C until needed. Ascospores were produced following the protocol of Li et al. (2004). Briefly, infested residue pieces were immersed in 10 ml sterile DI water in covered Petri plates for 1 h at 25°C under natural light and subsequently maintained at 4°C in the dark for 1 h. Ascospores were naturally ejected and collected by washing them from the inside of the Petri plate lid with DI water. Ascospores were suspended in 0.001% Tween 20 and the concentration adjusted to 10⁶ ascospores ml⁻¹ measured using a haemocytometer.
**Kabatiella caulivora**: A single isolate of *K. caulivora* Race 2 (WAC5757; Barbetti 1995), stored as a lyophilized culture in a glass ampoule was rehydrated using sterile DI water and plated onto PDA containing 100 mg liter$^{-1}$ of aureomycin hydrochloride. Cultures were maintained in the dark at 15°C and subcultured every 5 to 8 days to maintain the budding, yeast-type form required for inoculation (Cole and Couch 1959; Barbetti, 1995). A platinum wire loop full of conidia was scraped from the surface of each culture and used to inoculate 100 ml of sterile malt extract broth in 250 ml Erlenmeyer flasks that were placed on a rotary shaker at 20°C and 150 rpm for 72 h. The conidial suspensions were filtered through four layers of sterilized muslin cloth to remove any hyphae. Conidia were suspended in 0.001% Tween 20 and the concentration adjusted to $10^6$ conidia ml$^{-1}$ measured using a haemocytometer.

**Puccinia graminis f. sp. tritici**: Urediniospores of *P. graminis* f. sp. *tritici* [pathotyped as 34-1,2,7 [rather than the more recent 34-1,2,7 + Sr38 pathotype; Cuddy and Park (2013)], were provided by the Department of Primary Industries and Regional Development Western Australia and maintained at -80°C until needed. The spores had been collected from infested wheat plants using a handheld purpose-built vacuum collector and dried over silica gel prior to storage. The viability of the spores was confirmed to be 80% germination by checking their germination on PDA. Urediniospores were suspended in 0.001% Tween 20 and the concentration adjusted to $10^6$ urediniospores ml$^{-1}$ measured using a haemocytometer.

Effect of fungicides and disinfectants on germination of spores in aqueous suspension
For each treatment, 100 µl of the fungicides or disinfectant solution was added to a microplate well in a 36 well culture plate, to which was added 100 µl of a fungal conidia suspension and the two mixed using the pipette tip. Plates were covered and placed in 10 litre plastic boxes with moistened sterile paper towels to maintain high humidity, and incubated at 22 ± 1 °C in the dark for 6, 12, 24, 48, or 72 h. Separate plastic boxes were used for each time to maintain consistent high humidity for all treatments. At each sample time, a 50 µl aliquot of treated spores was placed on a glass microscope slide, covered with a glass cover slip and 100 conidia for each of the six replicates at each concentration were assessed under an Olympus (BX51) microscope to determine the number of conidia germinated, from which the optimal concentration and time of exposure needed to attain maximum reduction in germination rate was calculated.

Germination of spores on agar plates amended with fungicides and disinfectants

Autoclaved half-strength PDA cooled to 55°C was amended to achieve the optimal concentration of each fungicide or disinfectant based on the results from the previous section (as detailed in Table 1). For each treatment, 100 µl of a fungal conidia suspension prepared as previously described was spread evenly on the plates using a bent, sterile glass rod. Six replicate plates were included for each chemical/pathogen combination, and for the unamended PDA controls. The plates were sealed with Parafilm and maintained at high humidity as described above at 22 ± 1°C and in the dark for 48 h and germination of spores was assessed using an Olympus (BX51) microscope.
Decontamination of spore carrier materials with fungicides and disinfectants

Five different spore carrier materials: metal (steel), fabric (cotton jeans), wood (jarrah, *Eucalyptus marginata*), paper, and rubber (tire), were selected as test materials. They were chosen because they serve as common materials used in everyday life, found on farms and commonly used by travellers or in transport. Furthermore, all had been used in recent studies to define the role of different carrier materials on survival of conidia of *L. maculans* (Barua et al. 2018b), *K. caulivora* (Barua et al. 2017b) and *Puccinia graminis* f. sp. *tritici* (Barua et al. 2018a). All test materials were scrubbed under running tap water using a plastic brush for 5 min to remove any potential contaminants. Each of the carrier materials were cut into 0.5 cm squares, autoclaved and randomly placed into rows within sterile 48-well culture plates (Greiner sterile, Greiner Bio-One, Interpath Services Pty Ltd, West Heidelberg, Victoria, Australia). An aliquant of 10 µl of conidia suspension of each plant pathogen was placed individually on each carrier material. The samples were air-dried in a laminar flow cabinet for 1 h. Solutions of the test fungicides or disinfectants at the manufacturer recommended rates were applied so as to cover the inoculated carrier materials in the wells. The plates were covered with lids and all sides of the culture plates sealed with Parafilm. The plates were placed in 10 liter plastic boxes maintained at 22 ± 1°C in the dark for 48 h. Six replicate plates were used for each material/treatment combination; controls consisted of inoculated materials that were unsprayed. Conidia were recovered from the carrier materials as described by Barua et al. (2017a, b, 2018a,b). Briefly, 800 µl of 0.001% Tween 20 was added directly to each of the wells in the treatment
plates which were subsequently placed on a rotary shaker for 40 min at 700 rpm. After washing, the spore suspensions recovered from the carrier materials were examined and spore germination calculated as described above.

Microscopic examination of spore attachment to carrier materials

After washing the carrier materials to obtain the conidia suspensions, the carrier materials were prepared for scanning electron microscopy (SEM) studies to observe for attachment of the remaining conidia on the different carrier materials. Materials were fixed, dehydrated, dried and mounted on metal stubs following the standard SEM sample preparation technique at the Centre for Microscopy, Characterisation and Analysis (CMCA, UWA, Perth, Western Australia, Australia) and as described by Lešer et al. (2009). A microwave processing method (a BioWave microwave processor fitted with PELCO coldspot) was used to prepare samples for SEM. Two to three pieces from each treatment were transferred separately into glass vials with fresh 2.5% glutaraldehyde and fixed by microwave vacuum infiltration for 6 min (2 min on/2 min off/2 min on) at a power of 80 W. Before dehydration, specimens were subjected to buffer wash with deionized water at a power of 80 W for 40 s. Specimens were dehydrated with a series of ethanol solutions increasing in concentration from 50% to 90% and finally with 100% ethanol solution twice at a power of 250 W for 40 s. Thereafter, specimens were critical point-dried with liquid carbon dioxide (Polaron critical point dryer, E3000, Quorum, Laughton, Wast Sussex, England) and mounted on standard aluminium pin
mounts with carbon tabs (ProSci-Tech, Thuringowa Central, Queensland, Australia). Finally, samples were sputter-coated with 5 nm carbon, 3 nm platinum and surface imaging conducted with a field emission SEM (Zeiss Supra 55 VP, Zeiss, Sydney, New South Wales, Australia) to observe conidia attachment, shape and structure.

Experimental design and data analyses

All experiments were conducted three times, and all experiments were arranged using a completely randomized design, with six replications for each treatment. The results from the initial and repeat experiments were analysed by paired t-testing using GenStat [GenStat (18th edition, GenStat Procedure Library Release PL23.2; www.vsni.co.uk)] and checked for homogeneity of variances across the original and repeat experiments using Bartlett’s test (Snedecor & Cochran, 1989). There were no significant differences between the original and repeat experiments (P > 0.05) using the t test, and variances were similar based on Bartlett’s test. Therefore, data sets from initial and the two repeat experiments were pooled, re-analyzed and presented as a single data set. Single and multiple factor ANOVA were conducted as appropriate to determine whether there was an effect of treatment. Subsequently, means separation using Fisher’s least significant differences (Iisd, α = 0.05) was performed to explore the characteristics of differences among fungicide or disinfectant treatments on germination inhibition of the fungal spores. Treatment effects in relation to each pathogen were analysed using single factor ANOVA, while the interaction between
chemicals, pathogens and carrier materials were analysed using multiple factor ANOVA. As variance ratios (VRs) were greater for main effects than for interactions, comment was made both on main effects and interactions. The percent reduction in germination was calculated by using the formula:

\[ P = \frac{a}{b} \times 100 \]

Where \( P \) is the percentage of reduction, \( a \) is the amount of the reduction and \( b \) is the original amount.

Results

Germination of spores in suspensions treated with fungicides/disinfectants

**Overview:** There were significant effects \((P < 0.001)\) of fungicide/disinfectant, pathogen, and a fungicide/disinfectant \(\times\) pathogen interaction, on germination of spores following treatment with fungicides or disinfectants (Table 1). The maximum % germination was observed at 48 h in all controls and treatments, and there was no significant change in % germination of conidia after 48 h. At 48 h, the minimum % germination (10%) was observed when spores were treated with azoxystrobin, but germination varied with pathogen: 10% germination was observed with *M. oryzae*, and 20% for both *L. maculans* and *P. graminis* f. sp. *Tritici*, respectively. Minimum germination (20%) was observed for *K caulivora* when treated with propiconazole.
**Magnaporthe oryzae:** While up to 95% of untreated conidia germinated by 48 h, only 15% and 10% of conidia germinated at the manufacturer’s recommended rate (2.5% a.i.) with propiconazole and azoxystrobin, respectively. At 0.625% a.i. there was more germination with propiconazole (45%) and azoxystrobin (30%), respectively. The least germination with alkylbenzene sulfonic acid + coconut diethanolamide was 45% at the recommended rate of 10%, while with potassium peroxymonosulfate germination was 79%. Least germination (90%) was observed using didecyldimethyl ammonium chloride at the recommended rate of 1% a.i.

**Leptosphaeria maculans:** A mean of 90% of ascospores germinated under the control treatment after 48 h. In contrast, only 25% and 20% had germinated when treated with the recommended rates of 2.5% a.i. propiconazole azoxystrobin, respectively. At 0.625% a.i. of these products, germination increased to 48% and 24%, respectively. The lowest germination observed using alkali metal salts of alkylbenzene sulfonic acid + coconut diethanolamide was 70% (at the recommended concentration of 10%). Least germination using potassium peroxymonosulfate or didecyldimethyl ammonium chloride was 90% and 89%, respectively, and was at the recommended rate of 1% a.i. for both chemistries.

**Kabatiella caulivora:** Nearly all (97%) of conidia germinated in the control treatment within 48 h. Treatment with propiconazole or azoxystrobin at the recommended rate of 2.5% a.i. reduced germination to 20% and 27%, respectively. Higher concentrations of propiconazole or azoxystrobin did not reduce germination, but germination increased with lower concentrations of 1.25 and 0.625% a.i. Lowest germination (75%) with the alkali metal salts of alkylbenzene sulfonic acid + coconut diethanolamide was at the
recommended rate of 10%. Lowest germination using potassium peroxymonosulfate was 88%, and using didecyldimethyl ammonium chloride was 89%, respectively, both at the recommended rate of 1% a.i.

*Puccinia graminis* f. sp. *tritici*: Most urediniospores (88%) in the control treatment germinated within 48 h. Germination was least at the recommended dose of 2.5% a.i. with propiconazole (25% germination) or azoxystrobin (20%), respectively. Germination increased at one or more lower concentrations of both products. Germination when treated with alkali metal salts of alkylbenzene sulfonic acid + coconut diethanolamide was least (66%) at the recommended rate of 10%. Least germination using potassium peroxymonosulfate (70% germination) or didecyldimethyl ammonium chloride (95% germination) was at the recommended rate of 1% a.i.

Germination of spores on agar plates amended with fungicides/disinfectants

There were significant effects (*P* <0.001) of fungicide/disinfectant, pathogen, and a fungicide/disinfectant × pathogen interaction on germination of spores on half-strength PDA following fungicide/disinfectant treatments (Table 2). The maximum germination of conidia of *M. oryzae* on the control media was 98%. On propiconazole and azoxystrobin amended media it was 20%, but when amended with alkali metal salts of alkylbenzene sulfonic acid + coconut diethanolamide germination was 65%. Germination was also high when media was amended with potassium peroxymonosulfate (68%) or didecyldimethyl ammonium chloride (90%). Ascoposre germination of *L. maculans* was 95% on the control media, but significantly less in media incorporating propiconazole (28%) or azoxystrobin (25%), respectively. Other
Disinfectants were less effective with more germination on media amended with alkali metal salts of alkylbenzene sulfonic acid + coconut diethanolamide (70%), potassium peroxymonosulfate (80%) or didecyldimethyl ammonium chloride (90%). Maximum conidial germination with *K. caulivora* was on the control media (97%). Least germination was on media amended with propiconazole (25%) or azoxystrobin (30%). However, less effective reduction in germination was observed with the alkali metal salts of alkylbenzene sulfonic acid + coconut diethanolamide (80%), potassium peroxymonosulfate (81%) and didecyldimethyl ammonium chloride (90%), respectively. Maximum germination of urediniospore of *P. graminis* f. sp. *tritici* was on control media (95%). Germination was lower on media amended with propiconazole (30%) or azoxystrobin (25%). Again, the alkali metal salts were less effective in reducing germination on agar (germination on media amended with alkylbenzene sulfonic acid + coconut diethanolamide was 70%, with potassium peroxymonosulfate was 65%, and with didecyldimethyl ammonium chloride was 90%, respectively).

Decontamination of spore carrier materials with fungicides/disinfectants

There were significant effects (*P* < 0.001) of fungicide/disinfectant, pathogen, of carrier material and interaction of fungicide/disinfectant × pathogen, pathogen × carrier material, fungicide/disinfectant × carrier material, and also fungicide/disinfectant × pathogen × carrier material (Table 3). The maximum germination on half-strength PDA of spores recovered from control treatments after 48 h was: conidia of *M. oryzae* recovered from steel (62%), jarrah wood (60%), denim jeans (62%), paper (60%) and rubber tyre (60%);
ascospores of *L. maculans* recovered from steel (65%), jarrah wood (60%),
denim jeans (64%), paper (63%) and rubber tyre (60%); conidia of *K. caulivora*
recovered from steel (65%), jarrah wood (60%), denim jeans (60%), paper
(62%) and rubber tyre (63%); and urediniospores of *P. graminis* f. sp. *tritici*
recovered from steel (60%), jarrah wood (65%), denim jeans (63%), paper
(60%) and rubber tyre (60%).

**Steel:** With treatments of propiconazole and azoxystrobin, the germination
of conidia of *M. oryzae* was 12% and 15%, of ascospores of *L. maculans* was
15% and 18%, of conidia of *K. caulivora* was 25% and 25%, and of
urediniospores of *P. graminis* f. sp. *tritici* was 32% and 25%, respectively.
These germination rates are a relative reduction of 47 to 81% with
propiconazole and 54 to 73% with azoxystrobin when compared to the non-
treated control. In contrast, relative reduction in germination on steel was 0%
to 35% with the other three fungicides/disinfectants.

**Jarrah wood:** With treatments of propiconazole and azoxystrobin, the
germination of conidia of *M. oryzae* was 16% and 15%, of ascospores of *L. maculans* was
30% and 20%, of conidia of *K. caulivora* was 20% and 30%, and of
urediniospores of *P. graminis* f. sp. *tritici* was 30% and 25%, respectively. These germination rates are a relative reduction of 50 to 73%
with propiconazole and 58 to 76% with azoxystrobin when compared to the
non-treated control. In contrast, relative reduction in germination was 0% to
46% with the other three fungicides/disinfectants.

**Denim jeans:** With treatments of propiconazole and azoxystrobin, the
germination of conidia of *M. oryzae* was 15% and 15%, of ascospores of *L. maculans* was 24% and 20%, of conidia of *K. caulivora* was 20% and 30%,
and of urediniospores of *P. graminis* f. sp. *tritici* was 30% and 20%,
respectively. These germination rates are a relative reduction of 52% to 76% with propiconazole and 50 to 76% with azoxystrobin when compared to the non-treated control. In contrast, relative reduction in germination was 0% to 44% with the other three fungicides/disinfectants.

**Paper:** With treatments of propiconazole, azoxystrobin and potassium peroxymonosulfate, germination of conidia of *M. oryzae* was 17%, 15%, and 40%, respectively. With the same three fungicides/disinfectants, germination of ascospores of *L. maculans* was 30%, 20%, and 30%, respectively, and for germination of conidia of *K. caulivora* was 20%, 30% and 30%, respectively, and for urediniospores of *P. graminis* f. sp. *tritici* was 30%, 25%, and 38%, respectively. These germination rates are a relative reduction of 50% to 72% with propiconazole and 52 to 75% with azoxystrobin when compared to the non-treated control. In contrast, relative reduction in germination was 0% to 52% with the other three fungicides/disinfectants.

**Rubber tyre:** With treatments of propiconazole and azoxystrobin, the germination of conidia of *M. oryzae* was 17% and 16%, and of ascospores of *L. maculans* was 25% and 21%, respectively. With treatments of propiconazole, azoxystrobin and potassium peroxymonosulfate, germination of conidia of *K. caulivora* was 20%, 35%, and 36%, and of urediniospores of *P. graminis* f. sp. *tritici* was 30%, 30%, and 40%, respectively. These germination rates are a relative reduction of 50% to 72% with propiconazole and 44 to 73% with azoxystrobin when compared to the non-treated control. In contrast, relative reduction in germination was 0% to 43% with the remaining three treatments.

Microscopic examination of spore attachment to carrier materials
Scanning Electron Microscopy (SEM) revealed that some spores readily adhered to all the inert material types tested, even after washing with fungicides or disinfectants (Fig. 1A-H). Nearly all the spores from the untreated controls retained their natural shape. However, both conidia of *M. oryzae* and urediniospores of *P. graminis* f. sp. *tritici* disintegrated following spraying with propiconazole or azoxystrobin, as compared with little effect following application of potassium peroxymonosulfate (Fig. 1A-H).

**Discussion**

This study demonstrated that the germination of fungal conidia was inhibited, although not always significantly, by the various fungicides or disinfectants tested. However, the extent to which germination was inhibited was dependent on the specific fungicide/disinfectant treatment. Fungicides or disinfectants were more effective at reducing conidia germination when added directly to conidial suspensions when compared with being incorporated in solid agar media. Furthermore, all fungicides and disinfectants at least partially inhibited germination of conidia on the different carrier materials, the extent of inhibition varying with the pathogen and the material. Overall, maximum reductions in conidia germination occurred at the manufacturer’s recommended rate and it is noteworthy that increasing the fungicide/disinfectant concentration above the recommended rate generally did not significantly reduce germination beyond what was observed at the recommended rates. Of note, the concentrations of fungicides or disinfectants tested at below recommended rates were often less effective, emphasizing the
need to adhere to manufacturer’s recommendations. This study highlights the potential for increased use of fungicides, particularly demethylation inhibitor (triazoles, e.g. propiconazole) and QoI fungicides (strobilurins, e.g. azoxystrobin) and/or some other more efficacious disinfectants, to decontaminate carrier materials. Overall, azoxystrobin and propiconazole, were the most effective of the fungicides and disinfectants tested, while didecyldimethyl ammonium chloride had the least effect inhibiting spore germination.

The extent to which germination of fungal spores was inhibited, compared with the non-treated control, varied between the fungicides and disinfectants and also with the pathogen. For example, azoxystrobin and propiconazole effectively reduced germination of conidia of *M. oryzae* on half-strength PDA, while the alkali metal salts of alkylbenzene sulfonic acid + coconut diethanolamide and potassium peroxymonosulfate were more effective reducing germination of conidia of *M. oryzae* and *P. graminis* f. sp. *tritici* when compared with *L. maculans* or *K. caulivora*. Material contaminated with fungal conidia may be either fumigated or sprayed with fungicides or other more general-purpose disinfectants. Examples of plant pathogen control using fungicides includes applications to crops to control diseases in the field (e.g., to control *Cercospora zebrina* in *Trifolium subterraneum* (Barbetti 1987)); applications to infested crop residues (e.g., fluquinconazole, flutriafol, and glufosinate-ammonium to delay development of pseudothecia and decrease ascospore discharge in *L. maculans* (Wherrett et al. 2003)); or for treating infected or contaminated seeds (e.g., benomyl, carbathiin + thiram, iprodione, prochloraz, thiabendazole, tolclofos-methyl against seed borne diseases, including *L. maculans* (Kharbanda 1992)). Disinfectants are commonly used
in attempts to decontaminate materials entering a country (including potassium peroxymonosulfate), particularly for some high-risk plant and animal pathogens (Fretzel et al. 2013). In contrast, applications of foliar-applied fungicides in the field can be less efficacious as spores are released over extended periods during the growing season (Barbetti 1975; Kharbanda, 1992). Most fungicides, particularly those used in agriculture, are effective when applied prior to infection and generally involve inhibition of spore germination and/or suppression of germ tube and early hyphal growth (Taskeen-Un-Nisa et al. 2011). The current study demonstrates that the fungicides and disinfectants tested inhibited spore germination and/or suppressed germ tube and early hyphal growth, making them successful for treating spore-contaminated carrier materials. However, most fungicides are only registered for use on crops, and even if useful for decontaminating carrier materials, they would need to be specifically registered for this purpose.

We appreciate that there are a range of other decontamination measures not considered in this study and that may be effective in many situations. For example, fumigants are used to mitigate risks associated with invertebrate pests, yet some of these chemicals may be less well recognized for decontamination targeted at fungal plant pathogens (Fields and White 2002). Similarly, UV light and/or gamma irradiation treatments are used to decontaminate working surfaces, including laminar flow cabinets, from fungal and bacterial spore contamination and may offer some benefits for the decontamination of everyday products (Choi et al. 2012; Christofi et al. 2008). The chemicals used in our may not be suited to all situations relating to decontamination due to health concerns or their inability to control a broad
enough spectrum of microorganisms, leaving them most likely to be utilized in situations targeting movement of specific, destructive plant pathogens.

When added directly to fungal conidia/spores of all pathogen tested, azoxystrobin reduced germination between 72-89% and propiconazole reduced germination 72-84% respectively, depending on the pathogen. Quinone outside inhibitor (QoI) fungicides, including azoxystrobin, are extremely potent inhibitors of spore germination (Balba 2007). As demonstrated in the current study, while such fungicides have a broad spectrum fungicidal effect, and while they are generally considered ‘safer’ (Bartlett et al. 2002), their efficacy and safety need to be tested across a broader range of plant pathogens. The demethylation inhibitor class of fungicides, and specifically those within the triazole group which includes propiconazole, prevent production of sterol in fungi (Calonne et al. 2012), preventing cell wall formation and thus inhibits fungal growth (Oger et al. 2009). Hence, propiconazole is considered to be fungistatic or growth inhibiting rather than fungicidal. Pak et al. (2017) reported that while azoxystrobin and propiconazole effectively inhibited conidial germination of M. oryzae, azoxystrobin was more efficacious than propiconazole. This is likely as azoxystrobin is a broad spectrum fungicide and a member of the β-methoxyacrylates class originally derived from naturally occurring strobilurins, which are highly effective phytotoxic compounds (Balba 2007). Azoxystrobin is very effective against several major groups of fungi, including those in the Ascomycota, Deuteromycota, Basidiomycota and against Oomycota. Azoxystrobin is registered in 72 countries for use on 84 different crops (Bartlett et al., 2002; Rao et al. 2013). Similarly, propiconazole is a fungistatic agent that has protective and curative action with a broad range of activity (Ivic
Propiconazole is used to control a wide range of fungal diseases including powdery mildew, rust, scab and leaf spot diseases caused by plant pathogens of several different genera including *Puccinia* spp., *Erysiphe* sp., *Leptosphaeria* sp., *Pseudocercospora* *herpotrichoides*, and *Septoria* spp. in cereals, rice, brassicas, grasses, and many vegetable and fruit crops (Kurti 2004; Ali et al. 2013; Prasanna Kumar and Veerabhadraswamy 2014). That propiconazole and azoxystrobin act as effective systemic fungicides with curative and preventative actions (Ivic 2010), and that they can easily be applied as a spray to almost any carrier material surface, makes them ideal chemicals for decontamination. Propiconazole is also used for preserving wood used in millwork, shingles and shakes, siding, plywood, structural lumber and timbers and composites that are used in above-ground applications (Anonymous 2017; Baileys et al. 2003; Clausen and Green 2009), additionally highlighting its effectiveness across a wide range of different plant pathogens. Propiconazole used to protect and decontaminate wood is considered a safer alternative to traditionally used preservatives such as azaconazole and pentachlorophenols (Anonymous 2017; Kurti 2004). Although the fungicides and disinfectants tested did not completely inhibit germination of fungal spores the more effective ones, azoxystrobin and propiconazole, decreased the germination rate relative to the non-treated control by up to 89% and 84%, respectively for *M. oryzae*.

Treatments including azoxystrobin and propiconazole that demonstrated the greatest spore inhibition offer potential for commercial decontamination of different carrier materials. The importance of these chemicals as decontaminants should not be underestimated, as some fungal spores remain viable for long periods even at high temperatures when attached to carrier
materials (Barua et al. 2017a,b, 2018a,b). Furthermore, it has been reported that spread associated with contaminated clothing, footwear, baggage, plastic wrapping and the external surfaces of shipping containers constitutes significant risk to importation of exotic plant pathogens (Grgurinovic et al. 2006; Hill et al. 1995). Decontamination of carrier materials with fungicides and disinfectants offers a cost-effective, preventive measure and can be utilized as part of future recommendations in Integrated Pest Management (IPM) programs to control fungal plant pathogen spread and to reduce risks of introduction of exotic plant pathogens and/or exotic races of plant pathogens already endemic in a country or region. For example preventing spread of the bacterial pathogen citrus canker in Florida was the focus of major eradication and sanitation campaigns in Florida in the USA (Graham et al. 2004), with decontamination methods tested against the pathogen (Bock et al. 2011).

This study has confirmed that, although not absolute, substantial decontamination of long-term carrier materials harbouring viable spores of various plant pathogens is possible, including spores of *P. graminis* f. sp. *tritici*, *K. caulivora*, *L. maculans*, and *M. oryzae*. There remains a critical need to implement a practical commercial solution to deal with threats posed by the long-term viability of plant pathogens on inert materials associated with movement of humans, farming equipment, and commodities both nationally and internationally. The results from this study also demonstrate the larger biosecurity implications in relation to disinfestation of transported fungal plant pathogen spores on carrier materials in general, and highlight an urgent need to re-evaluate chemical (and other) treatments for effective decontamination of carrier materials that have historically been considered a low biosecurity risk in regards to the transport of fungal plant pathogens.
Acknowledgements

We appreciate the scholarship provided by the Plant Biosecurity Cooperative Research Centre, Bruce, ACT 2617, Australia, for project CRC62042: “Curtailing and managing exotic fungal spore incursion into Australia”, and the financial support by the School of Plant Biology at the University of Western Australia. We thank Mr Robert Creasy and Bill Piasini from the University of Western Australia for support and help with these controlled environment studies.

Compliance with ethical standards

Ethical statement: This research did not involve any animal and/or human participants.

Conflict of interest: The authors declare that they have no conflict of interests.
References


Table 1 Germination of Magnaporthe oryzae, Leptosphaeria maculans, Kabatiella caulivora and Puccinia graminis f. sp. tritici in spore suspensions 48 h after treatment with different concentrations of propiconazole (Tilt 250EC), azoxystrobin (Amistar 250EC), didecyldimethyl ammonium chloride (Sporekill), alkali metal salts of alkylbenzene sulfonic acid + coconut diethanolamide (Farmcleanse) or potassium peroxymonosulfate (Potassium peroxymonosulfate)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean germination (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M. oryzae</td>
<td>L. maculans</td>
<td>K. caulivora</td>
<td>P. graminis f. sp. tritici</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>95</td>
<td>90</td>
<td>97</td>
<td>88</td>
</tr>
<tr>
<td>Propiconazole</td>
<td>0.625</td>
<td>45</td>
<td>48</td>
<td>36</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>30</td>
<td>45</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>2.5*</td>
<td>15</td>
<td>25</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>3.75</td>
<td>15</td>
<td>25</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>15</td>
<td>25</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Azoxystrobin</td>
<td>0.625</td>
<td>30</td>
<td>24</td>
<td>45</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>30</td>
<td>24</td>
<td>45</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>2.5*</td>
<td>10</td>
<td>20</td>
<td>27</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>3.75</td>
<td>10</td>
<td>20</td>
<td>27</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>27</td>
<td>20</td>
</tr>
<tr>
<td>Alkali metal salts of alkylbenzene sulfonic acid + coconut diethanolamide</td>
<td>5</td>
<td>62</td>
<td>81</td>
<td>84</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>58</td>
<td>78</td>
<td>80</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>10*</td>
<td>45</td>
<td>70</td>
<td>75</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>45</td>
<td>70</td>
<td>75</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>45</td>
<td>70</td>
<td>75</td>
<td>65</td>
</tr>
<tr>
<td>Didecyldimethyl ammonium chloride</td>
<td>0.5</td>
<td>92</td>
<td>92</td>
<td>90</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>91</td>
<td>91</td>
<td>90</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>1*</td>
<td>90</td>
<td>89</td>
<td>90</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>90</td>
<td>89</td>
<td>89</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>90</td>
<td>89</td>
<td>88</td>
<td>87</td>
</tr>
<tr>
<td>Potassium peroxymonosulfate</td>
<td>0.5</td>
<td>87</td>
<td>90</td>
<td>90</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>80</td>
<td>90</td>
<td>90</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>1*</td>
<td>79</td>
<td>86</td>
<td>88</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>79</td>
<td>86</td>
<td>88</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>79</td>
<td>86</td>
<td>88</td>
<td>70</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>55</td>
<td>62</td>
<td>65</td>
<td>57</td>
</tr>
</tbody>
</table>

Main effects and interaction:  
- Chemical: F = 797.2, P = <0.001, l.s.d. (P < 0.05) = 0.7  
- Pathogen: F = 35.5, P = <0.001, l.s.d. (P < 0.05) = 0.6  
- Chemical x Pathogen: F = 9.7, P = <0.001, l.s.d. (P < 0.05) = 1.3

* Recommended concentration from the manufacturer
† Experiments were conducted three times, were arranged using a completely randomized design, with six replications, and date in table is a composite of data from all three experiments - see material & methods for details.
Table 2 Germination of *Magnaporthe oryzae*, *Leptosphaeria maculans*, *Kabatiella caulivora* and *Puccinia graminis* f. sp. *tritici* spores after 48 hours incubation on half strength potato dextrose agar (½ PDA) amended with propiconazole (Tilt 250EC), azoxystrobin (Amistar 250EC), didecyldimethyl ammonium chloride (Sporekill), alkali metal salts of alkylbenzene sulfonic acid + coconut diethanolamide (Farmcleanse) or potassium peroxymonosulfate (Virkon)†.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Mean germination (%)</th>
<th>M. oryzae</th>
<th>L. maculans</th>
<th>K. caulivora</th>
<th>P. graminis f. sp. tritici</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (½PDA)</td>
<td>98</td>
<td>95</td>
<td>97</td>
<td>95</td>
<td>96.3</td>
<td></td>
</tr>
<tr>
<td>Propiconazole</td>
<td>20</td>
<td>28</td>
<td>25</td>
<td>30</td>
<td>70.7</td>
<td></td>
</tr>
<tr>
<td>Azoxystrobin</td>
<td>20</td>
<td>25</td>
<td>30</td>
<td>25</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td>Alkali metal salts of alkylbenzene sulfonic acid + coconut diethanolamide</td>
<td>65</td>
<td>70</td>
<td>80</td>
<td>70</td>
<td>71.2</td>
<td></td>
</tr>
<tr>
<td>Didecyldimethyl ammonium chloride</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90.0</td>
<td></td>
</tr>
<tr>
<td>Potassium peroxymonosulfate</td>
<td>68</td>
<td>80</td>
<td>81</td>
<td>65</td>
<td>73.5</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>60.2</td>
<td>64.7</td>
<td>67.2</td>
<td>62.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Main effects and interaction:*  

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>P</th>
<th>l.s.d. (P &lt; 0.05 =)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical</td>
<td>22458.0</td>
<td>&lt;0.001</td>
<td>0.5</td>
</tr>
<tr>
<td>Pathogen</td>
<td>496.8</td>
<td>&lt;0.001</td>
<td>0.4</td>
</tr>
<tr>
<td>Chemical × Pathogen</td>
<td>149.7</td>
<td>&lt;0.001</td>
<td>1.0</td>
</tr>
</tbody>
</table>

† Experiments were conducted three times, were arranged using a completely randomized design, with six replications, and date in table is a composite of data from all three experiments - see material & methods for details.
Table 3  Germination of *Magnaporthe oryzae*, *Leptosphaeria maculans*, *Kabatiella caulivora* and *Puccinia graminis f. sp. tritici* spores washed from carrier materials 48 h after treatment with propiconazole (Tilt 250), azoxystrobin (Amistar 250), didecyldimethyl ammonium chloride (Sporekill), alkali metal salts of alkylbenzene sulfonic acid + coconut diethanolamide (Farmcleanse) or potassium peroxymonosulfate (Virkon).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Carrier material</th>
<th>Mean % germination</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>M. oryzae</em></td>
<td><em>L. maculans</em></td>
<td><em>K. caulivora</em></td>
<td><em>P. graminis f. sp. tritici</em></td>
</tr>
<tr>
<td>Control</td>
<td>Metal (steel)</td>
<td>62</td>
<td>65</td>
<td>65</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Jarrah Wood</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>Fabric (Denim jeans)</td>
<td>62</td>
<td>64</td>
<td>60</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>Paper</td>
<td>60</td>
<td>63</td>
<td>62</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Rubber tyre</td>
<td>60</td>
<td>60</td>
<td>63</td>
<td>60</td>
</tr>
<tr>
<td>Propiconazole</td>
<td>Metal (steel)</td>
<td>12</td>
<td>15</td>
<td>25</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Jarrah Wood</td>
<td>16</td>
<td>30</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Fabric (Denim jeans)</td>
<td>15</td>
<td>24</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Paper</td>
<td>17</td>
<td>30</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Rubber tyre</td>
<td>17</td>
<td>25</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>Azoxystrobin</td>
<td>Metal (steel)</td>
<td>15</td>
<td>18</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Jarrah Wood</td>
<td>15</td>
<td>20</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Fabric (Denim jeans)</td>
<td>15</td>
<td>20</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Paper</td>
<td>15</td>
<td>20</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Rubber tyre</td>
<td>16</td>
<td>21</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>Alkali metal salts of alkylbenzene sulfonic acid and coconut diethanolamide</td>
<td>Metal (steel)</td>
<td>40</td>
<td>42</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Jarrah Wood</td>
<td>45</td>
<td>55</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Fabric (Denim jeans)</td>
<td>50</td>
<td>56</td>
<td>58</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Paper</td>
<td>50</td>
<td>55</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Rubber tyre</td>
<td>52</td>
<td>55</td>
<td>58</td>
<td>55</td>
</tr>
<tr>
<td>Didecyldimethyl ammonium chloride</td>
<td>Metal (steel)</td>
<td>55</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Jarrah Wood</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>Fabric (Denim jeans)</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Paper</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Rubber tyre</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Potassium peroxymonosulfate</td>
<td>Metal (steel)</td>
<td>60</td>
<td>50</td>
<td>42</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Jarrah Wood</td>
<td>54</td>
<td>50</td>
<td>45</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Fabric (Denim jeans)</td>
<td>53</td>
<td>50</td>
<td>41</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Paper</td>
<td>40</td>
<td>30</td>
<td>30</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Rubber tyre</td>
<td>54</td>
<td>52</td>
<td>36</td>
<td>40</td>
</tr>
</tbody>
</table>

**Main effects and interactions:**

<table>
<thead>
<tr>
<th></th>
<th><em>F</em></th>
<th><em>P</em></th>
<th>l.s.d. (<em>P &lt; 0.05</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical</td>
<td>44689.1</td>
<td>&lt;0.001</td>
<td>0.23</td>
</tr>
<tr>
<td>Pathogen</td>
<td>615.1</td>
<td>&lt;0.001</td>
<td>0.19</td>
</tr>
<tr>
<td>Carrier material</td>
<td>145.9</td>
<td>&lt;0.001</td>
<td>0.21</td>
</tr>
<tr>
<td>Chemical × Pathogen</td>
<td>995.8</td>
<td>&lt;0.001</td>
<td>0.46</td>
</tr>
<tr>
<td>Pathogen × Carrier material</td>
<td>35.8</td>
<td>&lt;0.001</td>
<td>0.42</td>
</tr>
<tr>
<td>Chemical × Carrier material</td>
<td>284.6</td>
<td>&lt;0.001</td>
<td>0.52</td>
</tr>
<tr>
<td>Chemical × Pathogen × Carrier material</td>
<td>74.3</td>
<td>&lt;0.001</td>
<td>1.03</td>
</tr>
</tbody>
</table>
Fig. 1 Scanning electron micrographs (SEM) of fungal spores attached to carrier materials 48 h after spraying with the recommended concentration of the fungicides or disinfectants. A-D Magnaporthe oryzae spores on paper (A) control, (B) when sprayed with azoxystrobin, (Amistar 250EC) (C) when sprayed with propiconazole (Tilt 250EC), (D) when sprayed with potassium peroxymonosulfate (Virkon). E-H Puccinia graminis f. sp. tritici urediospores on steel (E) control, (F) when sprayed with azoxystrobin (Amistar 250EC), (G) when sprayed with propiconazole (Tilt 250EC), and (H) when sprayed with potassium peroxymonosulfate (Virkon).
CHAPTER 7

7.1. General Discussion

My studies are the first to demonstrate utilization of an Alamar Blue (Resazurin) reagent for determining the viability of fungal spores. They are also the first comprehensive investigations to confirm the critical importance of inert materials as long-term carriers of viable fungal ascospores, urediniospores, conidia and resting hyphae across different temperatures. One critical aspect of any plant disease cycle is the ability of the pathogen to survive during periods of unfavourable environmental conditions and, in particular, my studies assessed the role and significance of inert materials on survival of fungal spores in absence of an alternative host using controlled temperature conditions.

7.1.1. Rapid Fungal spore Viability Assay

Fungal spore viability is conventionally estimated by germination methods that are laborious, space and time-consuming processes that sometimes require specific media for different species. Further, not all viable spores germinate readily. Hence the development of a rapid biochemical Alamar Blue (Resazurin) method that measures the viability of fungal spores has been an important advance in monitoring presence of viable fungal spores (Chapter 2). While the Alamar Blue bioassay had been extensively utilised in medical research involving human or animal cells (Al-Nasiry et al. 2007; Gloeckner et al. 2001; Nakayama et al. 1997) to assess cell viability and cytotoxicity across an extensive number of cell types including bacteria, yeast, protozoa and cultured mammalian cells (Fai and Grant 2009; Rampersad 2012), my study
was its first application for a range of important fungal pathogens. I confirmed Alamar Blue as an excellent indicator of the metabolic capacity of cells (Freimoser et al. 1999) and my research highlights how it offers significant improvement as a novel and fast technique for testing for viability of fungal spores. Compared with traditional methods for assessing spore viability, such as trypan blue staining, use of a cell counter, or assessing germination of the spores under the microscope, my novel application of this methodology demonstrated greater reliability and more rapid outcomes, with only a short (<2 h) incubation period needed to measure viability of spores.

While there are many viability assays available, I initially chose the Alamar Blue assay for its advantage in maintaining cells alive for long-term monitoring (Al-Nasiry et al. 2007; Xiao et al. 2010), unlike the MTT, MS and ATP assays (Fotakis and Timbrell 2006; Page et al. 1993; Rampersad 2012) that cause cell death. I not only found the Alamar Blue assay a simple, rapid way to measure the viability of fungal spores, it is less expensive, non-radioactive and non-toxic to both pathogens and users and therefore did not require any special handling or disposal methods (Ahmed et al. 1994; Coban et al. 2014). Further advantage of this methodology is that it can be added directly to a homogenous or non-homogenous spore suspension sample of any size and easily be utilized in 96 to 384-well plates; with spore viability highlighted and recorded using either fluorescence or absorbance.

I compared this Alamar Blue assay to other methods of viability assessment such as trypan blue staining, use of a cell counter, or assessing germination methods, particularly Trypan blue that is commonly used to observe the viability of cells (Louis and Siegel 2011; Strober 2001). I showed Trypan blue to be a comparatively time consuming assay, requiring
microscopic observations and found it inappropriate where large numbers of samples need to be processed. Also, extended exposure time to Trypan blue can be toxic to live cells (Mascotti et al. 2000). However, Alamar Blue did not require microscopic investigation to observe or confirm the viability of spores. While cell counters have been used to count viable cells both in animals and plants, these mostly work best with a homogenous cell concentration range of $5 \times 10^4$ to $1 \times 10^7$ cells ml$^{-1}$ and with cell diameters in the range of 6-50 µm (Bio-Rad 2010). I found the TC10 cellometer cell counter was not really appropriate for use for ascospores of *L. maculans* as the spore size for this pathogen range from width of 5–8 µm and in length from 30-70 µm (Punithalingam and Holliday 1972). Compared to using a cell counter, I showed that the Alamar Blue assay worked well in a non-homogenous spore suspension and across various sizes of spores and even with very low spore concentrations. I also validated the assay further by assessing the viability of spores from three other fungal plant pathogens *Magnaporthe oryzae Puccinia striiformis* sp. *tritici*, and *Kabatiella caulivora* and also commercially available baker’s yeast- *Saccharomyces cerevisiae*.

The successful application of the Alamar Blue assay to measure fungal spore viability has important implications for biosecurity in relation to potential incursions of exotic fungal pathogens. In my studies I could readily determine viability of *L. maculans* ascospores, *K. caulivora* conidia and resting hyphae, and *P. graminis*. f.sp. *tritici* urediniospores on a range of test materials including steel, fabric, wood, paper, rubber and leather in order to better understand the retention of viable fungal spores by these spore-carrier materials over time, as discussed below. While this highlights how the Alamar Blue assay can easily be adapted and optimised to determine the viability of a
wide range of fungal pathogens, it will be important to confirm a wider range of pathogen ‘types’ in future studies to broaden its application.

7.1.2. The role of inert materials as way to spread natural spore inoculum to new areas

For the first time across a range of material types, including metals, plastics, fabrics and woods, my studies demonstrated the significant effect of different carrier material types and different temperatures on fungal spore viability. Together, these findings provide important new understanding of mechanisms for movement of viable fungal spores by ‘non-traditional’ means and open up new possible explanations of extended survival of fungal spores and the potential initiation subsequent disease epidemics. For example, as discussed later in this section, how some wheat rust epidemics occur in parts of Western Australia where no ‘green-bridge’ volunteers harbouring rust are known to occur over the hot dry summer. Also these findings are an important step in the development and utilization of effective decontamination strategies for fungal spore infestations on carrier materials, as also discussed later in this section.

Long term viability was demonstrated for *L. maculans* ascospores (Chapter 3), *K. caulivora* conidia and resting hyphae (Chapter 4) and *P. graminis*. f.sp. *tritici* urediniospores (Chapter 5) on a wide range of different carrier materials and under different temperature conditions. This highlights the significant potential for dissemination of these spore types on inert carrier materials and, in particular, the role of these materials as an alternative way to spread natural spore inoculum of plant pathogens to new areas. Some widely used material in day-to-day life, such as fabrics, metals, paper, rubber tyre, leather and wood, have historically been shown to be potential carriers of
viable fungal spores (Hughes et al. 2010). Examples include viable resting spores of *Synchytrium endobioticum* (the causal agent of potato wart) from vehicles (Hampson and Wood 1997); 65 fungal species from 39 genera isolated from shoes of travellers arriving at Honolulu International Airport from San Francisco (Baker 1966); and *P. coronata* collected from clothing and baggage of passengers arriving by air in New Zealand (Sheridan and Nendick 1988; Sheridan 1989). However, my studies highlight the unexpectedly long time-period that plant fungal pathogen spores can remain viable. For example, viable *L. maculans* ascospores and *K. caulivora* conidia and resting hyphae were recovered up to 240 days; and viable *P. graminis*. f. sp.* tritici urediniospores recovered up to 365 days from inert carrier materials.

My studies highlight the particular relevance and threat from survival of *L. maculans* ascospores and *P. graminis*. f. sp.* tritici urediniospores, as such surviving spores can cause infections that then rapidly produce large spore numbers that are then primarily an widely dispersed by wind (Li et al. 2006; Misra et al. 2014). For example, one *P. graminis*. f. sp.* tritici uredinium can produce about 10,000 urediniospores/day (Katsuya and Green, 1967), with the estimated mean rate of spread of *P. graminis*. f. sp.* tritici approximates 35 km d^{-1} (Aylor 2003) and with long-distance transport critical for distributing new genetic variants of rusts over long distances such as across the 2000km from Australia to New Zealand (Luig 1985; Rees 1972). Further, my studies confirm finding of Sheridan (1989) that clothing can carry many viable pathogenic fungi, including urediniospores of rusts such as *P. coronata* and *P. graminis*. f. sp.* tritici, *P. hordei*, consequent from conducting disease surveys of cereal crops. Such clothing could then spread plant fungal pathogens over even longer distances via air travel of contaminated passengers. The relevance of
this latter potential spread is highlighted by the estimated 70,000 viable rust urediniospores brought into New Zealand in 1982 on travellers’ clothing and baggage (Sheridan 1989). Similarly, high occurrence of viable microbes including fungi have also been recorded from soil removed from footwear carried by passengers arriving in New Zealand (McNeill et al. 2011). The potential impact on agriculture from such introductions is well illustrated by the human-aided-introduction of barley stripe rust into Columbia in 1975 (Roelfs and Bushnell 1985), of wheat stripe rust into Australia in 1979 (Wellings et al. 1987; Wellings 2007), and the detection of viable *P. psidii* urediniospores on kiln-dried *Eucalyptus* timber imports from Brazil, including the plastic wrapping and shipping container external surfaces even following a month sea journey to Australia in 2004 (Grgurinovic et al. 2006). While successful long term survival of fungal spores on carrier materials is promoted by dry air, the subsequent potential for pathogen establishment in the new environment will depend upon the interaction of many factors, including proximity of susceptible hosts, subsequent spore release timing and wind patterns (Savage et al. 2010). However, it is evident that viable fungal spores on carrier materials such as transport containers can spread and establish on commonly occurring weedy host species in transport depots, on road sides, and on neighbouring farms.

In addition, my studies provide new understanding of disease epemics of existing current crop pathogens in Australia. For example, while infected oilseed rape debris plays a critical role in survival of *L. maculans* and provides the important primary source of inoculum for successive dissemination across seasons and neighbouring regions (Huang et al. 2003; Li et al. 2007; West et al. 1999; West et al. 2001), the survival and viability of
ascospores subsequent to emission from pseudothecia is crucial in the
disease epidemiology (Savage et al. 2012). In Australia, *L. maculans*
ascospore emissions have a distinct diurnal pattern with a mid- to late-
afternoon peak in ascospore release that matches with meteorological
conditions of warmer temperatures and lower humidity (Savage et al. 2012). It
is evident from my studies that not only do such ascospores readily survive
longer periods under dry conditions, enabling both enhanced dispersal over
larger distances than for equivalent release at other times of the day, any such
spores landing on inert materials, and even soil (Li et al. 2006, 2007), are also
likely to survive for longer periods of time that would otherwise have been expected.

7.1.3. Challenges in removal of fungal spore contamination

Not all *L. maculans* ascospores, *P. graminis* f. sp. *tritici* urediniospores,
nor *K. caulivora* conidia and hyphae could be removed from the carrier
materials even after washing the materials. This highlights the challenges in
eliminating fungal spore contamination from equipment, clothes, travellers,
wood, crafts and toys, especially from washing contaminated clothing. That
this was the case, even after multiple washes, highlights the challenges in
eliminating fungal spore and hyphae contamination in general and from farm
machinery, clothes of regional, national and international travellers, in
particular. This poses an environmental and/or economic biosecurity threat in
regions where these pathogens, or specific race(s) of them, do not yet occur.
For example, *K. caulivora* Race 2 is predominantly found in the Esperance
region of Western Australian (Barbetti 1996; You et al. 2007) but could quickly
spread much further on inert materials such as farm equipment and clothing.
As many varieties of subterranean clover with resistance against \textit{K. caulivora} Race 1 are very susceptible to Race 2 (Barbetti 1995, 1996; Bayliss et al. 2002), any spread of Race 2 more widely across southern Australia threatens >1 m ha of varieties with effective resistance only against Race 1 (Barbetti and You 2014).

7.1.4. Effect of temperature on spore viability over time

My studies highlighted a major role of temperature on the viability of the fungal spores and that it had a significant interaction with material type. For example, for \textit{L. maculans}, while at 23°C/8°C day/night, ascospores remained viable on pine wood and Tasmanian oak for up to 240 days; at 36°C/14°C day/night, ascospores were viable for up to 180 days and then only on pine wood; but at 45°C/15°C day/night, ascospores remained viable for only up to 60 days, and only on jute. For \textit{K. caulivora}, at 23°C/4°C day/night, conidia and resting hyphae remained viable on steel, corrugated steel, galvanised steel and all tested fabrics, wood and random mixed materials for up to 240 days; but at 36°C/14°C day/night, only up to 240 days on cotton, denim, fleece, silk, leather, paper, plastic and all the wood materials; and at 45°C/15°C day/night, for up to 240 days but only on fleece wool, \textit{E. marginata} (jarrah wood) and on paper. For \textit{P. graminis} f. sp. \textit{tritici}, urediniospores remained viable on aluminium, cotton, denim, fleece, fibre polyester, silk, jarrah wood, Tasmanian oak, jute, leather, paper and rubber for up to 365 days at 23°C/4°C, up to 300 days (denim and jute) at 36°C/14°C, and up to 180 days (cotton and jute) at 45°C/15°C. While the number of viable ascospores, urediniospores or conidia and resting hyphae generally decreased over time and with increasing temperature, there remained clear potential for their survival for much longer
periods in the absence of a host than previously considered possible from past epidemiological studies with these pathogens. For example, Huang et al. (2003) found that ascospores of *L. maculans* could only survive a little more than one month even when exposed to dry air at 20°C. Importantly, my studies demonstrated that fungal pathogen spores could survive much higher temperatures than previously considered possible, particularly for pathogens like *K. caulivora*. However, urediniospores of some rusts are known to be relatively resistant to high temperatures (Aydoğdu and Boyraz 2012), for example, urediniospores of *P. psidii* could remain viable up to 10 days at 40°C with 35–55% relative humidity (Lana et al. 2012), but urediniospores of *Phakopsora pachyrhizi* could remain viable for only 4-6 hours at 40-50°C (Twizeyimana and Hartman 2010). It is particularly significant, from my studies, that temperatures up to 45°C did not kill all *P. graminis* f. sp. *tritici* urediniospores. This likely explains, for the first time, how some *P. graminis* f. sp. *tritici* urediniospores survive under the relatively hot and dry environmental conditions frequently occurring between cropping seasons in Western Australia to enable carryover of rust from one cropping season to the next with subsequent initiation of infections leading to epidemics from apparently inconspicuous initial levels of inoculum. Overall, while my studies demonstrate the significant differences in spore survival between the different test pathogens across different temperature regimes and different carrier materials, my findings clearly highlight the critical importance of materials as long-term carriers of viable fungal spores under widely different environmental conditions.
7.1.5. Relevance of extended spore survival

While one year was the longest time period for extended viability for *P. graminis* f. sp. *tritici* urediniospores, survival may have been even longer had the experiment not been terminated at that time. Such findings have important implications, not just for carryover across sequential cropping seasons to initiate stem rust epidemics as discussed above, but also for domestic and international movement of freight and commodities that clearly can effectively carry and retain viable urediniospores for at least a year. This, along with findings across the different pathogens, have major ramifications for both domestic and international movement of commodities contaminated with spores of one or more fungal pathogens that may pose serious environmental and economic biosecurity threats in regions where such pathogens *per se*, or one or more of their races or strains, do not yet occur (Carnegie and Cooper 2011; Wellings 2007).

7.1.6. Relevance of subsequent germination and host infection from surviving spores

A critical finding in my studies was that spores recovered from these inert materials were able to germinate on artificial media and/or infect relevant specific hosts. For example, at least 30% of intact *L. maculans* ascospores recovered from inert carrier materials were able to germinate on artificial growth media within 48 hours of recovery; and even ‘broken’ *L. maculans* ascospores still germinated, particularly from non-terminal end cells. Similarly, *K. caulivora* conidia and resting hyphae recovered after 240 days could be successfully re-established on PDA. These re-established colonies readily infected subterranean clover seedlings under glasshouse conditions. I also found that *P. graminis* f. sp. *tritici* urediniospores recovered even after one
year from the lower two temperature regimes (23°C/4°C and 36°C/14°C) successfully infected wheat seedlings when inoculated under glass house conditions. Although urediniospores recovered from 45°C/15°C were unable to directly infect wheat seedlings, 1% of urediniospores still successfully germinated on PDA. My findings at the lower two temperature regimes are particularly significant for *P. graminis* f. sp. *tritici*, as studies in the Netherlands have shown that even a single uredinium/hectare surviving the winter, a level of inoculum which is below detectable thresholds, was sufficient to generate a subsequent spring rust epidemic (Singh et al. 2002). Hence, even the relatively low numbers of viable *P. graminis* f. sp. *tritici* urediniospores I found attached to inert carrier materials after one year are sufficient to play an important role in the spread, initiation and subsequent development of rust epidemics. In terms of biosecurity management, such potential pathogen transfers over large distances pose highlight the potential risks to Australian cropping industries from introduction of exotic pathogens and/or their races. However, early identification and decontamination of a pathogen immediately at the time of its introduction offers the greatest opportunity for its successful control and/or eradication while numbers of spores and/or infections are limited (French et al. 2007; Wheelis et al. 2002), highlighting a need for chemical decontaminants.

**7.1.7. Chemical decontaminants**

Decontamination of fungal spores on introduced carrier materials is critical in preventing introduction and establishment of pathogens and their races. As a quarantine measure, suspected or known fungal-spore-contaminated materials can either be fumigated, or sprayed with fungicides or with one or
more general-purpose chemical agents or disinfectants. In my studies I determined the effects of five chemical fungicide/disinfectant treatments [Tilt 250 (propiconazole), Amistar 250 (azoxystrobin), Sporekill, (didecyldimethyl ammonium chloride), Farmcleanse (Alkali metal salts of alkylbenzene sulfonic acid and coconut diethanolamide) and Virkon (potassium peroxymonosulfate)] in preventing spore germination of *P. graminis* f. sp. *tritici*, *K. caulivora*, *L. maculans* and *Magnaporthe oryzae*. My results demonstrated that fungicides/disinfectants can significantly reduce germination of fungal spore contamination. Maximum reductions in spore germination were obtained at manufacturer’s recommended concentration, but increasing concentrations did not significantly further reduce germination. In contrast, concentrations below those recommended were less effective.

The more effective chemicals reduced germination of plant pathogen spores from carrier materials by between 70 and 88%. However, this depended on the pathogen, the chemical, and/or the type of carrier material. For example, azoxystrobin was most effective and reduced germination of *M. oryzae* by 90%, but *L. maculans* and *P. graminis* f. sp. *tritici* by only 80%; propiconazole was the most effective for *K. caulivora* (reduced by 80%). Overall, azoxystrobin and propiconazole were the most effective of the chemicals tested, while didecyldimethyl ammonium chloride was least effective. Most fungicides, predominantly those used in agriculture, are effective when applied prior to infection and generally involve inhibition of spore germination and/or suppression of germ tube and early hyphal growth (Taskeen-Un-Nisa et al. 2011). Propiconazole and azoxystrobin act as effective systemic fungicides with curative and preventative actions. Hence both these could be ideal chemicals that can easily be applied as a foliar spray.
to almost any carrier material surface for decontamination. While absolute decontamination carrier materials was not possible in my studies, my results confirmed that very significant levels of decontamination were possible in terms of impeding germination of the vast majority of viable spores of diverse but important plant pathogens from the test carrier materials.

7.2. Conclusion and the future

My findings have major implications, both highlighting how domestic and international movement of people and commodities can effectively carry and retain viable fungal spores for much longer time periods than previously considered possible, and also how the same applies for between growing seasons in the absence of a growing host. Importantly, significant differences between carrier materials in their abilities to retain ascospores, urediniospores, conidia and resting hyphae after washing were demonstrated. While there been much research on the impact of exotic fungal pathogens on agriculture and/or on natural ecosystems, studies into alternative entry pathways into a country or specific areas or regions have been, at best, very limited (Desprez-Loustau et al. 2007). Although previous research conducted demonstrated that it is possible to identify fungal spores on clothing materials (e.g., Wright et al. 2011), my studies are the first to include the critical aspect of viability of fungal spores.

My studies also demonstrated the benefits of novel application of the Alamar Blue assay over traditional methods of spore viability assessment; highlighting its advantages of being a non-toxic, more reliable and rapid method, where up to 96 - 384 samples in a micro-titre-based format can be processed at a single time. Alamar Blue assay methodology is a much-
improved means to assess fungal spore viability for future research studies to investigate patterns and changes in spore viability across wide ranging exotic and non-exotic fungal and oomycete pathogens. However, as the Alamar Blue assay needs to be optimised for each application and cell model (Lim et al. 2016; Rampersad 2012), it is recognised that this assay is not a substitute fungal diagnostic tool to identify fungi or to differentiate spores of quarantine versus non-quarantine fungal species. Despite this, the Alamar Blue assay can easily be supplemented with other detection and diagnostic tools of pathogen identification, such that it can quickly detect the presence of viable spores where pathogens of quarantine relevance are suspected or detected. For example this assay could be readily adapted to assess the viability of eucalyptus or guava rust (P. psidii), or new strains of stem rust (P. graminis f. sp. tritici) like Ug99 or its variants that pose serious environmental and economic biosecurity threats, respectively, if and when introduced into regions where they do not yet occur (Carnegie and Cooper 2011; Wellings 2007).

My results should not only prompt new evaluation into possible sources of carryover of fungal diseases in Australia, particularly in relation to their survival and spread from infested stubble, but a wider reconsideration of risks associated with transmission of fungal spores from one place to another involving human activities. My studies emphasise the broader biosecurity implications in relation to the overall transport and movement of fungal spores via these carrier materials, highlighting significant potential for fungal pathogens being spread widely in association with movement of humans, farming equipment, clothing and commodities across regions and continents. In particular, my studies highlight the dispersion potential of other important plant and animal/human pathogens through inert carrier materials. For
example, destructive ascomycete plant pathogens such as wheat scab and Dutch elm disease and human ascomycete pathogens such as Candida albicans (thrush) and the dermatophytes causing athlete's foot. While pathogens may be introduced with increasing international trade and tourism (Wellings, 2007; Holliday et al., 2013), my findings results indicate that there is a wide range of potential carrier materials that could facilitate entry and spread of exotic fungal spores.

It is clear that when applied as recommended, chemicals can provide effective decontamination of viable fungal spores on carrier materials. Decontamination of carrier materials with fungicides and disinfectants offers a cost-effective, preventive measure and can be utilized as part of future recommendations in Integrated Pest Management (IPM) programs to control fungal plant pathogen spread and, particularly, reduce risks of introduction of exotic plant pathogens and/or exotic races of already present plant pathogens. However, as I only used chemicals registered under APVMA, I realize that there are a range of other decontamination measures not considered in my studies that also could potentially be effective in many situations.

Finally, my studies have wider biosecurity implications in relation to the transportation of fungal-infested carrier materials in general, as they highlight an urgent need to re-evaluate potential carrier materials such as metals, plastics, fabrics and woods that have historically long been considered of low biosecurity risk in regards to the movement of fungal plant pathogens.
Bibliography


Fai, P. B., and Grant, A. 2009. A rapid resazurin bioassay for assessing the

of LDH, neutral red, MTT and protein assay in hepatoma cell lines

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a fast
and reliable method for colorimetric determination of fungal cell

French, N. P., Gemmell, N. J., and Budle, B. M. 2007. Advances in
Biosecurity to 2010 and beyond: towards integrated detection, analysis
and response to exotic pest invasions. Pages 255-255 New Zealand
Veterinary Association, New Zealand.

and cell growth in a hollow-fiber bioreactor by use of the dye Alamar

Grgurinovic, C. A., Walsh, D., and Macbeth, F. 2006. Eucalyptus rust caused
by Puccinia psidii and the threat it poses to Australia. EPPO Bulletin
36:486-489.

Hampson, M., and Wood, S. 1997. Detection of infective resting spores of

97:828-834.

Huang, Y. J., Fitt, B. D. L., and Hall, A. M. 2003. Survival of A-group and B-
group Leptosphaeria maculans (phoma stem canker) ascospores in air
and mycelium on oilseed rape stem debris. Ann. Appl. Biol. 143:359-
369.

transfer of non-native soil organisms into Antarctica on construction

Katsuya, K., and Green, G. 1967. Reproductive potentials of races 15B and

Lana, V., Mafia, R., Ferreira, M., Sartório, R., Zauza, E., Mounteer, A., and
Alfenas, A. 2012. Survival and dispersal of Puccinia psidii spores in

Li, H., Sivasithamparam, K., and Barbetti, M. J. 2006. Evidence supporting the
polycyclic natures of blackleg disease (Leptosphaeria maculans) of
oilseed rape in Australia and implications for disease management.
Brassica 8:65-69.


Appendix

Presentations, Proceedings, Media Release

International Scientific Conference papers from this project


Local scientific conference/presentation from this project


temperature and viability of spore over time. *UWA Rottnest summer school*, Rottnest, WA, Australia (Abstract & oral presentation).


**Internship, Press and Media Release**


Video interview, PBCRC education and training programs 2016, fungal spore incursions, defining the likelihood and means by which fungal spore incursions can enter Australia, as well as effective methods of decontamination of materials. [https://youtu.be/cltjCUWfSrY](https://youtu.be/cltjCUWfSrY)