

Lincoln University Digital Thesis

Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- you will use the copy only for the purposes of research or private study
- you will recognise the author's right to be identified as the author of the thesis and due acknowledgement will be made to the author where appropriate
- you will obtain the author's permission before publishing any material from the thesis.

***Pyrenophora tritici-repentis* the causal agent of tan spot:
characterisation of New Zealand populations**

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Master of Science

at
Lincoln University
by
Sean Kevin Weith

Lincoln University

2015

Abstract of a thesis submitted in partial fulfilment of the requirements for
the Degree of Master of Science

***Pyrenophora tritici-repentis* the causal agent of tan spot:
characterisation of New Zealand populations**

by

Sean Kevin Weith

Tan spot, caused by the ascomycete fungus *Pyrenophora tritici-repentis*, is one of the most economically significant and devastating foliar diseases of wheat. In this study, the distribution, genetic diversity and race structure, sensitivity to commonly used fungicides and virulence on different wheat cultivars of *P. tritici-repentis* was investigated.

A total of 15 different populations of *P. tritici-repentis* were collected during the 2013-2014 survey period. The survey showed that *P. tritici-repentis* was widely distributed throughout the South Island of New Zealand. A follow-up farmer questionnaire revealed that the re-use and sowing of untreated wheat seed and continuous sowing of wheat over 10-20 years may have exacerbated tan spot infestations.

A total of 12 single spore *P. tritici-repentis* isolates were chosen to represent the geographic distribution and diversity of *P. tritici-repentis* in New Zealand. The identities of all isolates were confirmed through sequencing of the internal transcribed spacer (ITS), β -tubulin regions and species specific (PtrUnique) PCR primer products. All New Zealand *P. tritici-repentis* isolates had the Ptr ToxA gene *ToxA*, but lacked the Ptr ToxB gene *ToxB*. This result indicated that the New Zealand isolates most likely belonged to virulence races 1 or 2. Results were confirmed by comparing New Zealand isolates against a range of fully characterised *P. tritici-repentis* international isolates.

Three different molecular methods (UP-PCR, RAPD and microsatellite analysis) were utilised in this study to assess genetic diversity of a range of New Zealand and international *P. tritici-repentis* isolates. A total of 13 different genotypes were detected with all three methods using 12 different primers from all of the New Zealand and international *P. tritici-repentis* isolates. Although all three methods provided different levels of information, they indicated that the genetic structure of the South Island populations of *P. tritici-repentis* was clonal. The New

Zealand isolates were genetically distinct when compared to a range of international *P. tritici-repentis* isolates. These results suggested that New Zealand populations of *P. tritici-repentis* either do not undergo frequent genetic recombination or were part of a recent incursion. However, the exact explanation could not be identified.

The *in vitro* fungicide bioassay showed that all ten of the assessed fungicides effectively reduced the *in vitro* mycelial growth of *P. tritici-repentis* to <50%. The fungicides isopyrazam, propiconazole and prothioconazole had the lowest EC₅₀ values which indicated they were the most effective fungicides against *P. tritici-repentis*.

The inoculation of ten wheat cultivars in a glasshouse environment with *P. tritici-repentis* revealed that at least eight of the ten cultivars were susceptible to tan spot. Of the ten wheat cultivars assessed, Empress and Duchess had the lowest disease severity and displayed symptoms characteristic of resistance towards *P. tritici-repentis*.

Overall, this study has provided a new insight into the current distribution, genetic diversity and race structure, sensitivity to a broad range of fungicides and virulence on a range of commonly cultivated wheat cultivars of *P. tritici-repentis* in New Zealand. The findings of this study will also provide wheat growers with valuable knowledge that will facilitate the development and implementation of future tan spot control strategies in New Zealand.

Keywords: Tan spot, *Pyrenophora tritici-repentis*, wheat, genetic diversity, race structure, UP-PCR, RAPD, microsatellites, fungicide sensitivity, cultivar susceptibility.

Acknowledgments

This project would never have been possible without help from a large number of important people. Firstly, I would like to extend my deepest gratitude and thanks to my main supervisor Dr Eirian Jones for her expert advice, guidance, patience and encouragement throughout this research project. Her passion for plant pathology gave me the inspiration to pursue research in the field of plant pathology. I would also like to express my deepest gratitude to my associate supervisor Dr Hayley Ridgway for her valuable advice, encouragement and expert mentorship during the molecular component of this research project. Her passion for molecular biology helped to give me the inspiration to want to learn molecular-based techniques. I would also like to thank my project advisors Dr Ian Harvey and Rob Craigie (Foundation for Arable Research) for their helpful advice and guidance.

I would also like to thank the Foundation for Arable Research for funding this project and providing me with a stipend to support me while I carried out this research. I am also grateful to Lincoln University for the 2014 Masters scholarship I was awarded which helped further support my study.

I extend my gratitude to the South Island wheat farmers for allowing access to their properties to take samples and for taking time to fill out questionnaires. I would like to thank David Weith, Roy Stieller, Lovisa Eriksson, Dr Han Eerans and Daniel Suddaby of Bayer CropScience NZ Ltd and Dr Matthew Cromey for their assistance collecting samples and advice. I would like to thank Bayer CropScience NZ Ltd and Grant Hagerty of BASF NZ Ltd for providing me with actives for my *in vitro* sensitivity study.

I would also like to thank Steve Shorter (PGG Wrightson Seeds Ltd), Andy Hay (Plant and Food Research) and Natalie Stocker (Seedforce NZ Ltd) for providing me with wheat seed for the cultivar experiment. I also would like to thank Dr Reem Aboukhaddour (University of Alberta, Canada) and Dr Caroline Moffat (Curtin University, Australia) for providing me with the DNA of international *Pyrenophora tritici-repentis* isolates which were very useful in this project.

I also wish to thank Candice Barclay, Shanika Tennakoon, Sandy Hammond, Simon Hodge, Brent Richards and Leona Meachen from Lincoln University for their assistance in this project. I would like to thank my colleagues for helping to create a friendly working environment and providing helpful advice.

Lastly, I would like to give a special thanks to my Mum and Dad for their love, helpful advice and encouragement which has been invaluable in motivating me to complete this research project.

Table of Contents

| | |
|---|-------------|
| Abstract | i |
| Acknowledgments | iii |
| Table of Contents | iv |
| List of Tables | viii |
| List of Figures | ix |
| | |
| Chapter 1 Introduction | 1 |
| 1.1 Importance of wheat | 1 |
| 1.2 Tan spot disease of wheat | 1 |
| 1.3 Biology and life cycle of <i>Pyrenophora tritici-repentis</i> | 2 |
| 1.4 Symptoms of tan spot disease | 4 |
| 1.5 <i>Pyrenophora tritici-repentis</i> host-specific toxins | 5 |
| 1.6 Race structure of <i>Pyrenophora tritici-repentis</i> | 6 |
| 1.7 Genetic structure of <i>Pyrenophora tritici-repentis</i> populations | 7 |
| 1.7.1 Genetic variability analysis using different fingerprinting methods | 7 |
| 1.8 Sensitivity of <i>Pyrenophora tritici-repentis</i> to fungicides | 10 |
| 1.9 Susceptibility of New Zealand wheat cultivars to <i>Pyrenophora tritici-repentis</i> | 11 |
| 1.10 Research context and objectives | 12 |
| | |
| Chapter 2 Determination of the distribution and race structure of <i>Pyrenophora tritici-repentis</i> in the South Island, New Zealand | 14 |
| 2.1 Introduction | 14 |
| 2.2 Materials and Methods | 15 |
| 2.2.1 Sampling and collection procedure for <i>Pyrenophora tritici-repentis</i> symptomatic plant material | 15 |
| 2.2.2 Wheat grower survey process | 16 |
| 2.2.3 Identification of tan spot leaf symptoms | 17 |
| 2.2.4 Isolation of <i>Pyrenophora tritici-repentis</i> from infected wheat tissue | 17 |
| 2.2.5 Single spore isolate production | 19 |
| 2.2.6 Molecular confirmation of New Zealand <i>Pyrenophora tritici-repentis</i> isolates | 20 |
| 2.2.6.1 Genomic DNA extraction from fungal mycelium | 20 |
| 2.2.6.2 Species-specific PCR identification | 21 |
| 2.2.7 Sequencing of ITS and β -tubulin genes | 22 |
| 2.2.7.1 PCR Amplification with ITS and β -tubulin gene primers | 22 |
| 2.2.7.2 Sequencing of ITS, β -tubulin gene, species specific primer, ToxA and ToxB gene regions | 23 |

| | |
|--|----|
| 2.2.8 Determination of race structure of New Zealand and international <i>Pyrenophora tritici-repentis</i> isolates | 24 |
| 2.2.8.1 <i>Ptr-ToxA</i> and <i>Ptr-ToxB</i> PCR screening procedure | 24 |
| 2.3 Results | 26 |
| 2.3.1 Determination of distribution and incidence of tan spot in South Island | 26 |
| 2.3.1.1 <i>Results of farmer questionnaire</i> | 26 |
| 2.3.3 Identification of <i>Pyrenophora tritici-repentis</i> based on colony and conidia morphology | 28 |
| 2.3.4 Molecular confirmation of <i>Pyrenophora tritici-repentis</i> | 28 |
| 2.3.5 Determination of race structure of New Zealand and International <i>Pyrenophora tritici-repentis</i> populations | 30 |
| 2.4 Discussion | 33 |
| 2.5 Conclusions | 38 |

Chapter 3 Determination of the genetic diversity of *Pyrenophora tritici-repentis* in New Zealand

| | |
|---|-----------|
| 3.1 Introduction | 39 |
| 3.2 Materials and Methods | 40 |
| 3.2.1 Selection of <i>Pyrenophora tritici-repentis</i> populations and isolates | 40 |
| 3.2.2 Genomic DNA extraction | 41 |
| 3.2.3 Genetic variation analysis using UP-PCR method | 41 |
| 3.2.3.1 <i>Testing of primers</i> | 41 |
| 3.2.3.2 <i>UP-PCR</i> | 42 |
| 3.2.3.3 <i>Electrophoresis of UP-PCR products</i> | 42 |
| 3.2.4 Genetic variation analysis using RAPD | 43 |
| 3.2.4.1 <i>Selection of RAPD primers</i> | 43 |
| 3.2.4.2 <i>RAPD</i> | 43 |
| 3.2.4.3 <i>Electrophoresis of RAPD products</i> | 43 |
| 3.2.5 Genetic variation analysis using microsatellites | 44 |
| 3.2.5.1 <i>Selection of microsatellite primers</i> | 44 |
| 3.2.5.2 <i>Microsatellite PCR</i> | 44 |
| 3.2.5.3 <i>Electrophoresis of microsatellite products</i> | 44 |
| 3.3 Results | 45 |
| 3.3.1 Characterisation of South Island <i>Pyrenophora tritici-repentis</i> isolates using UP-PCR | 45 |
| 3.3.2 Characterisation of international and New Zealand <i>Pyrenophora tritici-repentis</i> populations using UP-PCR | 46 |
| 3.3.3 Characterisation of New Zealand and international <i>Pyrenophora tritici-repentis</i> populations using RAPD | 47 |
| 3.3.4 Characterisation of New Zealand and international <i>Pyrenophora tritici-repentis</i> populations using microsatellites | 49 |

| | |
|---|------------|
| 3.4 Discussion | 50 |
| 3.5 Conclusions..... | 57 |
| Chapter 4 Evaluation and determination of sensitivity of <i>Pyrenophora tritici-repentis</i> to fungicides | 58 |
| 4.1 Introduction | 58 |
| 4.2 Materials and Methods | 59 |
| 4.2.1 Effect of fungicides on <i>in vitro</i> mycelial growth | 59 |
| 4.2.2 Statistical analysis and determination of EC ₅₀ values for fungicides | 60 |
| 4.3 Results | 61 |
| 4.3.1 Effect of fungicides on <i>in vitro</i> mycelial growth of <i>Pyrenophora tritici-repentis</i> | 61 |
| 4.4 Discussion | 65 |
| 4.5 Conclusions..... | 70 |
| Chapter 5 Determination of susceptibility of wheat cultivars to <i>Pyrenophora tritici-repentis</i> | 71 |
| 5.1 Introduction | 71 |
| 5.2 Materials and methods | 73 |
| 5.2.1 Fungal isolates..... | 73 |
| 5.2.2 Selection of wheat cultivars..... | 73 |
| 5.2.3 Preparation of plants..... | 73 |
| 5.2.4 Preparation of fungal inoculum..... | 74 |
| 5.2.5 Determination of <i>Pyrenophora tritici-repentis</i> inoculum viability | 75 |
| 5.2.6 Inoculation of plants | 75 |
| 5.2.7 Disease severity rating system..... | 75 |
| 5.2.8 Re-isolation assessment | 77 |
| 5.2.9 Statistical analysis of cultivar disease scores and lesion area | 77 |
| 5.3 Results | 77 |
| 5.3.1 <i>Pyrenophora tritici-repentis</i> conidia viability..... | 77 |
| 5.3.2 Visual observation of tan spot symptoms on cultivars | 78 |
| 5.3.3 Determination of cultivar susceptibility to <i>Pyrenophora tritici-repentis</i> | 78 |
| 5.4 Discussion..... | 80 |
| 5.5 Conclusions..... | 86 |
| Chapter 6 Concluding discussion | 87 |
| References | 93 |
| Appendix A | 104 |

| | |
|--|------------|
| A.1 V8-PDA Recipe..... | 104 |
| A.2 V8 Recipe | 104 |
| A.3 TAE used for gel electrophoresis | 104 |
| Appendix B | 105 |
| B.1 Questionnaire form sent to farmers | 105 |
| B.2 Details of the New Zealand <i>Pyrenophora tritici-repentis</i> isolates isolated from wheat used in the study | 106 |
| B.3 Codes allocated to name <i>Pyrenophora tritici-repentis</i> obtained in the 2013-2014 survey | 107 |
| B.4 Race, toxin produced and origin of the international <i>Pyrenophora tritici-repentis</i> isolates used in study | 108 |
| Appendix C | 109 |
| C.1 DNA sequences of <i>Pyrenophora tritici-repentis</i> isolates: ITS, β -tubulin <i>ToxA</i> and PtrUnique primer sites..... | 109 |
| C.1.1 ITS sequences of <i>Pyrenophora tritici-repentis</i> isolates | 109 |
| C.1.2 β -Tubulin sequences of <i>Pyrenophora tritici-repentis</i> isolates | 115 |
| C.1.3 <i>ToxA</i> sequences of <i>Pyrenophora tritici-repentis</i> isolates..... | 116 |
| C.1.4 PtrUnique primer sequences of <i>Pyrenophora tritici-repentis</i> isolates | 118 |
| C.2 Darkened and inverted RAPD agarose gel fingerprints..... | 120 |
| Appendix D | 121 |
| D.1 Analysis of variance of fungicide sensitivity of <i>Pyrenophora tritici-repentis</i> | 121 |
| D.1.1 Two way ANOVA results on EC ₅₀ values of fungicides on <i>in vitro</i> mycelial growth..... | 121 |
| D.1.2 Mean EC ₅₀ (mg a.i./ L) values with transformed log ₁₀ means for eight different fungicides on the <i>in vitro</i> inhibition of mycelial growth of four <i>Pyrenophora tritici-repentis</i> single spore isolates. | 122 |
| Appendix E..... | 123 |
| E.1 ANOVA of mean disease score and lesion area (mm) which develop on different wheat cultivars after inoculation with <i>Pyrenophora tritici-repentis</i> | 123 |
| E.1.1 One way ANOVA results for mean disease score for wheat cultivar susceptibility | 123 |
| E.1.2 One way ANOVA results for lesion area on different wheat cultivars | 123 |

List of Tables

| | |
|---|----|
| Table 2.1: New Zealand single spore isolates of <i>Pyrenophora tritici-repentis</i> used in study. | 21 |
| Table 2.2: Details of the international <i>Pyrenophora tritici-repentis</i> isolates used in this study..... | 25 |
| Table 2.3: Summary of the information provided by farmers to questions asked in the 2014 tan spot questionnaire | 27 |
| Table 3.1: Sequence and annealing temperature of UP-PCR primers used in this study..... | 42 |
| Table 3.2: Name and sequence of RAPD primers used in this study (from Singh & Hughes, 2006). | 43 |
| Table 3.3: Name and sequence of microsatellite primers used in this study (from Gurung <i>et al.</i> , 2013)..... | 44 |
| Table 3.4: Genotypes detected by UP-PCR primers AA2M2, AS15inv and Fok1, RAPD primers UBC598, UBC600, Operon H-19 and UBC517 and microsatellite primers SSRPtr01, SSRPtr12 and SSRPtr13 | 49 |
| Table 4.1: Fungicides tested against mycelial growth of <i>Pyrenophora tritici-repentis</i> | 59 |
| Table 4.2: The mean EC ₅₀ (mg a.i./ L) values for eight different fungicides on the <i>in vitro</i> inhibition of mycelial growth of four <i>Pyrenophora tritici-repentis</i> single spore isolates. Data presented are back transformed means..... | 61 |
| Table 5.1: Disease score (0-5 scale) based on lesion size and appearance used to assess severity of <i>Pyrenophora tritici-repentis</i> lesions which developed on inoculated wheat cultivars (modified from Lamari & Bernier, 1989a). | 76 |
| Table 5.2: The mean disease score (0-5 scale) and mean lesion area (mm ²) of ten different New Zealand wheat cultivars assessed 21 days after inoculation with <i>Pyrenophora tritici-repentis</i> | 79 |

List of Figures

- Figure 1.1:** Disease cycle of tan spot caused by *Pyrenophora tritici-repentis*. (Retrieved from <https://www.apsnet.org/edcenter>, 25/2/2014). 3
- Figure 1.2:** A) and B) Typical tan spot lesions caused by *Pyrenophora tritici-repentis* infection of wheat, with characteristic tan lesions surrounded by a yellow chlorotic border..... 5
- Figure 2.1:** Map of New Zealand's South Island showing the distribution and number of wheat growing farms where plant material showing symptomatic *Pyrenophora tritici-repentis* lesions were obtained during the 2013-2014 season. (Source: http://en.wikipedia.org/wiki/Template:Location_map_New_Zealand_South_Island_). 16
- Figure 2.2:** A characteristic tan spot lesion on a wheat leaf, A indicating chlorosis (yellow) and B indicating Necrosis (tan/brown). 17
- Figure 2.3:** a) Conidia of *Pyrenophora tritici-repentis* (400x magnification), b) characteristic culture of *Pyrenophora tritici-repentis* on potato dextrose agar (PDA). 18
- Figure 2.4:** Pie graph showing the frequency and name of wheat cultivars *Pyrenophora tritici-repentis* was recovered from during 2013-2014 season. 27
- Figure 2.5:** Examples of the appearances of different isolated colonies of *P. tritici-repentis* on MEA. 28
- Figure 2.6:** 1% agarose gel of PCR products generated by the species-specific PtrUniqueF2/PtrUniqueR2 primers using the genomic DNA of 12 *Pyrenophora tritici-repentis* South Island single spore isolates. From left to right, lanes 1 to 12 are *P. tritici-repentis* isolates S12a, S22a, S26a, M13c, M14d, G14a, G22a, F12d, W12a, W12c and K16a. M = 1 kb plus DNA ladder (Invitrogen), - ve = negative control. 29
- Figure 2.7:** 1% agarose gel of PCR products produced by amplifying three field isolates of *Pyrenophora tritici-repentis* using primers ITS4F and ITS5R. Lanes numbered 1 to 3 are *P. tritici-repentis* fungal populations G2Ptr1, W2Ptr1 and G1Ptr3. M = 1 kb plus DNA ladder (Invitrogen), - ve = Negative control. 29
- Figure 2.8:** 1% agarose gel of PCR products generated using primers ITS4 and ITS5 using the genomic DNA of 12 *Pyrenophora tritici-repentis* South Island single spore isolates. From left to right, lanes 1 to 12 are *P. tritici-repentis* single spore isolates S12a, S22a, S26a, M13c, M14d, G14a, G22a, F12d, W12a, W12c and K16a. M = 1 kb plus DNA ladder (Invitrogen), - ve = negative control. 30
- Figure 2.9:** 1% agarose gel of PCR products generated by *ToxA*-specific ToxAscreeningF/ToxAscreeningR primers using the genomic DNA of 12 New Zealand single spore of isolates *Pyrenophora tritici-repentis*. From left to right, lanes 1-12 are *P. tritici-repentis* isolates S12a, S22a, S26a, M13c, M14d, G14a, G22a, F12d, W12a, W12c and K16a. M = 1 kb plus DNA ladder (Invitrogen), - ve = negative control. 31
- Figure 2.10:** 1% agarose gel of PCR products generated by *ToxA*-specific ToxAscreeningF/ToxAscreeningR primers using the genomic DNA of New Zealand and international *Pyrenophora tritici-repentis* single spore isolates. From left to right, lanes 1 to 13 are *P. tritici-repentis* isolates S12a, S26a, G14a, G22a, F12d, K16a, ASC1, AB47-10, Alg3-24, AB33-1, AB39-2, AB39-8 and TS93-71B. M = 1 kb plus DNA ladder (Invitrogen), - ve = negative control. 31
- Figure 2.11:** 1% agarose gel of PCR products generated by *ToxA*-specific ToxAscreeningF/ToxAscreeningR primers using the genomic DNA of New Zealand and international *Pyrenophora tritici-repentis* single spore isolates. From left to right, lanes 1 to 6 are *P. tritici-repentis* isolates S12a, CCDM Meck 4, CCDM 239, CCDM 134, Alg3-24 and TS93-71B. M = 1 kb plus DNA ladder (Invitrogen), - ve = negative control. 32

| | |
|--|----|
| Figure 2.12: 1% agarose gel of PCR products generated by <i>ToxB</i> -specific TB10f and TB12r primers using genomic DNA of New Zealand and international <i>Pyrenophora tritici-repentis</i> single spore isolates. From left to right, lanes 1 to 13 are <i>P. tritici-repentis</i> isolates S12a, S26a, G14a, G22a, F12d, K16a, ASC1, AB47-10, Alg3-24, AB33-1, AB39-2, AB39-8 and TS93-71B. M = 1 kb plus DNA ladder (Invitrogen), - ve = negative control. | 32 |
| Figure 2.13: 1% agarose gel of PCR products generated by <i>ToxB</i> -specific TB10f and TB12r primers using genomic DNA of New Zealand and international <i>Pyrenophora tritici-repentis</i> single spore isolates. From left to right, lanes 1 to 13 are <i>P. tritici-repentis</i> isolates S12a, S22a, S26a, M13c, M14d, G14a, W12a, W12c, W15a, K16a, CCDM-239, CCDM-134, ASC1, CCDM-Meck4, AB39-2, 90-2, Alg3-24, AlgH2 and TS93-71B. M = 1 kb plus DNA ladder (Invitrogen), - ve = negative control..... | 33 |
| Figure 3.1: 1% agarose gels of UP-PCR fingerprints from genomic DNA of 12 <i>Pyrenophora tritici-repentis</i> South Island single spore isolates. a) AA2M2 primer, b) AS15inv primer and c) Fok1. From left to right <i>P. tritici-repentis</i> single spore isolates are S12a, S22a, S26a, M13c, M14d, G14a, G22a, F12d, W12a, W12c and K16a. M = 1 kb plus DNA ladder (Invitrogen), - ve = negative control..... | 46 |
| Figure 3.2: 1% agarose gels of UP-PCR fingerprints generated by genomic DNA of two New Zealand single spore isolates and seven <i>Pyrenophora tritici-repentis</i> international isolates. a) AA2M2 primer, b) Fok1, c) AS15 inv primer. From left to right, lanes 1-9 are S12a, G14a, ASC1, AB47-10, AB33-1, Alg3-24, AB39-2, AB39-8, TS93-71B. M = 1 kb plus DNA ladder (Invitrogen), - ve = negative control..... | 47 |
| Figure 3.3: 1.3% agarose gels of RAPD fingerprints generated by genomic DNA of New Zealand and international <i>Pyrenophora tritici-repentis</i> isolates. a) UBC598 primer, b) UBC600 primer, c) Operon H-19 primer and d) UBC517 primer. From left to right, lanes 1-17 are <i>P. tritici-repentis</i> isolates S12a, S26a, M13c, G14a, G22a, F12d, K16a, CCDM Meck4, CCDM 239, CCDM 134, ASC1, AB47-10, AB33-1, Alg3-24, AB39-2, AB39-8, TS93-71B. M = 1 kb plus DNA ladder, - ve = negative control..... | 48 |
| Figure 3.4: 1.3% agarose gels of microsatellite fingerprints generated by genomic DNA of New Zealand and international <i>Pyrenophora tritici-repentis</i> isolates. a) SSRPtr12 primer, b) SSRPtr13 primer and c) SSRPtr01 primer. From left to right, lanes 1-18 are <i>P. tritici-repentis</i> isolates S12a, S26a, M13c, G22a, W15a, W13c, M13c, K16a, 90-2, ASC1, AB47-10, AB33-1, Alg3-24, AlgH2, AB39-2, AB39-8, TS93-71B, CCDM Meck4 and CCDM 239,. M = 1 kb plus DNA ladder, - ve = negative control. | 50 |
| Figure 4.1: Growth and appearance of <i>Pyrenophora tritici-repentis</i> on PDA amended with different concentrations of the fungicides bixafen + prothioconazole, fluxapyroxad and isopyrazam. For each fungicide and day the 6 plates represent the increasing concentrations of the fungicide from left to right. | 62 |
| Figure 4.2: Growth and appearance of <i>Pyrenophora tritici-repentis</i> on PDA amended with different concentrations of the fungicides epoxiconazole, propiconazole and prothioconazole. For each fungicide and day the 6 plates represent the increasing concentrations of the fungicide from left to right..... | 63 |
| Figure 4.3: Growth and appearance of <i>Pyrenophora tritici-repentis</i> on PDA amended with different concentrations of the fungicides azoxystrobin and pyraclostrobin. For each fungicide and day the 6 plates represent the increasing concentrations of the fungicide from left to right. | 64 |
| Figure 5.1: Experimental set up for the cultivar susceptibility experiment with the different wheat cultivars grown in pots arranged in a randomized complete block design in the Lincoln University glasshouse | 74 |
| Figure 5.2: Representative tan spot symptoms observed on the different wheat cultivars inoculated with <i>Pyrenophora tritici-repentis</i> : a) Discovery, b) Empress, c) Duchess, d) Inferno, e) Saracen, f) Starfire, g) Torch, h) Reliance, i) Viceroy, j) Wakanui. | 78 |

Figure 5.3: Appearance of characteristic *Pyrenophora tritici-repentis* on PDA from tan spot lesions which developed on *P. tritici-repentis* inoculated wheat plants. a) surface and b) underside colony morphology. 80

Chapter 1

Introduction

1.1 Importance of wheat

Wheat (*Triticum aestivum* L.) is an important staple food source for almost 40% of the world's population (Weise, 1987). Wheat is the leading source of plant derived protein, carbohydrate, minerals and vitamins in the diet of humans worldwide. It is also second, only to rice, as the crop with largest total tonnage produced globally each year (Weise, 1987). Wheat also played a significant role in enabling the emergence and establishment of city-based human societies during the early periods of human history, thanks particularly to it being one of the few crops that possessed the ability to be cultivated intensively on a large scale.

In New Zealand, like many other cereals, wheat is a popular cash crop for arable farmers. However, since the 1970's the land area used to grow wheat in New Zealand has been in a decline. In 2010, approximately 88% of wheat was planted in Canterbury in the South Island of New Zealand (Millner *et al.*, 2013). The total annual production of wheat in New Zealand over the past 10 years has ranged from 285, 860 to 485, 617 tonnes (averaging around 365, 000 tonnes) (Millner *et al.*, 2013).

Like most of the commonly cultivated cereals, such as Barley (*Hordeum vulgare*) and oats (*Avena sativa*), wheat suffers from many fungal diseases that can affect its overall productivity. It has been estimated that roughly 20% of wheat yield is lost worldwide as a result of wheat diseases (Weise, 1987). Some of the most notable diseases to affect wheat include speckled leaf blotch (*Zymoseptoria tritici* syn. *Mycosphaerella graminicola*), glume blotch (*Parastagonospora nodorum* syn. *Phaeosphaeria nodorum*), *Rhizoctonia solani* root rot, take all (*Gaeumannomyces graminis* var. *tritici*) and leaf rusts (*Puccinia* spp.). Changes in factors such as global and regional climates, transcontinental exchange of wheat derived commodities and adoption of alternative agricultural practices, have led to changes in the genetic profile and ranges of these notable wheat pathogens (Manning *et al.*, 2013).

1.2 Tan spot disease of wheat

One of the major foliar diseases which can affect wheat is tan spot (synonym yellow leaf spot). Tan spot disease of wheat is a destructive and economically significant foliar disease of wheat which can cause significant yield losses (Gurung *et al.*, 2013). Tan spot is found globally in all major wheat growing regions (Ciuffetti & Tuori, 1999; Gurung *et al.*, 2013). Tan spot is caused by the necrotrophic ascomycete fungus *Pyrenophora tritici-repentis* (Died.) (teleomorph), *Drechslera tritici-repentis* (Died) (anamorph) (Gurung *et al.*, 2013; Manning, *et al.*, 2013). Since

its first discovery in 1823, *P. tritici-repentis* has been found globally, in all regions of the world where wheat is cultivated (Ciuffetti & Tuori, 1999; Gurung *et al.*, 2013).

Over the last few decades tan spot disease has experienced significant shifts in its distribution and severity worldwide (Manning *et al.*, 2013). The increase in the incidence of this disease has been linked to the widespread adoption of minimum tillage practices, such as direct drilling, continuous wheat growing systems and the increase in the global exchange of wheat seed (Ciuffetti & Touri, 1999; De Wolf, 2008; Antoni *et al.*, 2010). As a result, tan spot is now considered to be one of the most economically significant diseases to affect wheat crop yields worldwide. Infection of wheat plants by *P. tritici-repentis* leads to reductions in the total yield, number of grains per head, kernel weight, total biomass and the overall grain quality (Singh *et al.*, 2010). These effects being primarily a result of the pathogen reducing the plants total leaf photosynthetic area (Singh *et al.*, 2010; Wegulo *et al.*, 2012). Yield losses in wheat due to tan spot infection have been reported on average to range from 5-10% (Singh *et al.*, 2010). However, under favourable conditions yield losses of up to 50% have been reported (Rees *et al.*, 1982).

In New Zealand, tan spot disease is considered a relatively new disease to affect wheat. However, it is highly likely that this pathogen is not relatively new to this country. There are reports that *P. tritici-repentis* may have been present in New Zealand for some time and gone unnoticed. The first recorded identification in New Zealand was its isolation from infected certified wheat seed in 1976 (Hampton & Matthews, 1978). Until recently, there have been no reports of *P. tritici-repentis* either causing disease or being isolated from wheat seed.

Recent shifts to adopt conservative agricultural practices e.g. direct drilling and intensification of wheat cultivation on farms (Cromeey *et al.*, 2008; Williams *et al.*, 2013) may have contributed to the emergence and increase in severity of tan spot observed in New Zealand in recent years. For example, in a survey of 40 fields in Canterbury over an 11 year period to 2001 it was found that wheat or barley were sown as 45% of the main crop on arable farms (Cromeey *et al.*, 2008). At present, it is unknown how prevalent and problematic tan spot is throughout New Zealand wheat growing regions. Similarly, the factors which affect the prevalence and severity of *P. tritici-repentis* wheat infection under New Zealand conditions are also unknown.

1.3 Biology and life cycle of *Pyrenophora tritici-repentis*

Pyrenophora tritici-repentis can infect all classes of wheat, brome grasses (*Bromus* spp.) and rye (*Secale cereale*) (Wegulo *et al.*, 2012). The primary inoculum for tan spot disease can be from infected grain, infected over-wintering grasses or volunteers, infested stubble and other plant residues such as straw (Figure 1.1) (Gurung *et al.*, 2013; Ciuffetti & Tuori, 1999).

Figure 1.1: Disease cycle of tan spot caused by *Pyrenophora tritici-repentis*. (Retrieved from <https://www.apsnet.org/edcenter>, 25/2/2014).

Pyrenophora tritici-repentis is a homothallic fungus, and can reproduce sexually to produce ascospores in pseudothecia, and asexually to produce conidia on conidiophores, with these spores serving as primary and secondary inoculum, respectively (Figure 1.1). Sexual reproduction occurs once, at the end of the season on stubble during the fall and winter, while asexual reproduction occurs in many cycles throughout the wheat growing season in spring and summer (Ciuffetti & Tuori, 1999; Kadar, 2010).

Both spore types are disseminated by wind and water (Schilder & Bergstrom, 1992; Leisova *et al.*, 2008). However, due to their size and light weight, conidia are dispersed by wind over longer distances e.g. kilometres (Wegulo, 2011). A study by Wolf & Hoffmann (1993) found that under field conditions in southern Bavaria the conidia of *P. tritici-repentis* were spread by wind over great distances over wheat fields. In contrast, it was found that although the ascospores of *P. tritici-repentis* were actively discharged from mature pseudothecia, they only had short distribution distances. The authors also determined that a changing relative humidity (RH) was a key factor for conidial liberation, with the liberation of *P. tritici-repentis* conidia from stubble being nearly 100% at wind speeds of 3.3 meters / second and more than 60% at an RH of 35% with a wind speed less than 0.7 meters / second.

Typically, ascospores will serve as the primary inoculum for infection. It is common that lesions found on lower leaves of wheat in winter and early spring are the result of infection by ascospores released from pseudothecia (Kadar, 2010). Once they have landed on plant tissue, ascospores of *P. tritici-repentis* must be exposed to a temperature between the range of 10°C to 30°C and free moisture for 6 to 48 hours in order to germinate (Moreno *et al.*, 2012). In contrast, conidia need a temperature between 10-28°C with free moisture for 6 to 48 hours and a high RH in order to germinate (Bakina & Priekule, 2011; Moreno *et al.* 2012; Wegulo *et al.* 2012). The incubation period required for a *P. tritici-repentis* conidium to germinate and initiate an infection varies from 5 to 6 days, depending on the resistance of the wheat variety and meteorological conditions (Bakina & Priekule, 2011). It has also been reported that the development of tan spot in wheat is very temperature sensitive (Kadar, 2010). For example, a study by Lamari & Bernier (1994) found that the severity of *P. tritici-repentis* infection was significantly reduced when the temperature was above 27°C.

Pyrenophora tritici-repentis is also a competitive saprophyte, with the ability to overwinter and survive within a paddock on wheat stubble and other plant residues for long periods of time (Schilder & Bergstrom, 1992). A study conducted by Summerell & Burgess (1989) investigated the factors which influence the survival of *P. tritici-repentis* on wheat stubble. It was found that *P. tritici-repentis* was able to survive on stubble on the soil surface for at least two years, and potentially longer. It was also reported that the incorporation of wheat stubble into soil was inimical to the survival of *P. tritici-repentis* and production of pseudothecia, with *P. tritici-repentis* being recovered infrequently from stubble buried for 26 weeks. In contrast, *P. tritici-repentis* recovery was only reduced by 50% when stubble was retained on the soil surface. The authors also found that the incorporation of infected stubble into soil helped to effectively break the life cycle of *P. tritici-repentis* by preventing the ejection of ascospores onto the leaves of wheat plants.

1.4 Symptoms of tan spot disease

Tan spot disease consists of two distinct symptoms, necrosis (tan colour) and extensive chlorosis (yellow colour) (Figure 1.2). Tan spot lesions on leaves characteristically have small tan/brown centre, which is surrounded by a yellow circular border. Initially, lesions will appear as tan/brown flecks which will subsequently expand into a lens-shaped lesion. These then develop into tan blotches on the leaf which will later coalesce and kill the leaves (Kadar, 2010). As a plant matures, *P. tritici-repentis* will infect the stem where it will begin to develop pseudothecia (Weise, 1987).

The development of these symptoms is highly specific and a result of an interaction between the pathogen secreted host-specific toxins (HST) (or host selective toxins) and the target receptors of a toxin-sensitive host wheat plant (Singh *et al.*, 2010; Aboukhaddour *et al.*, 2011).

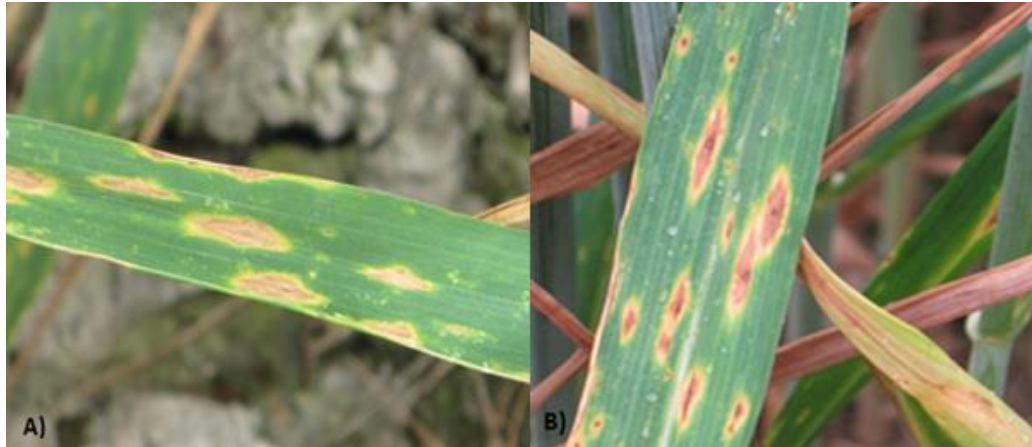


Figure 1.2: A) and B) Typical tan spot lesions caused by *Pyrenophora tritici-repentis* infection of wheat, with characteristic tan lesions surrounded by a yellow chlorotic border.

1.5 *Pyrenophora tritici-repentis* host-specific toxins

One unique feature of *P. tritici-repentis*, in contrast to other major wheat pathogens, is that it is one of a few necrotrophic fungi that possesses the ability to produce multiple HST (Ciuffetti *et al.*, 1999; Singh *et al.*, 2010). Each toxin interacts with a specific host-sensitivity locus, *Tsn1*, *Tsc2* and *Tsc1* (Faris *et al.*, 1996; Faris *et al.*, 2013). There are currently three HST that have been identified and described to date which are produced by *P. tritici-repentis*. These include the proteinaceous Ptr ToxA, which induces necrosis on susceptible wheat genotypes and is encoded by the gene *ToxA* (Ballance *et al.*, 1989; Tomás *et al.*, 1990; Tuori *et al.*, 1995), and the proteinaceous Ptr ToxB, which induces chlorosis on susceptible wheat genotypes and is encoded by a number of multi-copy genes such as *ToxB* (Strelkov *et al.*, 2002; Lamari *et al.*, 2003; Martinez *et al.*, 2004). There is also an, as yet uncharacterised, low molecular weight non-ionic Ptr ToxC, which can induce chlorosis on specific wheat genotypes (Effertz *et al.*, 2002). At present, it is possible to screen for the presence of both *ToxA* and *ToxB* using specific molecular primers (Antoni *et al.*, 2010). Additionally, there are also two other, uncharacterised HST, known as Ptr ToxD toxins, whose exact targets and functions have yet to be elucidated (Faris *et al.*, 2013). This *P. tritici-repentis* HST-wheat host interaction pathosystem is considered to be an accurate representative model of the 'classical' gene for gene concept. As a result, there has been considerable interest and focus on *P. tritici-repentis* and tan spot disease.

Despite the fact that *P. tritici-repentis* has existed in nature for a long time it has been recently proposed that the fungus has only relatively recently become pathogenic towards wheat. This change in pathogenicity has been speculated as being the direct result of the acquisition of the *ToxA* gene. Freisen *et al.* (2006) hypothesized that a horizontal gene transfer event involving the transfer of the *ToxA* gene may have occurred between *P. tritici-repentis* and the wheat pathogen *P. nodorum*. This hypothesis is mainly based on the recent discovery of a *ToxA* gene in the genome of *P. nodorum* which is similar to the *ToxA* gene in *P. tritici-repentis* (Manning *et al.*, 2013).

1.6 Race structure of *Pyrenophora tritici-repentis*

Based on the results of studies undertaken over the last few decades it is recognized that isolates of *P. tritici-repentis* differ widely in their virulence towards different cultivars of wheat (Lamari *et al.*, 2003; Faris *et al.*, 2013). In order to address the challenge of organising and differentiating the different virulences of *P. tritici-repentis* isolate populations from one another, a race grouping system has been developed.

This race designation system has been widely utilised in North American, South American and European countries. The race designation system is based on a rating system, which designates *P. tritici-repentis* isolates into races based on the presence, or absence and degree of the necrosis and chlorosis symptoms that an isolate induces when it is inoculated onto a set range of differential wheat cultivar lines (Aboukhaddour *et al.*, 2011). Using this system, isolates of *P. tritici-repentis* were initially grouped into four distinct pathotypes (Andrie *et al.*, 2007). A new classification system for organising *P. tritici-repentis* has recently been established. This new system places isolates into races based on their virulence patterns and the symptoms that the different *P. tritici-repentis* HST induce on three different wheat differential cultivars known as Glenlea, Salamouni and 6B-365 (Singh *et al.*, 2010; Faris *et al.*, 2013).

However, this current classification system has been found to often be plagued by a number of issues which can affect its overall accuracy and applicability. For example, the system has been found to be confusing when attempting to distinguish chlorosis and necrosis symptoms, too subjective and its overall accuracy depends largely on the knowledge and experience of the assessor (Lepoint *et al.*, 2010). In addition, a study by Andrie *et al.* (2007) reported that the phenotypic race classifications determined from the visual observations of symptoms induced by a range of *P. tritici-repentis* isolates on a set of differential wheat cultivars did not possess the HST associated with their specific race designation.

At present, eight races of *P. tritici-repentis* have been identified worldwide based on their ability to produce different HST, and thereby necrosis or chlorosis on a set of differential wheat cultivars (Ali *et al.*, 2010; Aboukhaddour *et al.*, 2013; Faris *et al.*, 2013). Isolates of *P. tritici-*

repentis belonging to races 1, 6 and 7 produce a combination of two different HST each (Ptr ToxA + Ptr ToxC; Ptr ToxB + Ptr ToxC; Ptr ToxA + Ptr ToxB, respectively). In contrast, isolates belonging to races 2, 3 and 5 produce a single HST each (Ptr ToxA, Ptr ToxC or Ptr ToxB, respectively). Lastly, isolates belonging to race 8 produce all three HST, in contrast, isolates of race 4 are non-pathogenic and do not produce any HST (Ali *et al.*, 2010; Aboukhaddour *et al.*, 2013).

The race structure of overseas populations of *P. tritici-repentis* has been extensively studied. Isolates belonging predominantly to races 1 and 2 were found to be present in Canada (Strelkov & Lamari, 2003; Aboukhaddour *et al.*, 2011). In the United States, isolates of races 1, 2, 3, 4 and 5 have been identified (Ali *et al.*, 2010). In North Africa, isolates belonging to races 5 and 6 have been identified (Lamari *et al.*, 1995; Strelkov *et al.*, 2002). Isolates belonging to races 1, 2, 5, 7 and 8 have been reported in Azerbaijan whilst isolates of 3, 5, 7 and 8 have been found in Syria (Lamari *et al.*, 2005). Isolates belonging predominantly to races 1 and 2 have been reported in Australia (Antoni *et al.*, 2010). The race structure, and therefore the HST present, in the New Zealand *P. tritici-repentis* isolate population has not been investigated.

1.7 Genetic structure of *Pyrenophora tritici-repentis* populations

Pyrenophora tritici-repentis reproduces by both sexual and asexual processes. Thus, genetic diversity within the international populations of *P. tritici-repentis* is likely to be a major factor contributing to the spread and severity of tan spot epidemics worldwide. Despite the fact that *P. tritici-repentis* is homothallic in nature, genetic studies have revealed that the population structure of *P. tritici-repentis* isolates is highly diverse (Manning *et al.*, 2013).

Considerable variation in growth, sporulation, virulence and fungicide sensitivity has been reported among different isolates of *P. tritici-repentis* (Hunger & Brown, 1987; Krupinsky, 1992). For example, a study by Kadar *et al.* (2009) reported significant variation in the growth, sporulation and virulence of isolates collected from winter wheat in Oklahoma in 1983, 1996 and 2006.

1.7.1 Genetic variability analysis using different fingerprinting methods

The use of molecular-based tools can help to determine the genetic variability of a fungal population. There are a number of PCR-based techniques available for the analysis of genetic diversity. Four common molecular methods are random amplified polymorphic DNA (RAPD), microsatellite analysis, amplified fragment length polymorphism (AFLP) and universally primed PCR (UP-PCR).

RAPD is a molecular technique which can be used for genotyping and identifying markers that vary between populations and species (Williams *et al.*, 1990). This method involves the

random amplification of DNA without any previous knowledge of the DNA sequences using different ten base pair (bp) primers that anneal to complementary sites distributed throughout the genome of a particular organism. Each RAPD primer produces several different bands that vary in size and position and can be polymorphic among different individuals within populations (Singh & Hughes, 2006; Baskarathevan, 2011).

RAPD is commonly utilised for population studies because it is simple and inexpensive in contrast to other PCR-based methods. However, RAPD does have some disadvantages. For example, RAPD requires specific reaction conditions e.g. low annealing temperature and reagents and equipment can affect the reproducibility of the results between different studies and laboratories (Tommerup *et al.*, 1995).

In a study by Singh & Hughes (2006) 30 RAPD primers were used to detect genetic variability among 33 isolates of *P. tritici-repentis*, and isolates of five other *Pyrenophora* species. The RAPD method revealed that all the isolates produced unique banding patterns; however, grouping of isolates based on banding patterns was independent of their race classification and point of geographic origin. One common trend observed in studies which have utilised methods like RAPDs (dos Santos *et al.*, 2002; Mironenko *et al.*, 2007), is that the methods are unable to link populations of *P. tritici-repentis* with their points of geographic origins. This may be related to the random nature of the molecular methods and the overall number of isolates selected for analysis. For example, RAPDs (Singh & Hughes, 2006) will only detect random variations which are distributed throughout the genome of *P. tritici-repentis*, whereas, the classification of races is based on specific loci.

Microsatellite (simple sequence repeats) analysis is a robust and reliable technique which can be used to detect genetic variation within different fungal populations. Simple sequence repeats are tandem repeat motifs composed of one to six nucleotides which are abundant, ubiquitous and highly polymorphic within the genomes of most eukaryotic organisms (Tautz & Renz, 1984; Moreno *et al.*, 2012). This technique involves the amplification of a target microsatellite region with PCR using a set of primers complementary to regions flanking the simple sequence repeats (SSR) (Zietkiewicz *et al.*, 1994). Due to stringent and high annealing conditions, microsatellite analysis, in contrast to RAPD, generates more specific and reproducible amplimers (Mereno *et al.*, 2012). However, one disadvantage of microsatellite analysis is that they require knowledge of sequences neighbouring the microsatellite in order to create primers and they are usually species specific.

Aboukhaddour *et al.* (2011) reported that SSR analysis of 80 geographically diverse *P. tritici-repentis* isolates revealed that isolates from different geographic origins had similar levels of genetic diversity. In a much larger study, Gurung *et al.* (2013) used 12 microsatellite markers to analyse and compare 439 *P. tritici-repentis* isolates collected from five different

continents. The results showed that there was a high level of genetic diversity present and a moderate to high population differentiation between continents.

AFLP is a robust and reliable technique which can be used to assess genetic diversity in fungal populations. AFLP is a PCR-based technique which uses restriction enzymes to digest specific DNA sequences to produce fragments. These fragments are then selectively amplified by complementary primers with the products being separated and visualised on polyacrylamide gel (Friesen *et al.*, 2005; Moreno *et al.*, 2012). A study conducted by Leisova *et al.* (2008) found that analysis of AFLP markers of 100 European isolates of *P. tritici-repentis*, showed that 98.1% of genetic variance occurred within local populations, in contrast to 1.9% among the *P. tritici-repentis* isolate populations studied. A study conducted by Friesen *et al.* (2005) using AFLP to examine 97 *P. tritici-repentis* isolates collected from throughout North America, South America and Europe, revealed that there were no grouping of isolates based on their races, or point of geographic origin.

Lastly, UP-PCR is a technique similar to RAPD, however it differs from RAPD due to the primers utilised being longer and requiring higher annealing conditions (Bulat *et al.*, 1998; Pottinger *et al.*, 2002). In contrast to RAPD primers, UP-PCR primers are not random primers and are designed to target the highly variable intragenic regions of the genome (Bulat *et al.*, 1998). Some advantages of UP-PCR, in contrast to RAPD, include the method having greater reproducibility and generating more variable and complex banding patterns. It is because of these features that UP-PCR has been regularly utilised to help study the genetic relatedness of different fungal populations including *Neofusicoccum parvum* (Baskarathavan *et al.*, 2012), *Ilyonectria liriodendri* (Pathrose *et al.*, 2014), *Spilocaea oleagina* (Obanor *et al.*, 2010), *Phaeomoniella chlamydospora* (Pottinger *et al.*, 2002) and *Sclerotium cepivorum* (Tyson *et al.*, 2002). UP-PCR has not been used to characterise the genetic diversity of *P. tritici-repentis* populations.

A common result obtained by some of the highlighted *P. tritici-repentis* molecular studies is that no genetic grouping based on either the race or geographic origin of the *P. tritici-repentis* isolate populations studied were identified (Mereno *et al.*, 2012; Manning *et al.*, 2013). It has been hypothesized that the high levels of genetic variation and population differentiation observed in *P. tritici-repentis* is related to its ability to be dispersed over long distances and then sexually reproduce within the new environments (dos Santos *et al.*, 2002; Singh & Hughes, 2006; Gurung *et al.*, 2013). Combined, these abilities would allow a given *P. tritici-repentis* isolate to quickly generate a large number of unique and diverse generations of progeny within a new environment over consecutive growing seasons (Gurung *et al.*, 2013). The genetic structure of the New Zealand *P. tritici-repentis* isolate population has not been studied and it is unknown whether it is more diverse, or more clonal than populations overseas.

1.8 Sensitivity of *Pyrenophora tritici-repentis* to fungicides

In the situation where a susceptible wheat cultivar is grown and the environmental conditions are conducive for infection by *P. tritici-repentis* the application of fungicides is usually necessary in order to help reduce damage to the overall yield of the crop during the growing season. Due to tan spot being a disease which can affect all leaves on the wheat plant, especially leaves 1 and 2 which are extremely important during grain filling period, the application of a fungicide which protects these leaves will generally result in a significant increase in the crop yield (Colson *et al.*, 2003). Research by Wolf and Hoffmann (1994) found that fungicides should be applied when symptoms of tan spot are observed on more than 5% of the upper leaves.

There are a wide range of fungicides, representing a number of different fungicide classes, which have been registered for controlling tan spot overseas but not yet in New Zealand. The first fungicide class is the strobilurins. The strobilurins belong to the Quinone outside inhibitors (QoI) fungicide class (Patel *et al.*, 2012). Some examples of common QoI fungicides which have been used both overseas and in New Zealand to control tan spot include azoxystrobin, fluoxastrobin, pyraclostrobin and trifyloxystrobin (Colson *et al.*, 2003; Wegulo *et al.*, 2011; Patel *et al.*, 2012). The Demethylation inhibitors (DMI), in particular the triazoles, is another fungicide class which have been used to control tan spot. Examples of DMI fungicides used both overseas and in New Zealand to control *P. tritici-repentis* include the fungicides prothioconazole, epoxiconazole and propiconazole (Colson *et al.*, 2003; Wegulo *et al.*, 2012; Harvey *et al.*, 2015). The succinate dehydrogenase inhibitors (SDHI) is another fungicide class which can control *P. tritici-repentis*. To date, there has been a limited work undertaken to investigate the effectiveness of SDHI fungicides for controlling *P. tritici-repentis* overseas (Wegulo *et al.*, 2012). However, the efficacy of the SDHI fungicides isopyrazam, fluxapyroxad and bixafen were recently investigated under field conditions in New Zealand (Harvey *et al.*, 2015).

There have been a number of studies conducted overseas to evaluate the effectiveness of different fungicides to control *P. tritici-repentis* in a field environment. Work by Colson *et al.* (2003) reported on the results of three separate field experiments conducted from 1988 to 1999 in Australia on the effectiveness of fungicides to control *P. tritici-repentis*. Within these three experiments, a total of 12 fungicides were evaluated for their ability to reduce the effect of tan spot on grain yield. Fungicides were applied at varying rates at a single spray timing at 90% flag leaf emergence. The severity of tan spot epidemics varied significantly between each year. In one experiment, it was reported that grain yield loss was significantly lowered following the application of 72 mL active ingredient per hectare (a.i/ ha) propiconazole, 125 mL a.i/ ha tebuconazole, 72 mL a.i/ ha epoxiconazole, 125 and 250 g a.i/ ha azoxystrobin. A study by Entz *et al.* (1990) reported that relative to untreated control, propiconazole reduced tan spot

infection in wheat in addition to other wheat diseases such as speckled leaf blotch (*Z. tritici*). Propiconazole was also reported to significantly increase grain yield and the amount of kernels in wheat. Research by Jorgensen & Olsen (2007) in Denmark showed that the fungicides propiconazole, prothioconazole, pyraclostrobin and picoxystrobin were the most effective fungicides for controlling tan spot disease, with efficacies reaching between 55-97%, depending on the dose and number of treatments used.

Due to tan spot only recently coming to the attention of the New Zealand wheat industry, there is at present limited research which has evaluated the performance of fungicides to control *P. tritici-repentis* under New Zealand field conditions. However, a recent study by Harvey *et al.* (2015) evaluated the effectiveness of fungicides at controlling tan spot. A total of eight different fungicides were applied at three different timings (GS-33, GS-39 and GS-65) with there also being a single treatment at GS-65 and again 10 days later. It was found that propiconazole, prothioconazole, isopyrazam, bixafen + prothioconazole and fluxapyroxad + epoxiconazole all gave similar levels of control when they were either applied alone or in mixtures. In contrast, the fungicides epoxiconazole and difenoconazole were the least effective. It was however, also noted by the authors that tan spot was not well controlled in the trial due to the overall severity of the epidemic being high.

In contrast to other wheat pathogens, such as *Z. tritici* and *P. nodorum*, the monitoring of strobilurin and triazole fungicide resistance in *P. tritici-repentis* field populations has been very limited to date. However, work conducted by Reimann & Deising (2005) found that *P. tritici-repentis* field isolates collected from Germany had developed reduced sensitivity to a range of strobilurin fungicides. Additionally, *P. tritici-repentis* field isolates resistant to strobilurins have also been found in Sweden and Denmark in both 2004 and 2005 (Frac, 2013). However, a recent study by Patel *et al.* (2012) reported that *in vitro* sensitivity testing of 28 North American *P. tritici-repentis* isolates collected prior 1997 and 136 North American isolates collected between 2007 and 2009, did not indicate any evidence of reduced sensitivity to the fungicide pyraclostrobin.

1.9 Susceptibility of New Zealand wheat cultivars to *Pyrenophora tritici-repentis*

In countries such as the USA, a range of wheat cultivars which have different levels of tolerance or resistance towards tan spot are available. The use of genetic resistance is the safest, sustainable and most economical method for tan spot management available at present (Rees & Platz, 1990; Reide *et al.*, 2003). It is also common practice, when growing resistant wheat cultivars to apply foliar protectant and systemic fungicides at lower rates than normal, in order to reduce any opportunities for *P. tritici-repentis* to establish a disease epidemic (De

Wolf, 2008; Wegulo *et al.*, 2012). This also helps to reduce the risks of the pathogen overcoming the resistance of the variety (Wegulo *et al.*, 2012; Singh *et al.*, 2010).

Currently in New Zealand, it has not been fully established which of the wide variety of commercial feed and milling wheat cultivars available on the market are either susceptible, tolerant, or resistant to *P. tritici-repentis*. Preliminary results from a cultivar rating trial conducted by the Foundation for Arable Research (FAR) during the 2012-2013 summer indicated that New Zealand wheat cultivars such as Saracen and Einstein are susceptible (FAR Trial report, Rob Craigie, Pers Comm). Results also indicated that cultivars such as Claire, Savannah, Excede, KWW45 and Wakanui were moderately susceptible.

At present in New Zealand, it is unlikely that there will be any commercially available wheat cultivars which complete resistance to tan spot disease. This is because tan spot has only recently become a disease of interest to the New Zealand wheat growing industry and therefore to date wheat breeders have not been selectively breeding wheat lines to possess resistance to tan spot. However, there is a possibility that there may be cultivars that possess moderate to partial levels of tolerance/ resistance towards tan spot, such as Empress (FAR trial report, Rob Craigie, Pers Comm). There is also a high likelihood that if tan spot epidemics continue to be frequent and severe, that over time the identification and inclusion of genes, which improve resistance to tan spot disease will be slowly introduced into selective wheat breeding programs in New Zealand.

1.10 Research context and objectives

Despite there being a large amount research conducted overseas over the last 30 years on the genetics, population structure and epidemiology of *P. tritici-repentis* there has been limited work carried out in New Zealand. To date, there have not been any comprehensive studies undertaken to investigate the distribution and severity of *P. tritici-repentis* infection in New Zealand and whether the current population structure of *P. tritici-repentis* is genetically diverse or clonal. Similarly, there is no or very limited information regarding the sensitivity of New Zealand *P. tritici-repentis* isolates to commercial fungicides used here and overseas and the susceptibility of wheat cultivars commonly grown in New Zealand to tan spot disease. One reason for this gap in knowledge can be attributed to *P. tritici-repentis* only being identified and confirmed as being present in New Zealand main wheat growing regions within the last three years. This gap in knowledge needs to be addressed in order to identify and develop the most effective strategies for controlling *P. tritici-repentis* in New Zealand. This will involve understanding the genetic structure, distribution, sensitivity to commonly used fungicides and practices that either promote, or discourage the fungus *P. tritici-repentis* in New Zealand. This study will attempt to address this issue, by investigating key features in the biology of

P. tritici-repentis, in order to provide data that can be quickly utilised by the New Zealand arable industry. This information will help to develop effective control strategies for tan spot.

The objectives of this research are as follows:

1. Determine the incidence and severity of tan spot caused by *Pyrenophora tritici-repentis* in the South Island wheat cropping areas of New Zealand.
2. Determine the race structure and genetic diversity of the New Zealand *Pyrenophora tritici-repentis* population.
3. Determine the susceptibility of commonly grown New Zealand wheat cultivars to *Pyrenophora tritici-repentis*.
4. Determine the sensitivity of *Pyrenophora tritici-repentis* to different fungicides.

Chapter 2

Determination of the distribution and race structure of *Pyrenophora tritici-repentis* in the South Island, New Zealand

2.1 Introduction

Pyrenophora tritici-repentis is a homothallic fungus which can reproduce sexually to produce ascospores in pseudothecia, and asexually to produce conidia on conidiophores, with these spores serving as primary and secondary inoculum, respectively. Sexual reproduction normally occurs once at the end of the season on stubble, while in contrast, asexual reproduction occurs in many cycles throughout the wheat growing season should conditions permit (Ciuffetti & Tuori, 1999).

Tan spot disease is characterised by two distinct symptoms, necrosis (tan colour) and extensive chlorosis (yellow colour); with the development of these symptoms being highly specific and a direct result of an interaction between the host-specific toxins (HST) released by *P. tritici-repentis* and the target receptors of the toxin-sensitive host wheat plant (Faris *et al.*, 2013).

To date, there have been three known HST produced by *P. tritici-repentis* which have been identified and had their biological functions elucidated (Leisova *et al.*, 2008; Faris *et al.*, 2013). These HST include the proteinaceous and ribosomally synthesized Ptr ToxA and Ptr ToxB. The HST Ptr ToxA, which induces necrosis on susceptible wheat genotypes, is a 13.2 kDa protein encoded by a single copy gene known as *ToxA* in isolates which are able to produce Ptr ToxA (Ballance *et al.*, 1989; Tuori *et al.*, 1995). The HST Ptr ToxB, which induces chlorosis on susceptible wheat genotypes, is a 6.6 kDa protein which has been found to be encoded by multi copy genes in isolates of the races that have the ability to produce Ptr ToxB (Strelkov *et al.*, 2002; Lamari *et al.*, 2003; Martinez *et al.*, 2004). There is also an, as yet to be characterised low molecular weight non-ionic HST referred to as Ptr ToxC which can induce chlorosis on specific wheat genotypes (Effertz *et al.*, 2002). Currently, there have been eight races of *P. tritici-repentis* identified worldwide, which have been defined by their ability to produce HST, and thereby necrosis or chlorosis on a set of differential wheat cultivars (Ali *et al.*, 2010; Faris *et al.*, 2013).

There have been several reports published detailing the race structure of *P. tritici-repentis* populations in different parts of the world (Lamari *et al.*, 1998; Ali & Francl, 2003; Friesen *et al.*, 2005; Aboukhaddour *et al.*, 2013). One common finding of studies undertaken in the last 5 years of *P. tritici-repentis* populations in the USA, Canada, South America and Australia is that Ptr ToxA has been the predominant HST found in isolates of *P. tritici-repentis*, with Ptr ToxB almost completely absent (Strelov & Lamari, 2003; Antoni *et al.*, 2010; Aboukhaddour *et al.*, 2013).

In New Zealand, tan spot disease is considered to be a relatively new disease that affects wheat. As a result of this, there has yet to be a large comprehensive study undertaken to investigate and determine what races, and therefore Ptr-HST, the New Zealand populations of *P. tritici-repentis* possess. In addition to this, the current distribution of *P. tritici-repentis* in New Zealand is also unknown and requires investigation. The aim of this study was to investigate and determine the distribution and race structure of *P. tritici-repentis* in the South Island of New Zealand.

2.2 Materials and Methods

2.2.1 Sampling and collection procedure for *Pyrenophora tritici-repentis* symptomatic plant material

A survey was conducted for tan spot on farms located throughout the South Island during the 2013-2014 wheat growing season. The sampling period began on 1st November 2013 and ended on 21st January 2014. A total of 15 sites were sampled in which symptoms characteristic of tan spot was observed on the wheat crop as shown in Figure 2.1. The *P. tritici-repentis* symptomatic plant material were sampled and collected by myself, Dr Matthew Cromeey (Plant and Food Research), FAR consultants, chemical representatives, regional commercial farm consultants, or the wheat growers themselves, depending on the geographic origin of the sample and time of sampling.

The Canterbury symptomatic plant material were collected from both Mid and South Canterbury locations and included two farms in Fairlie, one farm at Greendale, one farm near Geraldine, four farms around Waimate and four different paddocks on two different farm blocks owned by the same grower located near Methven. The Otago/Southland diseased plant material were collected from farms located in both, North Otago and Southland, these included one farm in Kakanui North Otago, one farm at Clinton and one farm in Wedonside Southland.

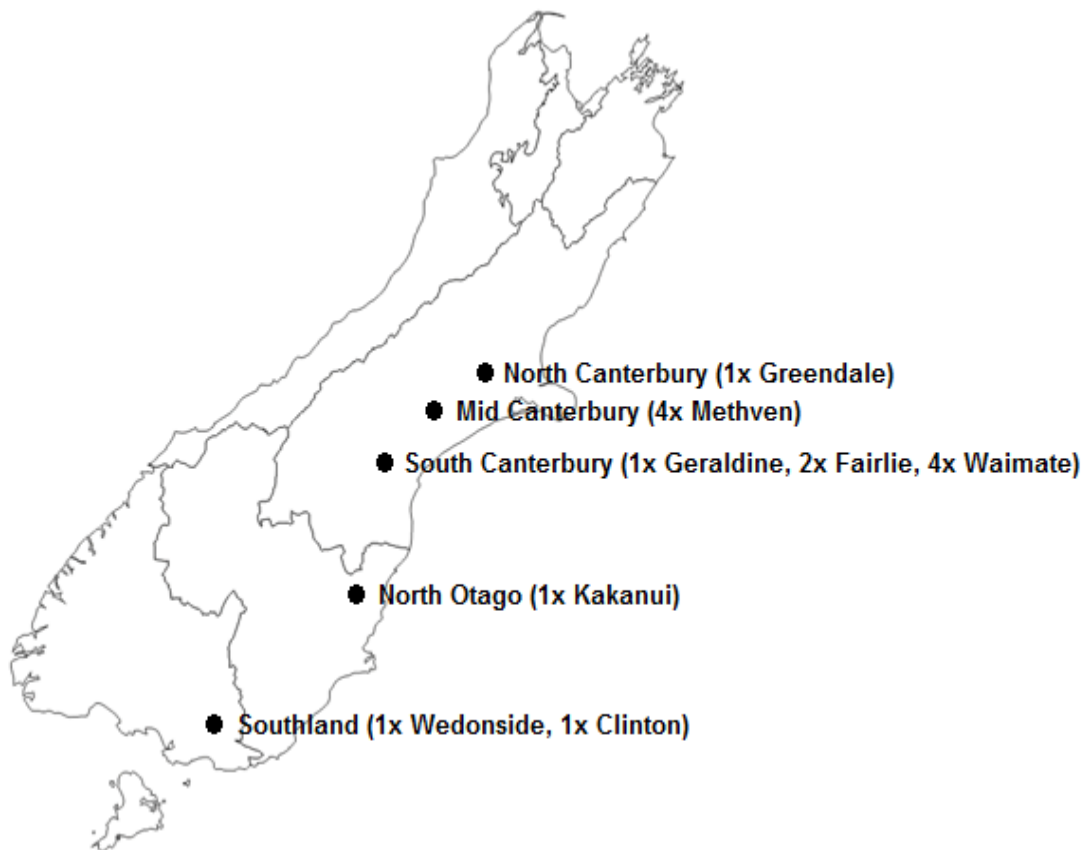


Figure 2.1: Map of New Zealand's South Island showing the distribution and number of wheat growing farms where plant material showing symptomatic *Pyrenophora tritici-repentis* lesions were obtained during the 2013-2014 season. (Source: <http://d-maps.com/m/oceania/nzelande/nzelande06.gif>).

2.2.2 Wheat grower survey process

A questionnaire (Appendix B.1) was sent to all of the farmers whose farms yielded a viable *P. tritici-repentis* isolate, during the tan spot sampling period. The questionnaire was completed either via email or over the phone.

Questions included in this questionnaire covered sowing date, cropping history (crop rotation history), farm practices (cultivation and post-harvest management methods), fungicide use and cultivar choices. The survey questionnaires were collected and the answers from each farmer to each particular question were compared in order to identify any trends relating to the way in which the farmer managed their property that may have attributed to the outbreak of tan spot on their farms during the 2013-2014 growing season.

2.2.3 Identification of tan spot leaf symptoms

Identification of presumptive *P. tritici-repentis* infection of wheat plants was based on symptom descriptions (Wegulo, 2011). These consisted of two distinct symptoms of tan spot; tan coloured necrosis and extensive yellow chlorosis halo on the leaves (Figure 2.2.). Leaves with distinct reasonably young tan spot lesions i.e. uncoalesced, were selected and placed into a paper bag labelled with the farms location, farmers name, wheat cultivar, date of collection, and plant and leaf number, if applicable. Diseased leaf samples were stored dry at 5°C for up to 2 weeks, until used for fungal isolation.

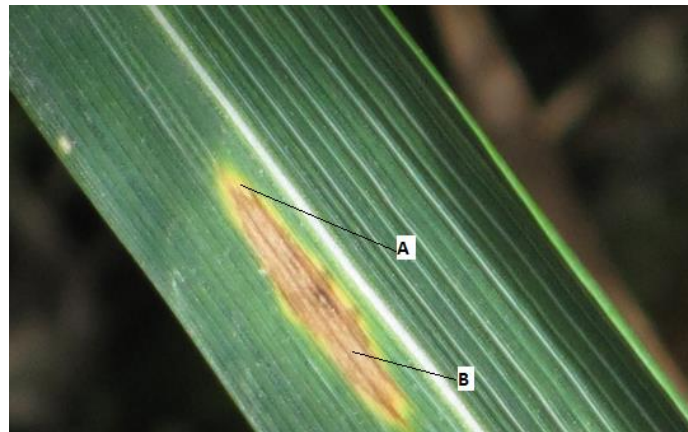


Figure 2.2: A characteristic tan spot lesion on a wheat leaf, A indicating chlorosis (yellow) and B indicating necrosis (tan/brown).

2.2.4 Isolation of *Pyrenophora tritici-repentis* from infected wheat tissue

Fungal isolations were carried out from individual tan spot lesions. Lesions were cut from the leaf by cutting along the margin between the chlorosis (yellow) and necrosis (tan) using a sterile scalpel. Preliminary experiments (data not shown) revealed that this method resulted in the highest degree of success in isolating *P. tritici-repentis* from infected plant tissue.

The lesions were sterilized by placing into a universal tube containing 2% bleach (NaOCl) (25 g a.i / 10 mL), and shaking vigorously by hand for 1 minute. The tissue was then washed three times in separate universals containing 25 mL sterile water which were also shaken vigorously by hand for 1 minute. The tissue was then removed and dried in a laminar flow hood on clean tissue paper for 30 seconds. Once dry, the sterilized lesion tissues of a single leaf were placed onto the surface of reduced strength PDA (30 g/ L; Difco) amended with 10 mg/ L of both

penicillin and streptomycin (penstrep) in Petri dishes. Plates were labelled and incubated at 25°C with a 12 hour light/dark cycle.

After 4-6 days of incubation, the PDA plates were observed for colonies characteristic of *P. tritici-repentis* growing from the leaf pieces. The initial identification of *P. tritici-repentis* was carried out based on the observation of characteristic anamorph morphology of colonies reported in the literature (Wegulo, 2011). A characteristic *P. tritici-repentis* colony was identified from other fungi, as having greenish-grey, dense and fluffy mycelium with a white colony margin (Figure 2.3). No conidia were produced on PDA or on malt extract agar (MEA) (Difco; 33.6 g/ L).

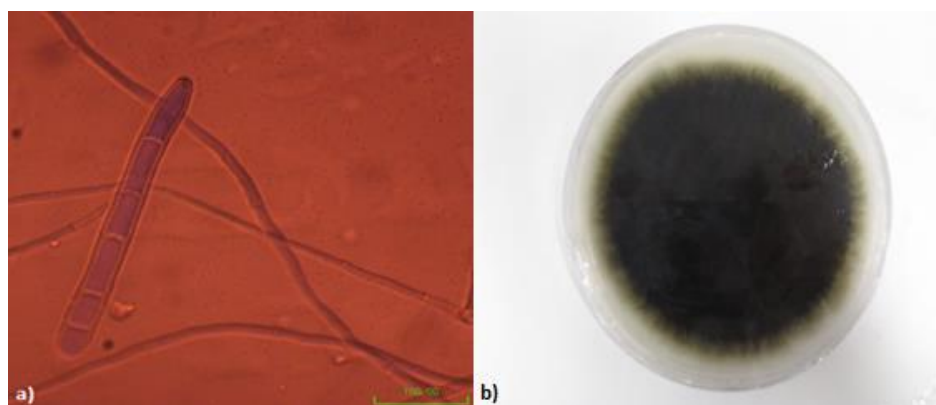


Figure 2.3: a) Conidia of *Pyrenophora tritici-repentis* (400x magnification), b) characteristic culture of *Pyrenophora tritici-repentis* on potato dextrose agar (PDA).

For production of conidia the characteristic *P. tritici-repentis* colonies were subcultured onto V8-PDA (Appendix A.1) or V8 (Appendix A.2) and incubated under near ultraviolet light for 12 to 24 hours followed by 12 to 24 hours darkness (Lamari & Bernier, 1989a). Conidia of *P. tritici-repentis* were observed by making a sellotape mount slide with a drop of lactoglycerol blue and visualized using a compound microscope (at x100 and x400 magnification). Characteristic *P. tritici-repentis* colonies were identified as being whitish to light grey with conidia being identified as being subhyaline, cylindrically shaped with 4-9 septa, with a conically tapered basal cell being particularly diagnostic for conidia of *P. tritici-repentis* (Figure 2.3) (Kadar, 2010). Colonies presumptively identified as *P. tritici-repentis* were subcultured onto MEA amended with 10 mg/ L penicillin and streptomycin. Plates were then incubated for 6-7 days at 25°C with a 12 hour light/dark cycle. After which, plates were removed and stored at 5°C until used for single spore isolation and further identification.

2.2.5 Single spore isolate production

The production of single-spore isolates is essential for any study investigating the genotypic diversity of fungi (Ho & Ko, 1997). Since a fungal colony which grows from a single conidium is derived from a single nucleus, it can therefore represent a genetic individual within a population (Ho & Ko, 1997).

For this study, single spore isolates were produced following modification of the method developed and described by Lamari & Bernier (1989a). Each isolate was subcultured onto V8-PDA agar. V8-PDA plates were wrapped in tinfoil and incubated at 20°C for 5 days in continuous darkness, or until the fungal colony was 5 cm in diameter. After 5 days incubation, the tinfoil was removed and the growing mycelium was then flooded with 5 mL of sterile water and flattened using a flame sterilized bent glass rod. The water was then decanted and the plates were then placed into an Adaptis CMP6010 growth cabinet (Convion) and incubated under white light for 12 hours at 22.5°C. After 12 hours incubation under white light, plates were placed into an incubator and incubated for a further 12 hours in continuous darkness at 16°C.

Conidia were harvested by flooding the plate with 5 mL of sterile water and then gently scrapping the conidia off the surface of the mycelium using a flame sterilized scalpel. The water in the plate was then swirled for 30 seconds, in order to ensure conidia in solution, and then 1.5 mL of the conidial suspension was then pipetted directly from the plate into a 1.7 mL Eppendorf tube. For each *P. tritici-repentis* isolate, 200 µL of conidial suspension was pipetted onto water agar (WA) (15 g Davis agar). The spores were spread using a flame sterilized hockey stick and the plates were incubated for 6 hours at 20°C.

After 6 hours incubation, plates were examined under a stereomicroscope (x 100 magnification) and the colonies originating from a single conidium were marked. A single conidium was cut from the WA using a flame sterilized scalpel and subsequently transferred to a full strength PDA plate (39 g PDA/ L) and then incubated for 6-7 days at 20°C with a 12 hour light/dark cycle. Single spore isolates were maintained on full strength PDA at 5°C for short term storage and in 20% glycerol at -80°C for long term storage prior to molecular confirmation of identification.

2.2.6 Molecular confirmation of New Zealand *Pyrenophora tritici-repentis* isolates

To confirm morphological identification (Section 2.2.4) of isolates *P. tritici-repentis* species-specific PCR was used. Of the total isolates recovered, 12 single spore *P. tritici-repentis* isolates representative of the geographic distribution of *P. tritici-repentis* were selected. The identity of all 12 *P. tritici-repentis* single spore isolates was confirmed by DNA sequencing of two taxonomically informative genes, the internal transcribed spacer region (ITS) and the β -tubulin gene region.

2.2.6.1 Genomic DNA extraction from fungal mycelium

Twelve *P. tritici-repentis* single spore isolates (S12a, S22a, S26a, M13c, M14d, G14a, G22a, F12d, W12a, W12c, W15a and K16a) (Table 2.1) were grown on potato dextrose broth (PDB; Difco) (24g/ L) and incubated for four days at 20°C in continuous darkness. The mycelium was harvested onto sterile Miracloth™, squeezed between paper towels to remove excess moisture, immediately wrapped with aluminium foil and transferred into liquid nitrogen to snap freeze. Harvested mycelium was stored at -80°C until used for DNA extraction.

Genomic DNA of each isolate was extracted from the mycelium using the plant tissue DNA isolation protocol of the PUREGENE® genomic DNA isolation kit (Gentra systems, USA). Approximately 100 mg of frozen mycelium was ground into a fine powder using a flash cooled mortar and pestle and then used for DNA extraction by following the manufacturer's instructions. During the final step of the procedure, the resulting DNA pellet was rehydrated in 30 μ L sterile water in a 1.7 mL tube.

DNA concentration was measured using a NanoDrop-ND-1000 spectrophotometer (NanoDrop Technologies, USA). All *P. tritici-repentis* genomic DNA samples were diluted to a working concentration of 10-20 ng/ μ L for PCR. DNA samples were stored at 4°C until for use.

Table 2.1: New Zealand single spore isolates of *Pyrenophora tritici-repentis* used in study

| Isolate Code | Date collected | Collection Location |
|--------------|--------------------------------|---------------------------------|
| S12a | 16 th January 2014 | Wedonside, Southland |
| S22a | 22 nd October 2013 | Clinton, Southland |
| S26a | 22 nd October 2013 | Clinton, Southland |
| M13c | 31 st December 2014 | Methven, Mid-Canterbury |
| M14d | 31 st December 2014 | Methven, Mid-Canterbury |
| G14a | 1 st November 2013 | Greendale, North Canterbury |
| G22a | 24 th November 2013 | Geraldine, South Canterbury |
| F12d | 19 th November 2013 | Fairlie basin, South Canterbury |
| W12a | 19 th November 2013 | Waimate, South Canterbury |
| W12c | 19 th November 2013 | Waimate, South Canterbury |
| W15a | 19 th November 2013 | Waimate, South Canterbury |
| K16a | 4 th December 2013 | Kakanui, North Otago |

For each code, the first letter represents the origin of collection; the second number denotes to number of farm; the number and letter at end represent the culture and number the isolate was sourced from (Appendix B.3)

2.2.6.2 Species-specific PCR identification

To confirm morphological identification of isolates as *P. tritici-repentis* their identities were confirmed by species specific PCR using the species specific primers PtrUniqueF2 (5'GGACTTTGGCTTTCTATTGTGC³) and PtrUniqueR2 (5'CTTGGTGAATGGTGAAGATGG³) designed by Antoni *et al.* (2010). The *P. tritici-repentis* species specific primers amplified a 490 bp product.

PCR was performed using 1 ng of the DNA for each single spore isolate which had been extracted using PUREGENE[®] genomic DNA isolation kit (Gentra systems, USA). Each reaction volume was made up to 25 μ L with sterile water. The PCR mixture contained 1 x PCR buffer (with 1.5 mM MgCl₂; Roche), 200 μ M dNTP's (dGTP, dCTP, dATP, dTTP), 10 μ M of primer, 1 U of FastStart Taq polymerase (Roche) and 1 μ L of template DNA. A negative control with 1 μ L sterile water instead of the template DNA was used in the PCR.

For all reactions the PCR amplification conditions were as follows: an initial denaturation at 94°C for 3 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 1 minute, with a final extension of 72°C for 10 minutes.

After amplification, 5 μL of each PCR product combined with 3 μL loading dye (40% (w/v) sucrose; 0.25% bromophenol blue; 0.25% xylene cyanol) was separated by electrophoresis in a 1% agarose gel (1 g agarose powder and 100 mL 1xTris-Acetate EDTA buffer) in 1x TAE buffer (at 10 V/cm) for 50 minutes. Eight μL of 1kb plus DNA ladder (0.1 μg / μL ; Invitrogen) was combined with 3 μL loading dye and used as a molecular weight marker. The gel was stained with ethidium bromide (0.5 μg / mL, AMRESCO®, OH, USA) for 15 minutes. The gel was then destained by rinsing for 5 minutes in tap water and was then photographed using a UV transilluminator (UVITEC Cambridge Imaging System, Total Lab Systems Ltd), where it was photographed under UV light using Firereader 16.04 (UVITEC Cambridge Imaging System, Total Lab Systems Ltd).

2.2.7 Sequencing of ITS and β -tubulin genes

2.2.7.1 PCR Amplification with ITS and β -tubulin gene primers

Confirmation of species identification consisted of two stages, initially *P. tritici-repentis* was identified with field isolates and later using single spore isolates. The internally transcribed spacer regions (ITS1 and ITS2) from the ribosomal (rRNA) gene of three randomly selected *P. tritici-repentis* field isolates and the 12 single spore isolates were amplified. The β -tubulin gene region of three randomly selected *P. tritici-repentis* single spore isolates was also amplified. The ITS region was amplified using the primers ITS4 (5'-TCCTCCGCTTATTGATATGC³) and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG³) (White *et al.*, 1990) and the β -tubulin gene was amplified using the primers Bt2b (5'-ACCCTCAGTG TAGTGACCCCTTGGC³) and T1 (5'-AACATGCGTGATTGTAAGT³) (Cabral *et al.*, 2012).

The ITS region of field *P. tritici-repentis* isolates was amplified using DNA extracted with a rapid method called the Extract N AMP™ Plant PCR kit (Sigma-Aldrich). In this process the DNA of each field isolate was extracted by gently scraping a small amount of mycelium off a colony using a sterile pipette tip and then immersing it in a tube containing 100 μL of Extraction solution. The tube was then incubated for 10 minutes at 95°C. The tube was then cooled to room temperature and 100 μL of Dilution solution was added and the tube vortexed. This solution was stored at 4°C prior to PCR.

Each reaction volume was made up to 20 μL . Each 20 μL PCR contained 10 μL Extract N AMP™ Plant PCR kit amplification solution (Sigma-Aldrich), 1 μL of a 5 μM solution of each primer (ITS4 and ITS5) and 4 μL extracted DNA. Each reaction volume was made up to 20 μL with sterile water. A negative control where 4 μL of a 1:1 mix of Extraction and Dilution solutions

was added instead of DNA to make sure no contamination was present. For the β -tubulin and ITS PCR with *P. tritici-repentis* single spore isolates purified DNA extracted using the method described in Section 2.2.6.1 was used. The ITS and β -tubulin gene regions from the single spore isolates were amplified in a 25 μ l reaction volume.

All PCR amplifications were performed in a Veriti Thermal Cycler-200. The ITS PCR conditions were as follows: an initial denaturation for 3 minutes at 94°C, followed by 35 cycles of denaturation for 1 minute at 94°C, annealing at 55°C for 30 seconds, and an elongation at 72°C for 1 minute, with a final extension period of 10 minutes at 72°C. The β -tubulin PCR amplification conditions were as follows: an initial denaturation of 94°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 80 seconds, and a final extension period of 7 minutes at 72°C. After amplification, 3-5 μ L of each PCR product combined with 3 μ L loading dye was separated by electrophoresis in a 1% agarose gel and visualized using the method described in Section 2.2.6.2.

2.2.7.2 Sequencing of ITS, β -tubulin gene, species specific primer, ToxA and ToxB gene regions

The ITS region of three randomly selected *P. tritici-repentis* field isolates and 12 single spore isolates were sequenced in order to initially confirm their identity. For β -tubulin and species specific primers, three representative *P. tritici-repentis* single spore isolates (S12a, M14d and G22a) were selected for sequencing in order to confirm the identities of the isolates.

The PCR product was sequenced directly in both directions in an ABI PRISM 310 genetic analyser (Applied Biosystems, Foster city, California) automated sequencer at the Bio-Protection Research Centre Sequencing Facility, Lincoln University.

The sequence data was analysed and edited to remove the ambiguous areas close to the sequencing primer using either Chromas LITE sequencing software (Technelysium Pty Ltd) or DNAMAN (Version: 4.0a; Lynnon Biosoft). The sequence data were submitted to GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the basic local alignment search tool (BLAST) function to confirm the identification of the isolates as *P. tritici-repentis*.

2.2.8 Determination of race structure of New Zealand and international *Pyrenophora tritici-repentis* isolates

2.2.8.1 *Ptr ToxA* and *Ptr ToxB* PCR screening procedure

The 12 New Zealand *P. tritici-repentis* single spore isolates (Table 2.1) were screened using PCR for the presence of the *Ptr ToxA* and *Ptr ToxB* genes using *ToxA* and *ToxB* specific primers, respectively. The *ToxA* primers were *ToxAscreeningF* (5'CCTCGTACTTCTTTTCAGCG3') and *ToxAscreeningR* (5'TCCTCTACCTTGAATTAAGCG3') which were designed by Antoni *et al.* (2010). For the *ToxB* screening PCR, the *ToxB* primers were *TB10f* (5'TATGCGACCCTAACCTAGCC3') and *TB12r* (5'GCCAGATAAAAAACCCCTATACC3') which were designed by Martinez *et al.* (2004) and used by Antoni *et al.* (2010).

The genomic DNA of 12 international *P. tritici-repentis* isolates was also used to provide DNA from isolates known to be *Ptr ToxA* and *Ptr ToxB* positive and negative. The inclusion of international isolates allowed confirmation that the PCR was working and provided a range of different *P. tritici-repentis* races to which the local New Zealand *P. tritici-repentis* populations could be compared against. The 12 international *P. tritici-repentis* isolates consisted of three Australian (CCDM-Meck4, CCDM-239 and CCDM-134) which were kindly provided by Dr Caroline Moffat (Curtin University, Perth, Australia) and nine isolates of varying geographic origin (90-2, ASC-1, AB47-10, AlgH2, Alg3-24, AB33-1, AB39-2, AB39-8 and TS93-71B) kindly provided by Dr Reem Aboukhaddour (University of Alberta, Canada) (Table 2.2).

Table 2.2: Details of the international *Pyrenophora tritici-repentis* isolates used in this study

| Isolate code | Race | Toxins | Region/ Country collected |
|--------------|----------|--------|--|
| 90-2 | R4 | - | Canada |
| ASC1 | R1 | A | Canada |
| AB47-10 | R1 | A | Morley region (Canada) |
| Alg3-24 | R5 | B | Algeria |
| AlgH2 | R6 | BC | Algeria |
| AB33-1 | R1 | A | Wilson region (Canada) |
| AB39-2 | R3 | C | Wilson region (Canada) |
| AB39-8 | R1 | A | Wilson region (Canada) |
| TS93-71B | R8 | ABC | Turkey-Syria |
| CCDM-Meck4 | R1 or R2 | A | Meckering, Western Australia (Australia) |
| CCDM-239 | R1 or R2 | A | Sea Lake, Victoria (Australia) |
| CCDM-134 | R1 or R2 | A | Biloela, Queensland (Australia) |

Notes: 90-2 and AlgH2 published in Aboukhaddour *et al.* (2011) and ASC1, Alg3-24 and AlgH2 in Aung (2001)

Each PCR was made up to 25 μ L and followed the method described in Section 2.2.6.2. The cycling parameters for both primer sets were as follows: initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and an elongation at 72°C for 1 minute, with a final extension at 72°C for 10 minutes. After amplification, 7-10 μ L of each PCR product combined with 3 μ L loading dye was separated by electrophoresis and visualized following the methods described in Section 2.2.6.2. To confirm primers amplified the correct target, the PCR products of three *P. tritici-repentis* single spore isolates (S12a, M14d and G22a) were selected for sequencing following methods described in Section 2.2.7.2.

2.3 Results

2.3.1 Determination of distribution and incidence of tan spot in South Island

Lesions characteristic of tan spot caused by *P. tritici-repentis* were observed on wheat plants from a total of 15 wheat fields (Figure 2.1).

2.3.1.1 Results of farmer questionnaire

Five tan spot questionnaires were completed by the South Island farmers whose farm had tan spot during the 2013-2014 season. A summary of the answers to the questions covered in the questionnaire is shown in Table 2.3. Three of the five farmers burned their wheat stubble after harvest. In contrast, two out of the five farmers ploughed, or worked (maxitilled) the wheat stubble after harvest of the crop. Two out of the five farmers surveyed continuously sowed wheat after wheat for over 5 years. Two out of five farmers rotated their wheat crops on an annual basis. Two out of the five farmers sowed the current years crop using wheat seed obtained from their previous crop. Two of the five farmers applied a chemical seed treatment to their wheat seed before sowing.

Four of the five farmers surveyed applied fungicides on four different timings. The most common fungicides applied by the farmers surveyed included epoxiconazole, azoxystrobin, prothioconazole, pyraclostrobin and tebuconazole. The efficacy of fungicides will be investigated in Chapter 4.

From the five farms surveyed, *P. tritici-repentis* was isolated from the tissue of the cultivars Wakanui (2 farms), Einstein (2 farms/ 3 crops), Empress (2 farms 3 crops), Torch (1 farm), Claire (1 farm) and Saracen (1 farm). Other cultivars which *P. tritici-repentis* was isolated from during the sampling period were Phoenix, Delphi, L45, A91 and A30 which all represent one wheat crop are shown in Figure 2.4. The relative susceptibility of different wheat cultivars will be assessed in Chapter 5.

Table 2.3: Summary of the information provided by farmers to questions asked in the 2014 tan spot questionnaire

| Farmer / Location | Burned wheat stubble | Plough / work stubble into soil | Has Continuously sown wheat for 5> years | Annually Rotate wheat crops | Re-use wheat cultivar seed | Apply chemical seed treatments | Applied fungicides at separate four timings |
|---------------------------|----------------------|---------------------------------|--|-----------------------------|----------------------------|--------------------------------|---|
| North Canterbury Farmer | No | Yes | Yes | No | No | No | Yes |
| South Canterbury Farmer 1 | Yes | No | No | Yes | No | No | No |
| South Canterbury Farmer 2 | Yes | No | No | Yes | Yes | Yes | Yes |
| South Canterbury Farmer 3 | Yes | No | No | No | No | Yes | Yes |
| Southland Farmer | No | Yes | No | No | Yes | No | Yes |

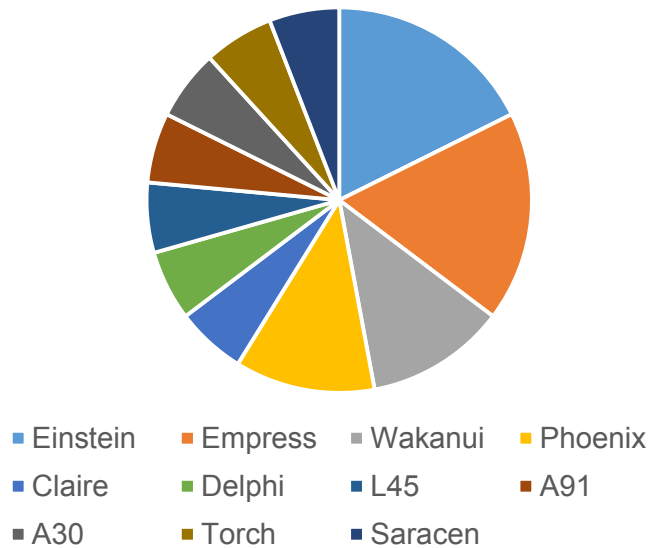


Figure 2.4: Pie graph showing the frequency and name of wheat cultivars *Pyrenophora tritici-repentis* was recovered from during 2013-2014 season.

2.3.3 Identification of *Pyrenophora tritici-repentis* based on colony and conidia morphology

Pyrenophora tritici-repentis was positively identified by observation of *in vitro* morphology of conidia from fungal populations collected at all 15 sites surveyed. Colonies typical of *P. tritici-repentis* were frequently recovered from lesions characteristic of tan spot on infected wheat leaf tissue taken from all of the farms surveyed. Fungal colonies matching the morphological description of *P. tritici-repentis* were isolated from 90% of the lesions selected for isolation. Isolates identified as *P. tritici-repentis* had grey-green fluffy mycelium with a white margin to the colony, with the underside of the colony being dark green. All colonies characteristic of *P. tritici-repentis* had similar morphological appearances and growth rates with the colony reaching the edge of the PDA or MEA plate after 7 days of growth at 25°C (Figure 2.5). Bacterial contamination of infected tissue reduced isolation success of *P. tritici-repentis* by 5%. In addition to *P. tritici-repentis*, fungal colonies characteristic of *Z. tritici* (Strukenbrock *et al.*, 2012) and *P. nodorum* (Cunfer, 2000) were also recovered (5% of lesions isolated) from lesions resembling tan spot.

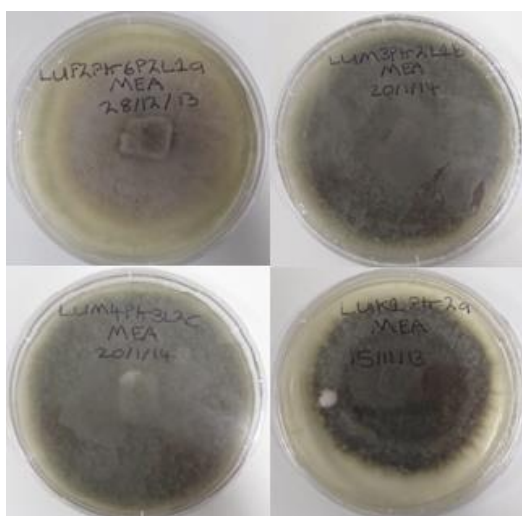


Figure 2.5: Examples of the appearances of different isolated colonies of *P. tritici-repentis* on MEA.

2.3.4 Molecular confirmation of *Pyrenophora tritici-repentis*

The *P. tritici-repentis* specific primers amplified a product of 490 bp from the genomic DNA of all twelve single spore isolates selected (Figure 2.6). The sequence of the PCR products of three randomly selected isolates were identical to *P. tritici-repentis* (XM_001930305.1) (Appendix C.1) sequences obtained from the NCBI database.

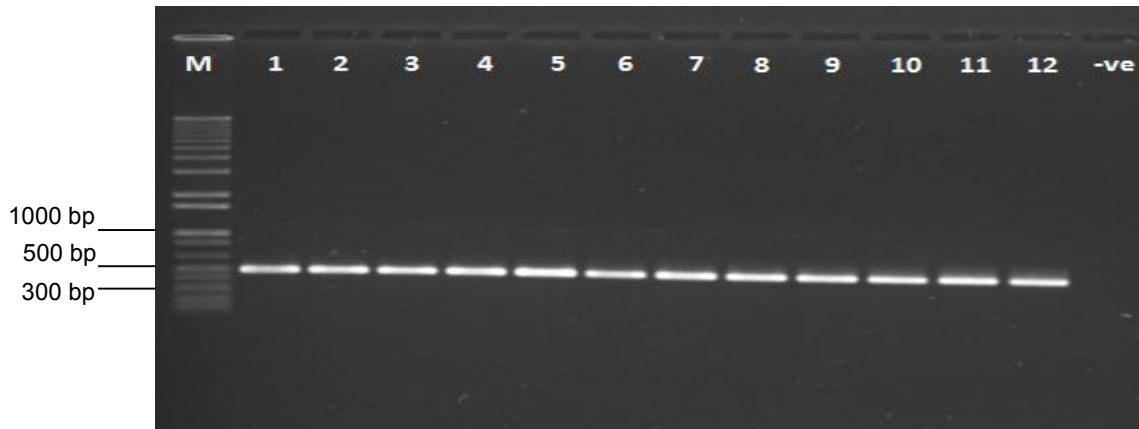


Figure 2.6: 1% agarose gel of PCR products generated by the species-specific PtrUniqueF2/PtrUniqueR2 primers using the genomic DNA of 12 *Pyrenophora tritici-repentis* South Island single spore isolates. From left to right, lanes 1 to 12 are *P. tritici-repentis* isolates S12a, S22a, S26a, M13c, M14d, G14a, G22a, F12d, W12a, W12c and K16a. M = 1 kb plus DNA ladder (Invitrogen), - ve = negative control.

A single PCR product of approximately 600 bp was amplified from the rRNA gene of all three of the randomly selected *P. tritici-repentis* field isolates (Figure 2.7) and all twelve single spore *P. tritici-repentis* isolates (Figure 2.8). A product of approximately 600 bp was amplified from the β -tubulin gene of three *P. tritici-repentis* single spore isolates. The results of the BLAST analysis confirmed all isolates as *P. tritici-repentis* with sequences identical to those obtained from the NCBI database (JQ314403.1; FJ907535.1) (Appendix C.1) for rRNA and β -tubulin.

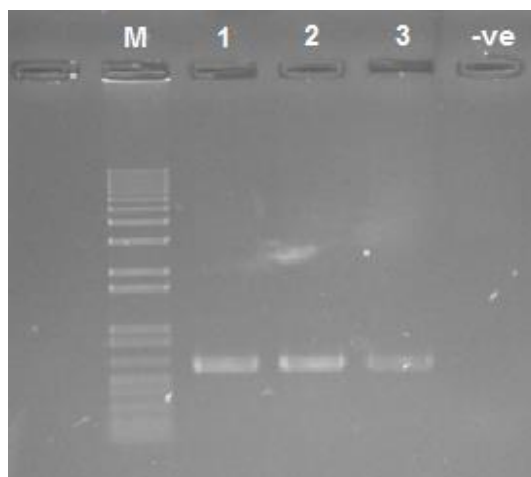


Figure 2.7: 1% agarose gel of PCR products produced by amplifying three field isolates of *Pyrenophora tritici-repentis* using primers ITS4 and ITS5. Lanes numbered 1 to 3 are *P. tritici-repentis* field isolates G2Ptr1, W2Ptr1 and G1Ptr3. M = 1 kb plus DNA ladder (Invitrogen), - ve = Negative control.

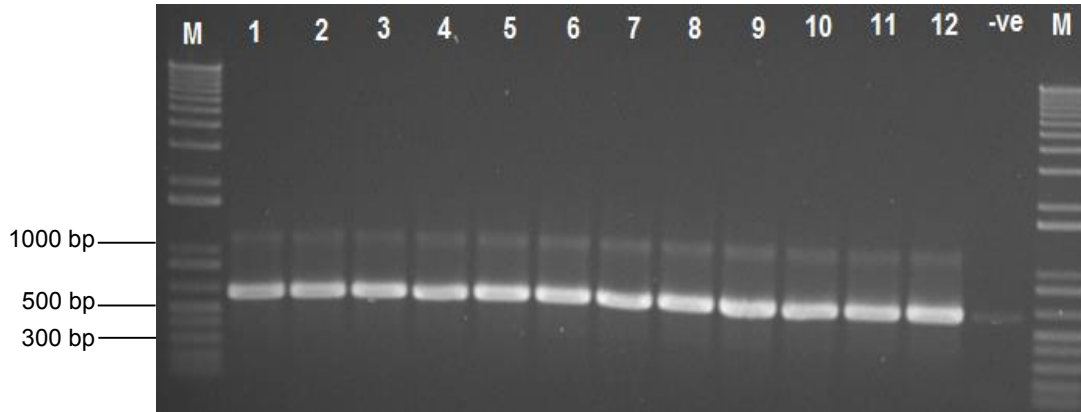


Figure 2.8: 1% agarose gel of PCR products generated using primers ITS4 and ITS5 using the genomic DNA of 12 *Pyrenophora tritici-repentis* South Island single spore isolates. From left to right, lanes 1 to 12 are *P. tritici-repentis* single spore isolates S12a, S22a, S26a, M13c, M14d, G14a, G22a, F12d, W12a, W12c and K16a. M = 1 kb plus DNA ladder (Invitrogen), -ve = negative control.

2.3.5 Determination of race structure of New Zealand and International *Pyrenophora tritici-repentis* populations

The *ToxA* specific primers amplified a band of 510 bp from the genomic DNA of all 12 New Zealand *P. tritici-repentis* isolates (Figure 2.9). A band of 510 bp was also amplified from all three Australian isolates (CCDM-Meck4, 239 and 134) (Figure 2.11) and five (ASC1, AB47-10, AB33-1, AB39-8 and TS93-71B) international *P. tritici-repentis* isolates (Figure 2.10) reported to be positive for the *ToxA* gene. No PCR products were obtained from the genomic DNA of the *P. tritici-repentis* isolates Alg3-24, AB39-2, AlgH2 (not shown) and 90-2 (not shown) using the *ToxA* specific primers (Figure 2.9). The PCR products of three randomly selected isolates were sequenced and the BLAST analysis confirmed that the products amplified were the Ptr *ToxA* encoding gene *ToxA* (HM234161.1) (Appendix C.1).

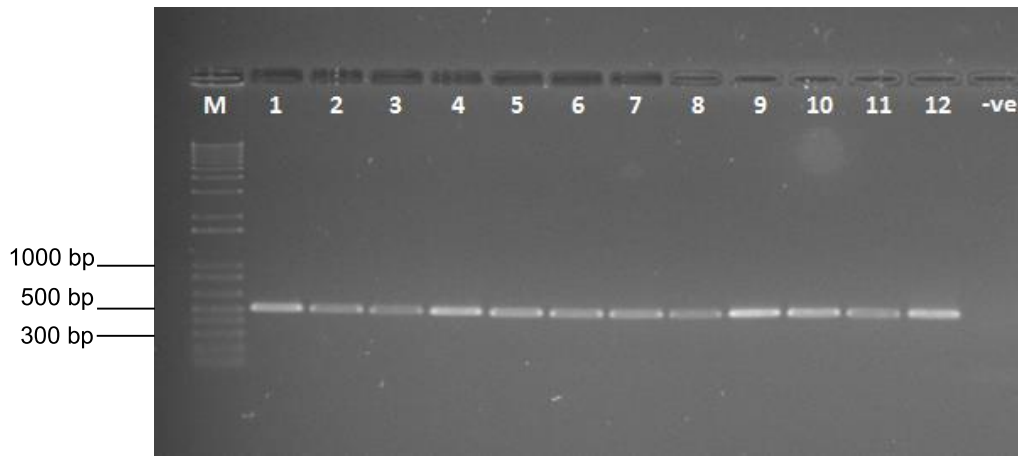


Figure 2.9: 1% agarose gel of PCR products generated by *ToxA*-specific ToxAscreeningF/ToxAscreeningR primers using the genomic DNA of 12 New Zealand single spore isolates of *Pyrenophora tritici-repentis*. From left to right, lanes 1-12 are *P. tritici-repentis* isolates S12a, S22a, S26a, M13c, M14d, G14a, G22a, F12d, W12a, W12c and K16a. M = 1 kb plus DNA ladder (Invitrogen), - ve = negative control.

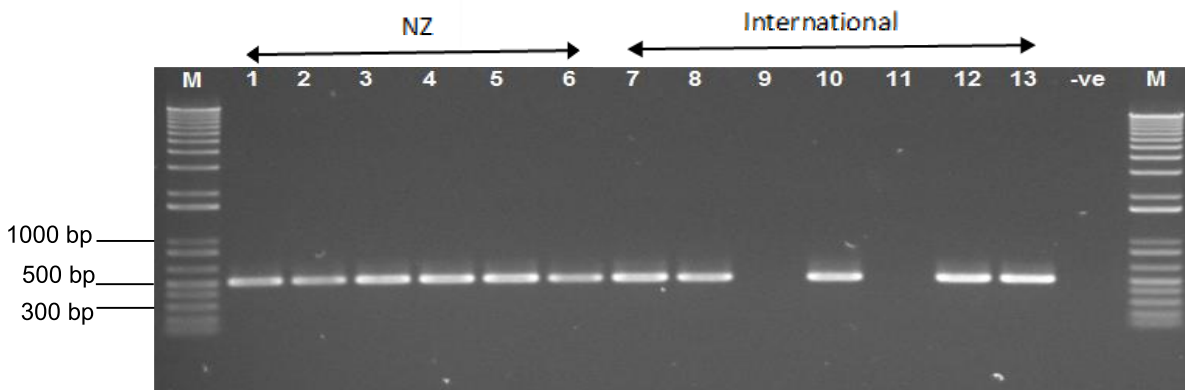


Figure 2.10: 1% agarose gel of PCR products generated by *ToxA*-specific ToxAscreeningF/ToxAscreeningR primers using the genomic DNA of New Zealand and international *Pyrenophora tritici-repentis* single spore isolates. From left to right, lanes 1 to 13 are *P. tritici-repentis* isolates S12a, S26a, G14a, G22a, F12d, K16a, ASC1, AB47-10, Alg3-24, AB33-1, AB39-2, AB39-8 and TS93-71B. M = 1 kb plus DNA ladder (Invitrogen), - ve = negative control.

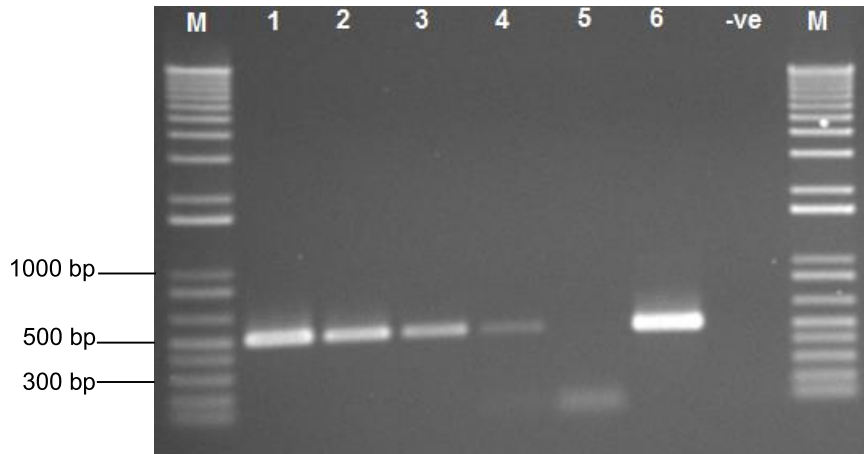


Figure 2.11: 1% agarose gel of PCR products generated by *ToxA*-specific ToxAscreeningF/ToxAscreeningR primers using the genomic DNA of New Zealand and international *Pyrenophora tritici-repentis* single spore isolates. From left to right, lanes 1 to 6 are *P. tritici-repentis* isolates S12a, CCDM Meck 4, CCDM 239, CCDM 134, Alg3-24 and TS93-71B. M = 1 kb plus DNA ladder (Invitrogen), - ve = negative control.

The *ToxB* specific primers amplified a band of 646 bp from the genomic DNA of the *P. tritici-repentis* isolates Alg3-24 (Race 5), TS93-71B (Race 8) and AlgH2 (Race 6). No PCR product was amplified using the *ToxB* specific primers for any of the New Zealand *P. tritici-repentis* isolates tested (Figures 2.12 and 2.13). The faint bands for isolates ASC1 (lane 7) and AB39-2 (lane 11) may be result of non-specific amplification by the primers.

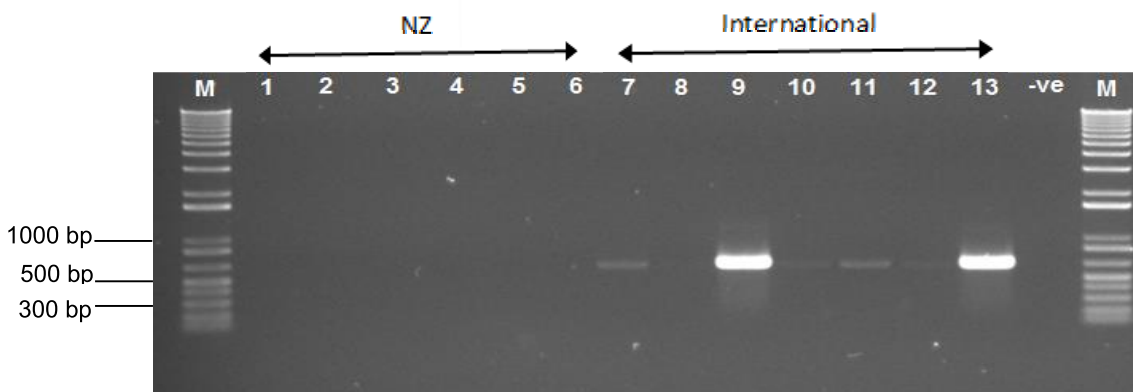


Figure 2.12: 1% agarose gel of PCR products generated by *ToxB*-specific TB10f and TB12r primers using genomic DNA of New Zealand and international *Pyrenophora tritici-repentis* single spore isolates. From left to right, lanes 1 to 13 are *P. tritici-repentis* isolates S12a, S26a, G14a, G22a, F12d, K16a, ASC1, AB47-10, Alg3-24, AB33-1, AB39-2, AB39-8 and TS93-71B. M = 1 kb plus DNA ladder (Invitrogen), - ve = negative control.



Figure 2.13: 1% agarose gel of PCR products generated by *ToxB*-specific TB10f and TB12r primers using genomic DNA of New Zealand and international *Pyrenophora tritici-repentis* single spore isolates. From left to right, lanes 1 to 13 are *P. tritici-repentis* isolates S12a, S22a, S26a, M13c, M14d, G14a, W12a, W12c, W15a, K16a, CCDM-239, CCDM-134, ASC1, CCDM-Meck4, AB39-2, 90-2, Alg3-24, AlgH2 and TS93-71B. M = 1 kb plus DNA ladder (Invitrogen), - ve = negative control.

2.4 Discussion

This study is the first investigation of the population distribution and race structure of *P. tritici-repentis* in New Zealand. Although *P. tritici-repentis* was first recorded in New Zealand in 1976 as *Drechslera tritici-repentis* (Hampton & Matthews, 1978), the identification was based on morphology with the cultures being unavailable for use in the current study. Thus, this study is the first to combine the use of molecular and morphological identification to study the population structure of *P. tritici-repentis*.

From the survey results of this study, it is clear that *P. tritici-repentis* is widely distributed throughout the South Island of New Zealand. However, whether or not *P. tritici-repentis* is also present in the North Island of New Zealand is currently unknown. To date, the confirmed distribution of *P. tritici-repentis* populations ranges from Southland, to as far as Greendale, North Canterbury covering most of the wheat growing area of the South Island. It is difficult to determine whether the present distribution pattern of *P. tritici-repentis* in the South Island was the result of a single incursion over a short period of time i.e. 2-3 years, or multiple introduction events over a long period of time i.e. 10-30 years. This will be investigated further in Chapter 3.

Geographic factors such as altitude and topography, which play important roles in influencing abiotic factors such as temperature and precipitation, may also have affected where populations of *P. tritici-repentis* were able to successfully establish and distribute themselves (Hosford *et al.*, 1990). Temperature and the availability of free moisture can greatly influence

the germination of *P. tritici-repentis* conidia. A wet period of 6-12 hours is required for successful infection, however, with an increased wet period the overall severity of disease will increase (Kadar, 2010). For example, Larez *et al.* (1986) reported that 95% conidial germination was observed at 20°C with a 6 hour period of wetness. An especially wet and cold southerly weather event may have also helped to generate favourable conditions which would have greatly increased the long distance spread and rate of infection of wheat plants with tan spot during the 2012-2013 and 2013-2014 growing season in the South Island. Schilder & Bergstrom (1992) reported that viable conidia of *P. tritici-repentis* have the potential to be spread over long distances without infested residues. In the current study, one of the wheat farms surveyed where tan spot was successfully recovered had never observed tan spot disease until the 2013-2014 season. In addition to this, tan spot wasn't identified in the infected paddock until after a strong southerly storm in late 2013.

Cultural factors, such as differences in farmer management practices, may have also played a significant role in determining where *P. tritici-repentis* was able to establish. For example, results from the questionnaire from the farmers where *P. tritici-repentis* populations were recovered revealed that there were variations in the cultural practices that were implemented to manage the infected wheat paddocks throughout the duration of the wheat growing season. These included differences in post-harvest management practices e.g. burning or direct drill and rates and classes of fungicides selected to control the outbreak of tan spot. Some of these management practices may have either helped to promote, or prevent, the ability of *P. tritici-repentis* to initiate infestations in the wheat paddocks.

Two factors that were highlighted by the questionnaire to have potentially contributed to tan spot disease were the continuous cropping of wheat in a paddock or where seed from a previous crop was used. The one farm where wheat was continuously sown for over 10 years in the same paddock was observed to have an extremely high disease severity, while for the two farms where own seed was reused disease severity/incidence was also high. It is well known that one source of inoculum for tan spot disease is seed infected with *P. tritici-repentis* (Lamari & Bernier, 1995). It has been hypothesized that seed infected with *P. tritici-repentis* may have played a major role in allowing the fungus to be spread between wheat growing regions on different continents around the world (dos Santos *et al.*, 2002; Singh & Hughes, 2006; Gurung *et al.*, 2013). Schilder & Bergstrom (1995) found that wheat seed infected with *P. tritici-repentis* was a notable source for tan spot epidemics and the long distance dispersal of *P. tritici-repentis* isolates to new areas. To date, whether or not *P. tritici-repentis* was able to spread to new wheat farms via transmission on infected wheat seeds is unknown and therefore requires further investigation. However, it should be noted that the first published report of *P. tritici-repentis* in New Zealand was where it was isolated

from certified wheat seed in 1976 (Hampton & Matthews, 1978). This would further suggest that tan spot can be spread between wheat farms via seed transmission in New Zealand.

There was also a number of farms, which did not complete the questionnaire, which reported that they direct drilled their wheat crops, rather than burn or plough their stubble after harvest. Over the last 10 years there has been a growing trend for New Zealand cereal farmers switching from conventional cultural practices such as ploughing and stubble burning, to more conservative practices such as direct drilling (Williams *et al.*, 2013). These conservative practices can greatly enhance the survival of necrotrophic fungi like *P. tritici-repentis* in the paddock post-harvest (Perello *et al.*, 2003). If wheat is continuously cultivated, the likelihood and severity of a subsequent tan spot infestation in the following wheat growing seasons can be increased (Summerell & Burgess, 1989).

A study conducted by Summerell & Burgess (1989) investigated the factors which influence the survival of *P. tritici-repentis* after the harvest of wheat. It was found that the incorporation of wheat stubble was inimical to the survival of *P. tritici-repentis* and production of pseudothecia, with *P. tritici-repentis* being recovered infrequently from stubble buried for 26 weeks. In contrast, *P. tritici-repentis* recovery was only reduced by 50% when stubble was retained on the surface of the soil. Sutton & Vyn (1990) reported that there was a positive correlation between the severity of tan spot disease and the amount of infected wheat residues which was present in a field.

All 12 New Zealand isolates, randomly selected to represent the geographic distribution of the pathogen in this study, contained the *ToxA* gene but none had the *ToxB* gene. Based on these results, the New Zealand populations of *P. tritici-repentis* tested could be classified as either virulence races 1 (Ptr ToxA + Ptr ToxC), or 2 (Ptr ToxA) (Aboukhaddour *et al.*, 2013). Thus, the New Zealand *P. tritici-repentis* population race structure appears to be similar to that reported for Australian *P. tritici-repentis* populations, which are also yet to be tested for *ToxC* (Antoni *et al.*, 2010). However, it should be noted that Antoni *et al.* (2010) reported that a low frequency (2%) of Australian *P. tritici-repentis* isolates have been found to possess *ToxB*. Unfortunately, the gene(s) which encode for the production of Ptr ToxC are currently unknown (Faris *et al.*, 2013), and therefore their presence could not be determined using PCR.

International *P. tritici-repentis* isolates, with different geographic origins, known to be positive or negative for either *ToxA* or *ToxB*, were also included in this study to verify that the PCR for *ToxA* and *ToxB* had worked. When the New Zealand isolates were compared to the international *P. tritici-repentis*, only eight of the twelve international isolates had a *ToxA* band. In contrast, no *ToxA* products were obtained from the genomic DNA of the *P. tritici-repentis* isolates Alg3-24, AlgH2, 90-2 and AB39-2. This result was expected as Alg3-24, AB39-2, AlgH2 and 90-2 are reported to be members of the virulence races 5 (Ptr ToxB), 3 (Ptr ToxC),

6 (Ptr ToxB and Ptr ToxC) and 4 (no HST) respectively, which do not possess the *ToxA* gene (Lamari *et al.*, 2003; Faris *et al.*, 2013). For all the international isolates screened, only Alg3-24, AlgH2 and TS93-71B possessed *ToxB*. However, non-specific amplification by primers, low DNA quality or DNA not being sourced from single spore isolates resulted in faint bands being produced with *ToxB* primers for isolates ASC1 and AB39-2.

The New Zealand populations of *P. tritici-repentis* screened in this study were observed in the field of being capable to induce chlorosis on all of the wheat cultivars sampled. However, whether this observation was indeed the result of a Ptr ToxC-sensitive wheat reaction, or just an indirect cultivar specific reaction to *P. tritici-repentis* requires further investigation. The development of Ptr ToxC-specific PCR primers would greatly facilitate the elucidation and identification of the race(s) the New Zealand populations of *P. tritici-repentis* may be classified as in future. As a result, further work is required in order to investigate and verify whether the populations of *P. tritici-repentis* do indeed possess the ability to produce Ptr ToxC. However, it has also been suggested by Andrie *et al.* (2007) that it may not be possible to accurately identify the race structure of a *P. tritici-repentis* population with just a molecular-based system. These authors suggested an established wheat differential system, which were currently not available in New Zealand.

The results obtained in this study are consistent with the findings of a range of previous studies, in which races 1 and 2 are the predominant *P. tritici-repentis* races present in wheat growing areas in Australia (Antoni *et al.*, 2010), Canada (Lamari *et al.*, 1998; Lamari *et al.*, 2005; Aboukhaddour *et al.*, 2013), USA (Friesen *et al.*, 2005) and South America (Gamba *et al.*, 2012). Antoni *et al.* (2010) reported that 119 Australian *P. tritici-repentis* isolates were positive for *ToxA*, whilst none were positive for *ToxB*. Similarly, Aboukhaddour *et al.* (2013) reported that from a total of 45 Canadian *P. tritici-repentis* isolates tested, 62% were classified as race 1 (Ptr ToxA + Ptr ToxC), 36% as race 2 (Ptr ToxA) and 2% as race 3 (Ptr ToxC).

The results of this study also imply that the *ToxA-Tsn1* gene interaction must be an important factor in the pathogenicity relationship between *P. tritici-repentis* and wheat in New Zealand. It is well understood that a dominant allele located on the *Tsn1* locus is responsible for conferring and determining the sensitivity of a wheat line to Ptr ToxA (Haen *et al.*, 2003; Strelkov & Lamari, 2003; Faris *et al.*, 2005; Faris *et al.*, 2013). Thus, it can be assumed that the majority of the New Zealand wheat cultivars sampled in this study must possess this dominant *Tsn1* gene. This is due to the *ToxA* possessing isolates of *P. tritici-repentis* screened in the current study being recovered from plants which were expressing necrosis in the field. From these results, it could also be postulated that the continued introduction of wheat lines possessing the *Tsn1* locus over the last 50 years, combined with favourable weather conditions, may have helped to promote the fast appearance and spread of *P. tritici-repentis* throughout the South Island during the 2013-2014 season. Friesen *et al.* (2006) proposed that

the sudden emergence and increase in severity of tan spot in the early 1940s was the result of *P. tritici-repentis* acquiring the *ToxA* gene from *P. nodorum* as a result of an interspecific horizontal gene transfer event as recently as 1941. However, the likelihood that an increase in the frequency of *Tsn1* gene possessing cultivars was a significant factor in the sudden emergence and spread of tan spot in New Zealand is unlikely. This is due to the frequency and trend of *Tsn1* possessing wheat cultivars being introduced over time not being found to be significantly linked to the emergence and spread of tan spot disease overseas.

A recent study by Oliver *et al.* (2007) investigated whether the emergence and establishment of tan spot disease in Australia was the result of widespread planting of *Tsn1* wheat lines. To investigate this, wheat cultivars which were released in Australia between the years 1911 and 1986 were tested for their sensitivity to *ToxA*. It was found that the majority of wheat cultivars both released before and during the emergence and spread of tan spot in Australia were *ToxA*-sensitive. As a result, the authors concluded that the spread of tan spot could not be causally linked to the deployment of *ToxA*-sensitive cultivars. Therefore, it is much more likely that an increased frequency of *Tsn1* possessing wheat cultivars in New Zealand in recent years would have been one factor with a combination of other factors that helped to further promote the incidence of tan spot infection in the field.

The results of this study also imply that the *ToxB-Tsc2* gene interaction must be an insignificant factor determining the pathogenicity of *P. tritici-repentis* towards wheat in New Zealand. The same result was also obtained by Antoni *et al.* (2010), where it was found that the *ToxA-Tsn1* interaction was the dominating factor in the Australian *P. tritici-repentis* population, with the *ToxB-Tsc2* being an insignificant factor.

This result could have important practical implications for how the breeding of wheat in New Zealand is approached in future. For example, New Zealand wheat breeders could selectively breed wheat lines which lack the *Tsn1* gene in order to confer resistance to *ToxA* possessing isolates of *P. tritici-repentis* (Faris *et al.*, 2013). By reducing the amount of wheat lines possessing *Tsn1* it may be possible to manage and reduce the incidence of tan spot infection. However, currently the susceptibility of wheat cultivars commonly grown in New Zealand to tan spot is not known and this will be investigated in Chapter 5.

Despite all of the New Zealand *P. tritici-repentis* isolates screened in this study possessing *ToxA* and the severity of tan spot epidemics increasing in recent years, the results obtained in this study do not indicate whether the *P. tritici-repentis* isolates studied were part of a recent single incursion or multiple incursions. The genetic diversity and potential origin of the New Zealand *P. tritici-repentis* isolates in this study will be investigated in Chapter 3. E

2.5 Conclusions

This study represents the first major attempt to investigate the distribution and characterise the race structure of *P. tritici-repentis* in the South Island of New Zealand. At present, *P. tritici-repentis* is widely distributed throughout the South Island of New Zealand, with its estimated range currently stretching from Clinton, Southland to as far as Greendale North Canterbury. A survey of the farms sampled for *P. tritici-repentis* during the 2013-2014 wheat growing season revealed that the re-use and sowing of untreated wheat seed and continuous sowing of wheat over 10-20 were potentially responsible for exacerbating tan spot infestations. The New Zealand *P. tritici-repentis* isolates were positive for Ptr ToxA, but negative for Ptr ToxB. At present, the New Zealand *P. tritici-repentis* isolates tested could be classified as either virulence races 1, or 2, which are the predominate virulence races reported worldwide to infect wheat crops. Further work is needed to investigate and verify whether New Zealand populations of *P. tritici-repentis* possess the ability to produce Ptr ToxC in order to accurately characterise the race structure of New Zealand populations. Currently, the *ToxA-Tsn1* interaction is a dominating factor in the New Zealand *P. tritici-repentis*-wheat interaction, with the *ToxB-Tsc2* being currently an insignificant factor in the *P. tritici-repentis*-wheat interaction.

Chapter 3

Determination of the genetic diversity of *Pyrenophora tritici-repentis* in New Zealand

3.1 Introduction

The genetic diversity of a fungal population is an extremely important feature of its biology. This is because a detailed knowledge of the genetic diversity of a pathogen population can provide information about its origin, dispersal and provide information relevant to strategies for its control (Reeleder, 2003). For example, knowledge of a fungal pathogen's genetic structure can have important implications for programs designed to screen the efficacy of new fungicides and plant cultivars (Leisova *et al.*, 2005). If a fungal population possesses a large degree of genetic variation it is capable of evolving rapidly in response to changes in its surrounding environment. It may also overcome more easily the pressures generated by the use of fungicides and the resistance of host plants (Singh & Hughes, 2006). In contrast, a fungal population which possesses a low degree of genetic variation is likely to adapt and evolve more slowly in response to environmental pressures.

With regards to genetic studies of fungal populations, the fungus *P. tritici-repentis* is an interesting pathogen to study. For example, it is a homothallic fungus within a genus which is predominately composed of heterothallic fungal species, most notably the barley pathogen *Pyrenophora teres* f. *teres* (Leisova *et al.*, 2005; Aboukhaddour *et al.*, 2011). In addition, it is also the only fungus in the genus *Pyrenophora* which is found worldwide, even in the regions where wheat is not cultivated (Aboukhaddour *et al.*, 2011).

Another important aspect of the biology and genetics of *P. tritici-repentis* is that, in addition to infecting wheat, it can infect a wide range of grass species (Aboukhaddour *et al.*, 2011). At present, it is reported that *P. tritici-repentis* has the widest host range of all of the species in the genus *Pyrenophora*, to date it is known to infect 26 different grass species (Singh *et al.*, 2010; Aboukhaddour *et al.*, 2011).

Like many important wheat pathogens, such as *M. graminicola* and *P. nodorum*, the biology and genetic structure of *P. tritici-repentis* is currently well understood. As such, it has been recognized that *P. tritici-repentis* has an enormous variability in its pathogenicity, morphology and population genetics (dos Santos *et al.*, 2002). To date, the results of a number of molecular population studies have shown that the global population structure of *P. tritici-repentis* is diverse in nature (dos Santos *et al.*, 2002; Aboukhaddour *et al.*, 2011; Manning *et al.*, 2013).

For example, research by Friesen *et al.* (2005) using an AFLP method to examine 97 *P. tritici-repentis* isolates collected from throughout North America, South America and Europe, revealed that there were no apparent genetic groupings of isolates based on their races, or point of geographic origin. Singh & Hughes (2006) utilised a RAPD method to detect genetic variability and establish the relationships between genetic similarity, race classification and geographical origin among 33 different isolates of *P. tritici-repentis*. Results revealed that all of the isolates tested had unique banding patterns, with the clustering being independent of their race classification or geographic origin.

In a more recent study, Gurung *et al.* (2013) used 12 microsatellite markers to analyse and compare 439 *P. tritici-repentis* isolates collected from five different continents. Results showed that there was a high level of genetic diversity present and a moderate to high population differentiation between continents. A common trend discovered in the results of the studies highlighted, was that the researchers were unsuccessful in grouping the detected genotypes of *P. tritici-repentis* based on their point of origin, or race classification.

At present, the genetic structure of *P. tritici-repentis* populations in New Zealand is uncharacterised. Thus, there is a need for a comprehensive study to determine whether the genetic structure of *P. tritici-repentis* populations present in New Zealand wheat growing regions is either more diverse or more clonal than overseas populations. In addition to this, other tools such as UP-PCR have not been used to characterise *P. tritici-repentis* populations. The aim of this study was to investigate and determine the genetic diversity of *P. tritici-repentis* populations in New Zealand. Three different methods to investigate genetic diversity, being UP-PCR, RAPD and microsatellites, were used.

3.2 Materials and Methods

3.2.1 Selection of *Pyrenophora tritici-repentis* populations and isolates

Twelve single spore *P. tritici-repentis* isolates were selected for analysis of genetic diversity from the *P. tritici-repentis* populations collected as part of the survey. Isolates were initially selected to best represent the geographic distribution of *P. tritici-repentis* to enable the inter-paddock and inter-regional genetic diversity to be determined. The New Zealand *P. tritici-repentis* isolates were S12a, S22a, S26a, M13c, M14d, G14a, G22a, F12d, W12a, W12c, W15a and K16a (Appendix B.2). To enable comparison with international populations the DNA of twelve international *P. tritici-repentis* isolates (90-2, ASC1, AB47-10, Alg3-24, AlgH2, AB33-1, AB39-2, AB39-8, TS93-71B, CCDM-Meck4, CCDM-239 and CCDM-134) were

obtained (Appendix B.4). The Australian isolates (CCDM-Meck4, CCDM-239 and CCDM-134) were kindly provided by Dr Caroline Moffat (Curtin University, Perth, Australia) whilst the other international isolates were kindly provided by Dr Reem Aboukhaddour (University of Alberta, Canada).

3.2.2 Genomic DNA extraction

Twelve *P. tritici-repentis* single spore isolates were selected based on their geographic origin in order to best represent the distribution of the current *P. tritici-repentis* population. The isolates were grown on potato dextrose broth (PDB) (24 g PDB and L distilled water) and incubated for four days at 20°C in continuous darkness. The mycelium was harvested following the method described in Section 2.2.6.1.

Genomic DNA of each isolate was extracted from the mycelium using the plant tissue DNA isolation protocol of the PUREGENE® genomic DNA isolation kit (Gentra systems, USA) following the method described in Section 2.2.6.1.

DNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, USA). All *P. tritici-repentis* genomic DNA samples were diluted to a working concentration of 10-20 ng/ µL for UP-PCR, RAPD and microsatellite PCR reactions.

3.2.3 Genetic variation analysis using UP-PCR method

3.2.3.1 Testing of primers

Five UP-PCR primers (AA2M2, Fok1, L45, L15 and AS15inv) were selected from a total of 11 available UP-PCR primers for the detection of genetic diversity in 12 *P. tritici-repentis* single spore isolates. In addition, three of the five selected UP-PCR primers (AA2M2, Fok1 and AS15 inv) were tested using the genomic DNA of two representative New Zealand isolates and seven international isolates in order compare New Zealand isolate banding patterns with international isolates. Primers were selected on the basis of the ones that had given the greatest number of polymorphic bands in previous studies on other pathogens (Obanor *et al.*, 2010; Baskaratheven *et al.*, 2012; Pathrose *et al.*, 2014). The sequences of the UP-PCR primers and their respective annealing temperatures are listed in (Table 3.1).

Table 3.1: Sequence and annealing temperature of UP-PCR primers used in this study.

| Primer name | Primer sequence | Annealing (°C) | Reference |
|-------------|------------------------------------|----------------|------------------------------|
| AA2M2 | 5'CTGCGACCCAGAGCGG ^{3'} | 50 | Lübeck <i>et al.</i> , 1998 |
| AS15inv | 5'CATTGCTGGCGAATCGG ^{3'} | 52 | Cumagun <i>et al.</i> , 2000 |
| L15 | 5'GAGGGTGGCGGTTCT ^{3'} | 52 | Tyson <i>et al.</i> , 2002 |
| L45 | 5'GTAAAACGACGGCCAGT ^{3'} | 51 | Bulat <i>et al.</i> , 1994 |
| Fok1 | 5'GGATGACCCACCTCCTAC ^{3'} | 52 | Lübeck <i>et al.</i> , 1999 |

3.2.3.2 UP-PCR

Each UP-PCR was done in a 25 µl reaction volume. Each PCR contained 1x PCR buffer (Roche Diagnostics, Basel, Switzerland), 200 µM each of dGTP, dCTP, dATP and dTTP, 20 pmol of UP-PCR primer, 2.5 mM MgCl₂, 1.25 U FastStart *Taq* polymerase (Roche Diagnostics, Mannheim, Germany) and 10-20 ng template DNA of one of the *P. tritici-repentis* isolates. A negative control with 1 µL sterile water instead of template DNA was also included in the PCR in order to ensure there was no contamination of the PCR reagents.

PCR amplifications were performed in a Veriti Thermal Cycler-200 as follows: an initial denaturation at 94°C for 5 minutes, 5 cycles of denaturation at 94°C for 50 seconds, annealing at respective primer temperature (Table 3.1) for 2 minutes and an extension at 72°C for 1 minute. This was followed by 34 cycles of denaturation at 94°C for 50 seconds, annealing at respective primer temperature (Table 3.1) for 90 seconds, and an extension at 72°C for 1 minute, with a final extension of 72°C for 10 minutes.

3.2.3.3 Electrophoresis of UP-PCR products

All UP-PCR amplification products were separated on a 1% agarose gel (1 g agarose powder and 100 mL 1xTris-Acetate EDTA buffer). Ten µL of PCR product was combined with 3 µL loading dye (40% (w/v) sucrose; 0.25% bromophenol blue; 0.25% xylene cyanol) before loading into wells. Eight µL of 1kb plus DNA ladder (0.1 µg / µL; Invitrogen) was combined with 3 µL loading dye and used as a molecular weight marker. The PCR products were run at 100 volts (at 10 V/cm) for 50 minutes in a 1 x TAE buffer. The agarose gels were stained and photographed as described in Section 2.2.6.2.

3.2.4 Genetic variation analysis using RAPD

3.2.4.1 Selection of RAPD primers

The RAPD primers UBC 517, UBC 598, UBC 600 and Operon H-19 used by Singh & Hughes (2006) were selected for use in this study. Primers were selected on the basis of the number of polymorphic bands for which they had produced. The sequences of the RAPD primers are listed in Table 3.2.

Table 3.2: Name and sequence of RAPD primers used in this study (from Singh & Hughes, 2006).

| Primer name | Primer sequence |
|-------------|----------------------------|
| UBC 517 | 5'GGTCGCAGCT ^{3'} |
| UBC 598 | 5'ACGGGCGCTC ^{3'} |
| UBC 600 | 5'GAAGAACCGC ^{3'} |
| Operon H-19 | 5'CTGACCAGCC ^{3'} |

3.2.4.2 RAPD

PCR was done in a 25 µl reaction volume. The reaction mix was as described in Section 3.2.3.2 except that the 20 pmol of UP-PCR primer was replaced with 10 pmol of RAPD primer.

The PCR amplifications were performed in a Veriti Thermal Cycler-200 as follows: an initial denaturation at 94°C for 6 minutes followed by 45 cycles of denaturation at 92°C for 1 minute, an annealing temperature of 36°C for 1 minute and an extension at 72°C for 1 minute, and a final extension cycle was 72°C for 6 minutes.

3.2.4.3 Electrophoresis of RAPD products

All RAPD amplification products were separated by electrophoresis on a 1.3% agarose gel (1.3 g agarose powder and 100 mL 1xTris-Acetate EDTA buffer). PCR products and 1 kb plus ladder were mixed with loading dye as described in Section 2.2.6.2. The PCR products were run at 100 volts (at 10 V/cm) for 50 minutes in a 1 x TAE buffer. The agarose gels were stained and photographed as described in Section 2.2.6.2.

3.2.5 Genetic variation analysis using microsatellites

3.2.5.1 Selection of microsatellite primers

A total of three microsatellite primer pairs used by Gurung *et al.* (2013) were selected for the detection of genetic diversity in New Zealand and international *P. tritici-repentis* single spore isolates. Primers were selected on the basis of the number of polymorphic bands for which they had amplified in Gurung *et al.* (2013). The sequences of the microsatellite primers are listed in Table 3.3.

Table 3.3: Name and sequence of microsatellite primers used in this study (from Gurung *et al.*, 2013).

| Primer name | Primer sequence |
|-------------|---------------------------------|
| PtrSSR01 | F 5' TTTGGGAGATGGGGAAA 3' |
| | R 5' TTGCGTCTGTGCGACATG 3' |
| PtrSSR12 | F 5' AAGAGGTGTCTCGACTAGCGTTT 3' |
| | R 5' GGCTTAATTTAAGCGCGTG 3' |
| PtrSSR13 | F 5' TCGTGGGTATAAAACGGCTCT 3' |
| | R 5' TTCGGCGGCTTCATATTACT 3' |

3.2.5.2 Microsatellite PCR

Each PCR was done in a 25 µl reaction volume. The reaction mix was as described in Section 3.2.3.2 except that 20 pmol of UP-PCR primer was replaced with 10 pmol of microsatellite primer.

The PCR amplifications were performed in a Veriti Thermal Cycler-200 as follows: an initial denaturation at 94°C for 5 minutes followed by 32 cycles of denaturation at 95°C for 30 seconds, an annealing temperature of 55°C for 20 seconds and an extension at 72°C for 30 seconds, and a final extension cycle was 72°C for 10 minutes.

3.2.5.3 Electrophoresis of microsatellite products

All microsatellite amplification products were initially run and separated by electrophoresis on a 1.3% agarose gel (1.3 g agarose powder and 100 mL 1xTris-Acetate EDTA buffer). PCR

products and 1 kb plus DNA ladder were mixed with loading dye as described in Section 2.2.6.2. The PCR products were run at 100 volts (at 10 V/cm) for 1 hour in a 1 x TAE buffer. The agarose gels were stained and photographed as described in Section 2.2.6.2.

3.3 Results

3.3.1 Characterisation of South Island *Pyrenophora tritici-repentis* isolates using UP-PCR

Examples of UP-PCR fingerprint profiles generated with all twelve South Island *P. tritici-repentis* isolates and different UP-PCR primers are shown in Figure 3.1. Generated bands for all primers ranged from approximately 350 bp to 2500 bp. Among the tested primers, no polymorphic bands were generated. Poor amplification of isolate DNA occurred in lanes 3 and 12 due to low quality DNA. In addition, a small amount of amplification occurred in the negative control due to contamination, however, bands do not align with any of the amplification bands of the isolates tested.

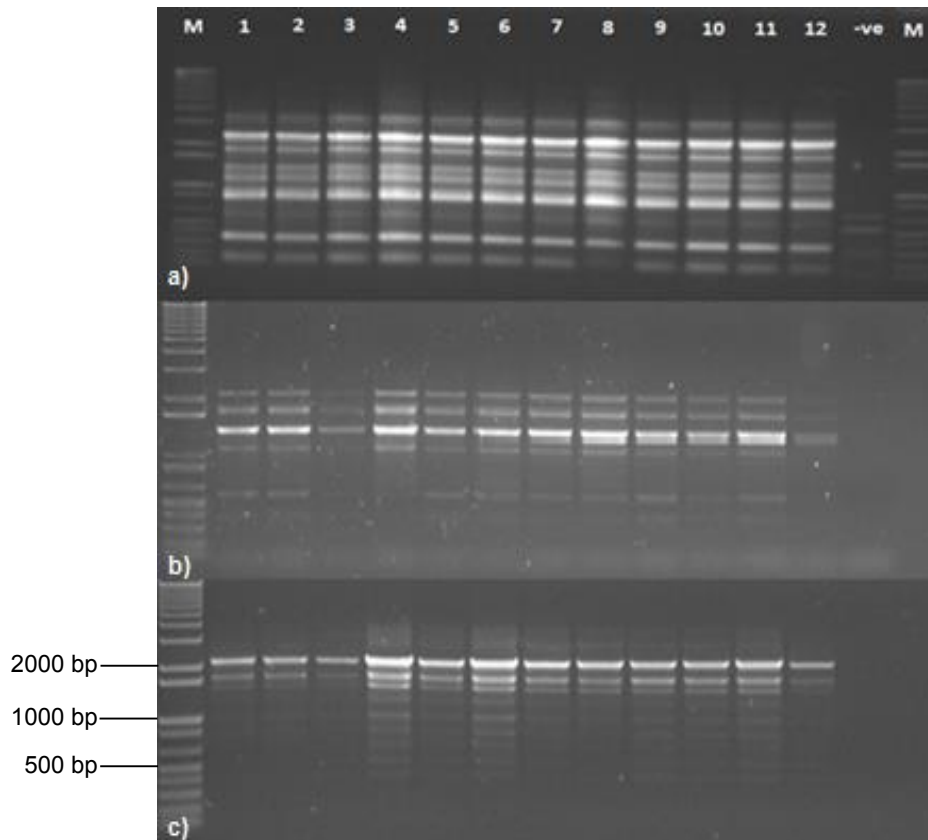


Figure 3.1: 1% agarose gels of UP-PCR fingerprints from genomic DNA of 12 *Pyrenophora tritici-repentis* South Island single spore isolates. a) AA2M2 primer, b) AS15inv primer and c) Fok1. From left to right *P. tritici-repentis* single spore isolates are S12a, S22a, S26a, M13c, M14d, G14a, G22a, F12d, W12a, W12c and K16a. M = 1 kb plus DNA ladder (Invitrogen), -ve = negative control.

3.3.2 Characterisation of international and New Zealand *Pyrenophora tritici-repentis* populations using UP-PCR

Examples of UP-PCR fingerprint profiles generated by each UP-PCR primer are shown in Figure 3.2. Generated bands for all primers ranged from approximately 350 bp to 2500 bp. Among the tested primers, no polymorphic bands were generated for any of the New Zealand or international *P. tritici-repentis* isolates tested (Table 3.4). Poor amplification occurred in lanes 3 and 9 due to low quality DNA.

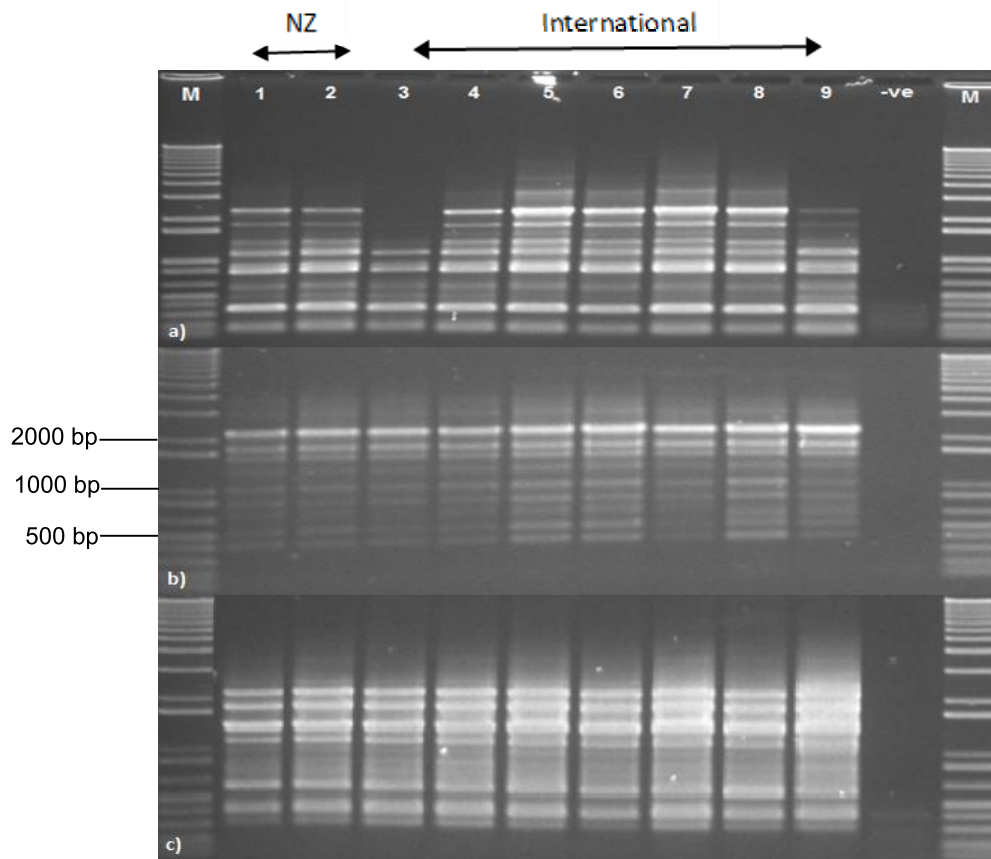


Figure 3.2: 1% agarose gels of UP-PCR fingerprints generated by genomic DNA of two New Zealand single spore isolates and seven *Pyrenophora tritici-repentis* international isolates. a) AA2M2 primer, b) Fok1, c) AS15 inv primer. From left to right, lanes 1-9 are S12a, G14a, ASC1, AB47-10, AB33-1, Alg3-24, AB39-2, AB39-8, TS93-71B. M = 1 kb plus DNA ladder (Invitrogen), - ve = negative control.

3.3.3 Characterisation of New Zealand and international *Pyrenophora tritici-repentis* populations using RAPD

A total of nine genotypes were detected from the four RAPD primers utilised across all *P. tritici-repentis* assessed (Table 3.4). Generated bands ranged from approximately 400 bp to 2000 bp. No polymorphic bands were generated for any of the New Zealand *P. tritici-repentis* isolates (S12a, S26a, M13c, G14a, G22a, F12d and K16a) assessed (Figure 3.3). Polymorphic bands were generated by the international isolates Alg3-24, AB47-10, AB33-1, AB39-8, AB39-2, CCDM-Meck4, CCDM-134 and TS93-71B with different RAPD primers (Figure 3.3). In contrast, the international *P. tritici-repentis* isolates ASC1 and CDMM-239 had similar fingerprints to the New Zealand isolates which varied depending on the primer utilised. Due to poor DNA quality, bands in lane 9 were not clearly visible. Inverted darkened photos of primer fingerprints were also used to help distinguish poorly visible bands (Appendix C.2).

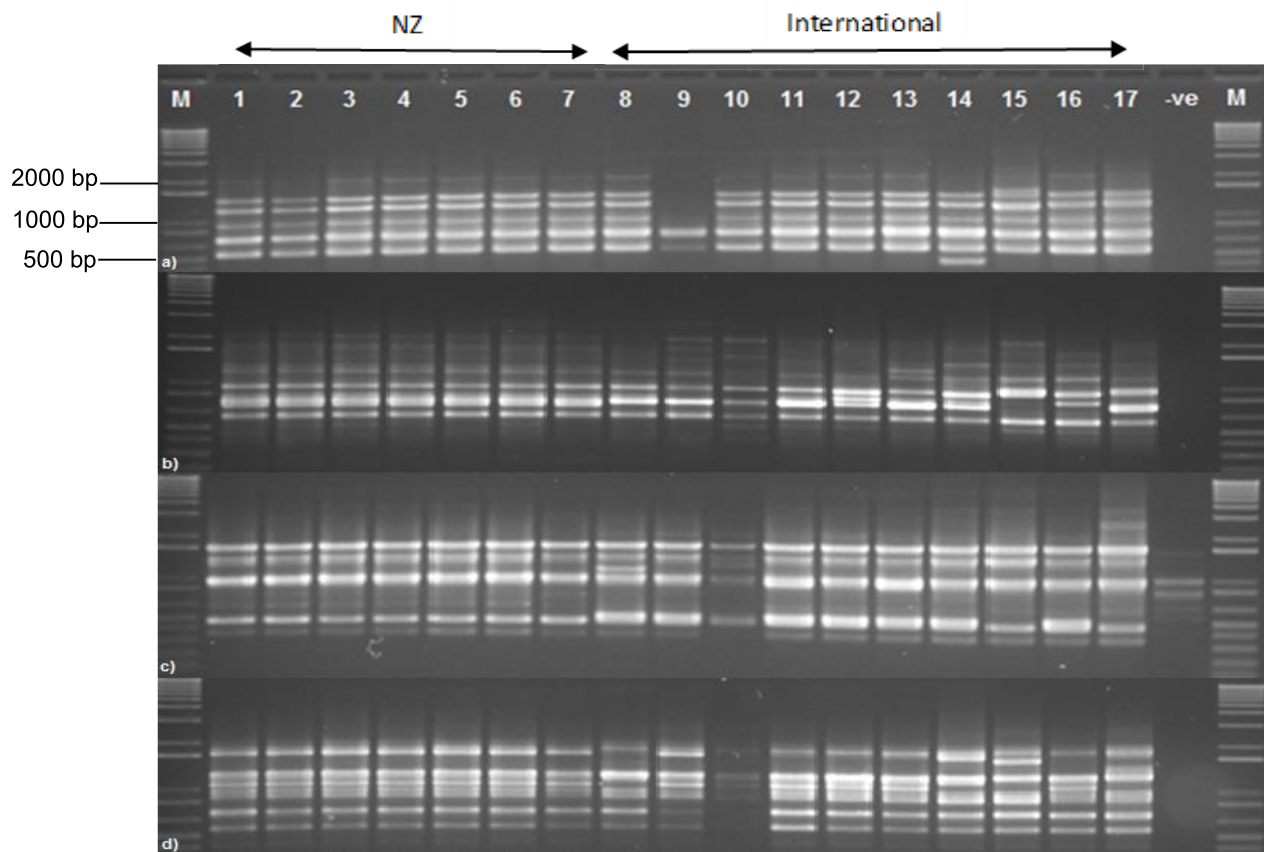


Figure 3.3: 1.3% agarose gels of RAPD fingerprints generated by genomic DNA of New Zealand and international *Pyrenophora tritici-repentis* isolates. a) UBC598 primer, b) UBC600 primer, c) Operon H-19 primer and d) UBC517 primer. From left to right, lanes 1-17 are *P. tritici-repentis* isolates S12a, S26a, M13c, G14a, G22a, F12d, K16a, CCDM Meck4, CCDM 239, CCDM 134, ASC1, AB47-10, AB33-1, Alg3-24, AB39-2, AB39-8, TS93-71B. M = 1 kb plus DNA ladder, - ve = negative control.

Table 3.4: Genotypes detected by UP-PCR primers AA2M2, AS15inv and Fok1, RAPD primers UBC598, UBC600, Operon H-19 and UBC517 and microsatellite primers SSRPtr01, SSRPtr12 and SSRPtr13.

| Isolate | Genotype group | UP-PCR** | RAPD primers | | | | Microsatellite primers | | |
|-------------------|----------------|----------|--------------|--------|-------------|--------|------------------------|----------|----------|
| | | | UBC598 | UBC600 | Operon H-19 | UBC517 | SSRPtr01 | SSRPtr12 | SSRPtr13 |
| S12a* | 1 | A | A | A | A | A | A | A | A |
| CCDM Meck4 | 2 | NT | A | A | B | B | B | B | B |
| CCDM-239 | 3 | NT | A | A | A | A | C | C | C |
| CCDM-134 | 4 | NT | A | A | C | A | NT | NT | NT |
| ASC1 | 5 | A | A | A | A | A | D | D | D |
| AB47-10 | 6 | A | A | B | A | A | E | E | E |
| AB33-1 | 7 | A | A | C | A | A | F | F | F |
| Alg3-24 | 8 | A | B | D | A | C | G | G | G |
| AB39-2 | 9 | A | C | E | D | D | H | H | H |
| AB39-8 | 10 | A | A | F | A | A | I | I | I |
| TS93-71B | 11 | A | A | G | D | E | J | J | J |
| AlgH2 | 12 | NT | NT | NT | NT | NT | K | K | K |
| 90-2 | 13 | NT | NT | NT | NT | NT | L | L | L |

NT not tested

* Representative of all New Zealand isolates which were genetically identical

** Primers AA2M2, AS15inv and Fok1

3.3.4 Characterisation of New Zealand and international *Pyrenophora tritici-repentis* populations using microsatellites

The microsatellite primer SSRPtr13 amplified a band of approximately 400 bp from genomic DNA of all New Zealand isolates. The primers SSRPtr12 and SSRPtr01 amplified a band of approximately 300 bp from the genomic DNA of all New Zealand isolates. Bands ranging from approximately 200 to 300 bp were amplified by all primers from the genomic DNA of all international isolates (Figure and Table 3.4). No polymorphic bands were generated for any of the New Zealand *P. tritici-repentis* isolates. Polymorphic bands were generated for all international *P. tritici-repentis* isolates with all microsatellite primers.

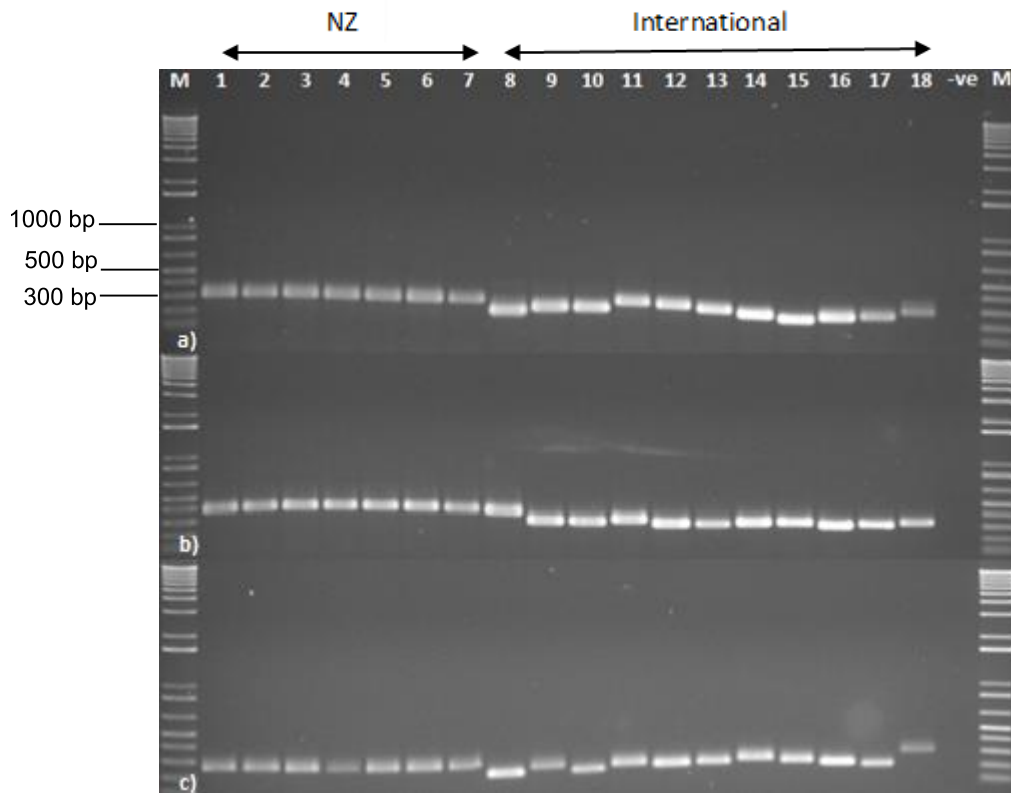


Figure 3.4: 1.3% agarose gels of microsatellite fingerprints generated by genomic DNA of New Zealand and international *Pyrenophora tritici-repentis* isolates. a) SSRPtr12 primer, b) SSRPtr13 primer and c) SSRPtr01 primer. From left to right, lanes 1-18 are *P. tritici-repentis* isolates S12a, S26a, G22a, W15a, W12c, M13c, K16a, 90-2, ASC1, AB47-10, AB33-1, Alg3-24, AlgH2, AB39-2, AB39-8, TS93-71B, CCDM Meck4 and CCDM 239,. M = 1 kb plus DNA ladder, - ve = negative control.

3.4 Discussion

The principal aim of this study was to characterise and determine the genetic diversity of *P. tritici-repentis* populations found in the South Island of New Zealand. In addition to being the first study to characterise New Zealand populations of *P. tritici-repentis*, this study is also represented the first attempt to assess the genetic diversity of *P. tritici-repentis* using UP-PCR.

For analysis of genetic diversity representative isolates were selected from different regions as this was likely to encompass the maximum diversity within the population. The results generated by the UP-PCR, RAPD and microsatellite methods showed that the 12 *P. tritici-repentis* isolates belonged to single genotype (clonal). Therefore, no more New Zealand isolates were included in this study because the UP-PCR and RAPD results strongly indicated the absence of genetic diversity within the New Zealand populations. This was further verified by the microsatellites.

The UP-PCR, RAPD and microsatellite methods detected 13 different genotypes using a total of 12 primers within all of the New Zealand and international *P. tritici-repentis* isolates. Using UP-PCR there were no polymorphic bands within the international isolates of *P. tritici-repentis*. In contrast, the RAPD method identified nine genotypes from the fingerprints generated by four primers across all *P. tritici-repentis* assessed in this study. Lastly, the microsatellite method successfully differentiated New Zealand and international *P. tritici-repentis* isolates from one another, with the New Zealand isolates genetically identical to each other. However, the amplicons were displayed on agarose and greater resolution would have occurred using polyacrylamide, potentially showing some polymorphism between isolates. Overall, based on these results it is clear that there were different outcomes produced depending on the molecular method utilised.

UP-PCR had not been applied to *P. tritici-repentis* before and was used to see if it was more informative than other methods. The method of UP-PCR is similar to genotyping methods such as RAPD because it amplifies random genetic elements distributed throughout the genome of a particular organism (Lübeck *et al.*, 1998). However, unlike RAPD, it differs due to the primers which are utilised being longer and targeting the more variable intergenic spacer (IGS) regions of the genome of a fungus (Bulat *et al.*, 1998; Pottinger *et al.*, 2002).

In the current study, no polymorphic bands were generated by the UP-PCR method despite the isolates originating from four different countries. This result indicated there was no inter-continental diversity between the New Zealand isolates and international populations tested using this method which was highly unlikely.

This result is interesting, because other studies utilizing UP-PCR in other phytopathogenic fungi have reported that the method can detect genetic diversity at varying levels. For example, a study conducted by Yli-Mattila *et al.* (1997) using UP-PCR to assess the genetic variation of 22 *Fusarium avenaceum* isolates isolated from wheat and barley in Finland showed that each isolate assessed could be distinguished from the others and that the isolates tested clustered into two main groups. In another study, Obanor *et al.* (2010) used UP-PCR to determine the genetic structure of 98 different New Zealand isolates of *Spilocaea oleagina*. They showed low levels of gene and genotypic diversity in all populations, with 76% of the loci being polymorphic. The authors concluded that the low levels of genetic diversity were a strong indication that *S. oleagina* reproduced predominately by asexual means. Lastly, a similar study by Tyson *et al.* (2002) investigated genetic diversity in 231 New Zealand and 25 international isolates of the onion white rot fungus *Sclerotium cepivorum* using eleven UP-PCR primers and was able to detect six different genetic groups. When the UP-PCR results of other studies are compared to those presented here, although relatively small in the case of Obanor *et al.* (2010) and Tyson *et al.* (2002), higher levels of genetic diversity were detected using the same primers in *P. chlamydospora*, *F. avenaceum* and *S. cepivorum* than *P. tritici-repentis*. The results of the

highlighted studies are intriguing because all the fungal pathogens studied are fungi that are known to reproduce predominantly asexually, or have rare sexual states (Pottinger *et al.*, 2002; Tyson *et al.*, 2002; Obanor *et al.*, 2010; Baskathevan *et al.*, 2012). Therefore, these fungi would not be expected to have higher levels of genetic variation than *P. tritici-repentis*, a fungus that is known to frequently undergo sexual reproduction (Aboukhaddour *et al.*, 2011).

One hypothesis to explain why more genetic diversity was detected with UP-PCR in fungi such as *S. cepivorum* than in *P. tritici-repentis*, is that it may be possible that fungi such as *S. cepivorum* and *P. chlamydospora* have more variability present in the IGS and other genes where UP-PCR primers target, than *P. tritici-repentis* (Bulat *et al.*, 1998; Pottinger *et al.*, 2002). It is possible that, in contrast to these fungi, *P. tritici-repentis* has more genetic variation present in other less conserved and variable regions of the genome where other molecular methods, such as AFLP, have been able to successfully detect genetic variation (Friesen *et al.*, 2005).

The method of RAPD was used to assess the genetic variation in *P. tritici-repentis* for this study. This method is quick and affordable and utilizes short primers which can bind to multiple sites within the genome of a target organism (Aung, 2001). In this study, the RAPD primers detected nine different genotypes. However, the method was unable to differentiate the New Zealand isolates. This further confirmed the UP-PCR results and further indicated that the genetic structure of the New Zealand isolates of *P. tritici-repentis* sampled in this study are uniform in nature. This result is in contrast to the current knowledge of the genetic structures of populations of *P. tritici-repentis* around the world that suggests this pathogen has a high degree of genetic variation between different populations.

Several studies have utilised RAPD to study the genetic diversity of *P. tritici-repentis* (Aung, 2001; Singh & Hughes, 2006; Mironenko *et al.*, 2007). In all of these studies 30-45 RAPD primers were tested and detected low to high levels of genetic diversity between different isolates of *P. tritici-repentis* from different geographic origins. A common result reported by these studies was that the most genetic diversity was observed within the isolate populations rather than between them. In addition, the researchers were unable to group the RAPD genotypes detected based on their race or geographic origins. In this study, the intra-paddock genetic diversity of the sampled *P. tritici-repentis* populations was not investigated due to no levels of genetic diversity being detected between isolates. Further work in future could investigate whether intra-paddock diversity of *P. tritici-repentis* populations could be higher than inter-paddock and inter-regional diversity in New Zealand.

Correlation of some of the banding patterns generated by the different RAPD primers with the known virulence races indicate that this method may also group different *P. tritici-repentis* isolates based on their virulence race classifications. The banding patterns generated by RAPD primers Operon H-19 and UBC517 appeared to be the same for isolates of races 1 (ASC1, AB33-1, CCDM-Meck4, CCDM-239 and CCDM-134), including the New Zealand

isolates (S12a, S26a, M13c, G14a, G22a, F12d and K16a). Whereas, international isolates of races 3 (AB39-2), 5 (Alg3-24) and 8 (TS93-71B) had unique bands with all of the tested RAPD primers.

Grouping of isolates based on their virulence races by RAPD was observed in a similar *P. tritici-repentis* study conducted by Aung, (2001). In that study eight RAPD primers, not the same as the primers in this study, were utilised to assess the genetic variability of 53 *P. tritici-repentis* single spore isolates, including two isolates included in this study (Alg3-24 and ASC1), from varying global origins and representing six virulence races. A total of 59 polymorphic bands were generated, with an analysis of molecular variance (AMOVA) revealing that 36% of the genetic diversity detected was attributed to differences between races which possessed or lacked the necrosis inducing *ToxA* gene. Further investigation revealed that isolates of races 1 and 2 were in fact part of the same population, with it being further suggested that the lack of genetic structure between races 1 and 2 may indicate they had a recent origin. In contrast to isolates of races 1 and 2, there were significant genetic differences between isolates of races 3 and 5, with it being concluded that virulence may be a major driving force in the evolution and variation of *P. tritici-repentis*.

It is difficult to explain why RAPD only detected genetic variation between some of the *P. tritici-repentis* isolates of different virulence races, rather than genetic variation between isolates of the same race or point of geographic origin. There are a number of explanations for why this may have occurred which can be potentially linked to the method itself. One issue in particular which surrounds the use of RAPD for genetic diversity studies is that it is difficult to locate and elucidate the regions targeted i.e. HST gene sites which are amplified by RAPD primers. This is due to the fact RAPD can target any random location within the genome of a particular organism (Lübeck *et al.*, 1998; Lübeck *et al.*, 1999). Thus, it is because of this that it was not worthwhile in respect to time and money to further investigate the sites which the RAPD primers used in this study targeted.

Another issue with RAPD is that the results produced can vary widely between different studies due to reproducibility of the amplified bands being low (Lübeck *et al.*, 1998). This could potentially explain why other related studies investigating the genetic diversity of *P. tritici-repentis* with the same RAPD primers used in this study reported varying results to this study. For example, Singh & Hughes (2006) found that from a total of thirty three isolates of *P. tritici-repentis* a cluster analysis of the RAPD amplification patterns, including UBC598, 517, 600 and Operon H-19 used in the current study, revealed that all isolates assessed had unique banding patterns, with the clustering being independent of the isolates race or origin. One explanation for the differences between this study and Singh & Hughes (2006), is that in contrast to the four RAPD primers utilised in this study, Singh & Hughes (2006) utilised 30 different primers. Thus, Singh & Hughes (2006) sampled more loci than this study.

Lastly, the results produced by RAPD can also vary due to factors relating to the quality of the DNA and the equipment such as the PCR cycler machine utilised (Lübeck *et al.*, 1998; Bulat *et al.*, 1998). For example, in the current study the DNA of the isolates studied was isolated and purified using three different DNA isolation and purification methods. This could have led to variations in the DNA quality and amplification product visibility during the RAPD-PCR amplification procedure used in this study. For instance, in this study it was found that the DNA quality of Australian isolates CCDM-239 and 134 appeared to be quite low.

Microsatellite analysis was also selected to assess the genetic variation in *P. tritici-repentis*. In this study, no polymorphic bands were detected between the New Zealand *P. tritici-repentis* isolates. However, in this study the PCR products were only run on agarose gel and not on acrylamide. The greater resolving power of acrylamide (single base pairs) has the potential to detect size polymorphisms that were not detected by agarose. In contrast, polymorphic bands were generated for all of the international *P. tritici-repentis* isolates tested. This result indicated that the New Zealand isolates tested were genetically identical (clonal) to one another. This result also showed that the selected microsatellite primers could differentiate different isolates of *P. tritici-repentis* from one another, regardless of their race, or point of geographic origin.

Several studies have shown the versatility of microsatellite markers for the analysis of genetic diversity in *P. tritici-repentis* (Aboukhaddour *et al.*, 2011; Gurung *et al.*, 2013). In all of these studies a wide range of different microsatellite primers were able to detect a high level of genetic diversity between different isolates of *P. tritici-repentis* from different geographic origin. The microsatellite results seen in this study are very similar to that reported by Aboukhaddour *et al.* (2011) for a diverse collection of 80 isolates of *P. tritici-repentis* examined with thirty one different SSR markers. A significant level of genetic differentiation was detected among the different isolates examined, with isolates from different origins grouping and having similar levels of genetic diversity.

There are also a number of possible explanations which relate to the biology and environment of New Zealand which could account for why a low degree of genetic variation was detected within the New Zealand isolates of *P. tritici-repentis* assessed in this study. The results revealed by the RAPD, UP-PCR and microsatellite methods suggest that the New Zealand isolates present in the South Island are clonal. This clonal genetic structure may indicate that asexual reproduction is the predominant reproductive strategy of *P. tritici-repentis* under New Zealand field conditions. Additionally, this finding could also suggest that the current distribution pattern of *P. tritici-repentis* populations in New Zealand may be the result of a rapid widespread dispersal of asexual conidia across the South Island. For example, a study by Wolf & Hoffmann (1993) found that under field conditions the conidia of *P. tritici-repentis* were spread by wind over great distances. In contrast, it was also found that although the

ascospores of *P. tritici-repentis* were actively discharged from mature pseudothecia, they only had short distribution distances.

This result may suggest that *P. tritici-repentis* is not undergoing sexual reproduction, or undergoing genetic differentiation and recombination events at an extremely slow rate. This result is interesting, considering *P. tritici-repentis* is known to be a homothallic fungus (Lepoint *et al.*, 2010; Gurung *et al.*, 2013). Therefore there should be no barriers preventing New Zealand populations of *P. tritici-repentis* from undergoing sexual reproduction.

It is difficult to explain why *P. tritici-repentis* would not be able to freely undergo sexual reproduction when New Zealand's environment appears to favour its biology and life cycle. It is possible that the lack of sexual reproduction in New Zealand *P. tritici-repentis* populations is due to frequent interruptions in the sexual reproduction component of the pathogens life cycle. Some factors that could potentially be linked to this interruption is that the New Zealand environment, wheat type and alteration of cultural farm practices in recent years may have directly affected the rate of population differentiation within the New Zealand populations surveyed in this study. For example Gurung *et al.* (2013) speculated that changes in wheat type and cultural practices may have resulted in little gene flow between sub-populations of *P. tritici-repentis* isolates recovered from winter wheat in Arkansas and spring and winter wheat in North Dakota in North America. In addition, recent studies by Adhikari *et al.* (2008) and Gurung *et al.* (2011) reported significant population differentiation in the wheat pathogens *M. graminicola* and *P. nodorum* which were collected from two distinct wheat producing regions in the USA which each had its own diverse climatic conditions, wheat cultivar classes and cropping practices.

It is possible that the milder climatic conditions and more intensive post-harvest practices such as frequent stubble burning, ploughing and crop rotations may have also slowed down, or interrupted the life cycle of *P. tritici-repentis* by preventing it from undergoing sexual reproduction. Abiotic factors such as temperature and moisture can play important roles in the production of disease inoculum (Hosford *et al.*, 1987). It is well known that the development of tan spot in wheat is very temperature sensitive (Kadar, 2010). A study by Lamari & Bernier (1994) reported that the severity of *P. tritici-repentis* infection was significantly reduced when temperatures were higher than 27°C. It is therefore possible that regular periods of temperatures exceeding 27°C during the summer of 2012-2013 in South, Mid and North Canterbury could have prevented the fungus from fully colonising infected wheat plant tissue and subsequently form pseudothecia during autumn and winter. In addition, the lack of reports and observations during the period of this study for the presence of the resulting sexual state pseudothecia of *P. tritici-repentis* in South Island wheat fields infected with tan spot during the post-harvest period may also help to support this hypothesis.

In contrast to the New Zealand isolates, the diverse populations of *P. tritici-repentis* examined by Gurung *et al.* (2013), Aung (2001) and Singh & Hughes (2006) would have arrived between 50-100 years prior to the introduction of modern disease management practices such as fungicides, crop rotations and seed treatments. Therefore, they would have had the ability to undergo sexual reproduction in the absence of intentional human interference (Gurung *et al.*, 2013). For example, tan spot was first observed and officially reported between the years 1934 and 1941 in countries like Canada, India and United States (Oliver *et al.*, 2007). Whereas, the first active multi-site fungicidal seed treatments were not introduced on a large commercial scale until sometime between 1940 and 1960 (Staub, 2008). This would therefore have provided these international populations sufficient time to spread from their point of introduction and then subsequently undergo recombination to generate new diverse populations before the first significant *P. tritici-repentis* global population study was conducted by Friesen *et al.* (2005).

Another hypothesis which may also explain the clonal nature of New Zealand *P. tritici-repentis* isolates is that *P. tritici-repentis* has not been present in New Zealand long enough for the population to differentiate. It is difficult to pinpoint the exact time, site of introduction or origin, from which *P. tritici-repentis* was introduced into New Zealand. The earliest known report of *P. tritici-repentis* in New Zealand was by Hampton & Matthews (1978) as part of a survey of certified wheat seed. Based on this report, *P. tritici-repentis* would have had sufficient time to undergo population differentiation via sexual reproduction, as it has been shown that *P. tritici-repentis* has a polycyclic sexual cycle (Bankina, 2005; Cox *et al.*, 2004). However, the isolates described by Hampton & Matthews (1978) were identified by morphology and not by DNA sequencing and therefore may have been incorrectly identified. If *P. tritici-repentis* was introduced to New Zealand within the last 10-20 years it is possible a slow rate of population differentiation may have delayed the generation of genetic variation and resulted in the clonal population structure observed in the current study. However, if *P. tritici-repentis* was introduced 40-50 years ago, a more diverse population structure would be expected.

In contrast to New Zealand, the occurrence and study of tan spot disease in Australia may provide a good exemplary timeline to compare New Zealand populations against in order to help estimate the time it takes for the generation of population genetic variation to occur. Tan spot was first reported in Australia in 1953, however, it was only considered to be an economically significant disease in the 1970s (Oliver *et al.*, 2007). To date the genetic structure of Australian *P. tritici-repentis* populations has been described as having low to moderate levels of genetic variation. For example, a linkage disequilibrium (*LD*) analysis of 67 Australian isolates by Gurung *et al.* (2013) revealed that the Australian populations assessed had significant *LD* values, with the authors concluding asexual reproduction played a more important role in determining the genetic structure of the Australian populations studied.

The current race structure of Australian populations of *P. tritici-repentis* is mainly composed of race 1 and 2 isolates (Antoni *et al.*, 2010). Therefore, based on the Australian introduction, *P. tritici-repentis* may require between 50-70 years to generate a moderate level of genetic variation, provided frequent sexual reproduction and differentiation was permitted. In contrast, considering the report of Hampton & Matthews (1978), the New Zealand isolates of *P. tritici-repentis* may have only been present in New Zealand for 30 to 40 years and still appear to possess a clonal population structure. This suggests New Zealand may have some unique environmental factors, or conditions, which are not present in Australia that decrease the rate of genetic recombination. Further research is required in order to help investigate the nature of these particular New Zealand factors.

3.5 Conclusions

This study used three molecular methods (UP-PCR, RAPD and microsatellite analysis) to demonstrate that the *P. tritici-repentis* isolates selected from diverse geographic locations were clonal and genetically distinct from other international isolates of *P. tritici-repentis*. The clonal genetic structure of the New Zealand isolates is unique when compared to the other global populations of *P. tritici-repentis* characterised to date. UP-PCR was not suitable for detecting genetic diversity between isolates of *P. tritici-repentis*. RAPD could differentiate some *P. tritici-repentis* isolates based on their race and point of geographic origin. Microsatellite analysis could differentiate international isolates from one another, regardless of their race, or point of geographic origin. The results suggest that New Zealand populations of *P. tritici-repentis* do not undergo frequent genetic recombination, but the factors contributing to this were not identified. Possible explanations for this absence of genetic variation are the recent nature of the incursion and the life cycle of *P. tritici-repentis* being interrupted by both abiotic and cultural factors in the New Zealand wheat growing environment. Further investigation is needed in order to elucidate the nature of these unique New Zealand factors.

Chapter 4

Evaluation and determination of sensitivity of *Pyrenophora tritici-repentis* to fungicides

4.1 Introduction

In recent years the severity and incidence of tan spot infestations have been observed to have increased (Gurung *et al.*, 2013; Manning *et al.*, 2013). This increase has been linked to there being a major shift in the preference of minimum tillage over conventional tillage practices (Patel *et al.*, 2012). Thus, the application of chemical fungicides has become more necessary in order to ensure that the potential biological yield of wheat crops are protected.

To date, there has been a range of fungicides, representing a number of different fungicide classes which have been reported to reduce tan spot infection in New Zealand. However, none of the currently available fungicide treatments have been reported to completely control tan spot in the field (Harvey *et al.*, 2015). Of these fungicides, the most commonly applied fungicides are the strobilurins (QoI), DMI's and SDHI's (Harvey *et al.*, 2015).

A decrease in the sensitivity of a *P. tritici-repentis* population towards fungicides is one potential risk which can threaten the longevity of use for fungicides. Decreases in sensitivity towards a particular fungicide will often occur as a result of selection pressure induced by the fungicide on a fungal population if it is frequently applied at below lethal doses over long periods of time (Beard *et al.*, 2009). Once the onset of insensitivity to a particular fungicide has occurred in a fungal population it will often be permanent and be visually expressed through the loss or decrease of satisfactory disease control of the fungicide whenever it is applied (Beard *et al.*, 2009; Cools & Fraaije, 2012).

The degree of sensitivity exhibited by *P. tritici-repentis*, and the closely related barley pathogen *P. teres* f. *teres* have been reported to vary *in vitro* when exposed to different fungicides (Beard *et al.*, 2009). There is currently a limited number of studies undertaken to investigate the sensitivity of *P. tritici-repentis* populations to different fungicides (Hunger & Brown, 1987; Beard *et al.*, 2009). In addition, these studies also only tested a small number of DMI fungicides such as tebuconazole, epoxiconazole and propiconazole. In all of these studies it was found that growth of *P. tritici-repentis* was inhibited below 50% at varying concentrations by all the tested DMI fungicides. In contrast to *P. tritici-repentis*, there has been more studies undertaken to investigate the fungicide sensitivity of *P. teres* f. *teres* (Sheridan & Nendick, 1987; Peever & Milgroom, 1994) to fungicides in Australia and the USA.

To date, there been no comprehensive studies undertaken to evaluate and establish a baseline sensitivity of New Zealand populations of *P. tritici-repentis* towards a range of commonly used fungicides. Thus, the aim of this study was to determine the variation in sensitivity of New Zealand *P. tritici-repentis* isolates to a range of commonly used fungicides.

4.2 Materials and Methods

4.2.1 Effect of fungicides on *in vitro* mycelial growth

A total of eight different fungicides belonging to three different fungicide classes (Table 4.1) were tested *in vitro* to evaluate their inhibitory effect on the mycelial growth of four single spore isolates of *P. tritici-repentis* (S12a, G14a, G22a and K16a; Table 2.1). The isolates were selected to best represent the geographic range and current genetic diversity of *P. tritici-repentis* populations in New Zealand based on the results of Chapter 3.

Table 4.1: Fungicides tested against mycelial growth of *Pyrenophora tritici-repentis*.

| Active ingredient | Trade name | Chemical class | Conc. range (mg a.i./ L) | Manufacturer |
|----------------------------------|----------------|------------------------|--------------------------|------------------------------|
| Azoxystrobin | Amistar® | Strobilurin | 0.003 – 3.0 | Syngenta Crop Protection Ltd |
| Bixafen + Prothioconazole | Aviator Xpro® | SDHI & DMI Triazole | 0.003 – 3.0 | Bayer NZ Ltd |
| Epoxiconazole | Opus® | DMI Triazole | 0.003 – 3.0 | BASF New Zealand Ltd |
| Fluxapyroxad | Sercadis® | SDHI & DMI Triazole | 0.003 – 3.0 | BASF New Zealand Ltd |
| Isopyrazam | Seguris Flexi® | SDHI | 0.003 – 3.0 | Syngenta Crop Protection Ltd |
| Propiconazole | Tilt® | DMI Triazole | 0.003 – 3.0 | Syngenta Crop Protection Ltd |
| Prothioconazole | Proline® | DMI Triazole | 0.003 – 3.0 | Bayer NZ Ltd |
| Pyraclostrobin | Comet® | Strobilurin | 0.003 – 3.0 | BASF New Zealand Ltd |

Each fungicide was tested at five different concentrations. The test range of the active ingredient (a.i) concentrations was from 0.003 to 3000 mg a.i. / L (Table 4.1). Concentrations were determined based on the reported activity range and formulated concentrations of the chosen fungicides. Each fungicide bioassay experiment consisted of four replicate plates for

each combination of fungicide concentration and *P. tritici-repentis* isolate. Unamended control plates were included for each fungicide and *P. tritici-repentis* isolate.

Each of the tested fungicides was suspended in 100 mL of sterile water to make a stock concentration of 1 g/ L. The fungicides were then further diluted to double of the required concentration by diluting a measured amount of the stock fungicide solution in 250 mL of sterile water. The suspended fungicide was then combined with 250 mL of double strength molten PDA (78 g/ L) at a temperature of 50°C, mixed thoroughly and poured into 90 mm Petri dishes. Control plates consisted of unamended PDA (39 g/ L). Each plate was inoculated centrally with a 5 mm diameter mycelial disc of the selected *P. tritici-repentis* isolate.

Plates were placed into an incubator and arranged on shelves corresponding to their assigned replicate in a complete randomized design. The control plates for each fungicide and *P. tritici-repentis* isolate were evenly distributed between the replicate shelves. Plates were incubated at 25°C in continuous darkness. After three, five and seven days of incubation mycelial growth was measured. Mycelial growth was determined with a digital calliper by measuring across the two perpendicular diameters of each colony with the mean mycelial growth inhibition being calculated as a percentage relative to the control plates.

4.2.2 Statistical analysis and determination of EC₅₀ values for fungicides

The mycelial growth for each isolate fungicide concentration combination was calculated as a percentage of the mycelial growth of the corresponding isolate control (on unamended PDA). Prior to statistical analysis, mycelial growth diameter data was transformed to Log₁₀ values. The transformed log values were used to calculate EC₅₀ (effective concentration which reduced mycelial growth below 50%) for each fungicide and isolate combination using probit analysis option within the Generalised Linear Model (Genstat version 16). EC₅₀ values were analysed with two way ANOVA (Genstat version 16) to determine fungicide and *P. tritici-repentis* isolate effect. Means were separated using Fisher's protected LSD at $P \leq 0.05$.

4.3 Results

4.3.1 Effect of fungicides on *in vitro* mycelial growth of *Pyrenophora tritici-repentis*

Only the day five mycelial measurement data was used to determine the EC₅₀ for each fungicides. All of the tested fungicides were effective at reducing the mycelial growth of *P. tritici-repentis* below 50% (Figures 4.1, 4.2 and 4.3). There was a significant effect of fungicide ($P>0.001$) on mean EC₅₀ values (Appendix D.1.1). The mean EC₅₀ values of all tested fungicides ranged from 0.02 to 2.96 mg/ L of active ingredient, with there being a significant difference ($P=0.05$; Appendix D.1.2) in the EC₅₀ values of the fungicides, apart for prothioconazole and bixafen + prothioconazole. The overall EC₅₀ value of *P. tritici-repentis* isolate S12a was significantly lower (0.15 g a.i. / L) compared with the other isolates (G14a, G22a and K16a). The significant ($P>0.001$; Appendix D.1.1) isolate-fungicide interaction was associated with the growth of some *P. tritici-repentis* isolates differing for some fungicides. The log₁₀ transformed and untransformed values for EC₅₀ data are presented in Appendix D.1.2.

Table 4.2: The mean EC₅₀ (mg a.i./ L) values for eight different fungicides on the *in vitro* inhibition of mycelial growth of four *Pyrenophora tritici-repentis* single spore isolates. Data presented are back transformed means.

| Fungicide | <i>Pyrenophora tritici-repentis</i> isolate | | | | Fungicide mean EC ₅₀ |
|--------------------------------------|---|---------------|---------------|---------------|---------------------------------|
| | S12a | G14a | G22a | K16a | |
| Isopyrazam | 0.014 b | 0.022 bc | 0.026 cd | 0.007 a | 0.02 A |
| Propiconazole | 0.037 de | 0.048 ef | 0.053 ef | 0.057 efg | 0.05 B |
| Bixafen + Prothioconazole | 0.086 gh | 0.086 gh | 0.136 i | 0.120 hi | 0.10 C |
| Prothioconazole | 0.067 fg | 0.127 hi | 0.145 i | 0.161 ij | 0.12 C |
| Epoxiconazole | 0.175 ijk | 0.237 jkl | 0.228 jkl | 0.274 klm | 0.23 D |
| Fluxapyroxad | 0.313 lm | 0.507 no | 0.569 no | 0.518 no | 0.47 E |
| Pyraclostrobin | 0.679 o | 0.710 o | 0.579 no | 0.426 mn | 0.59 F |
| Azoxystrobin | 2.529 p | 2.559 p | 2.472 p | 4.775 q | 2.96 G |
| Isolate mean effect | 0.15 A | 0.20 B | 0.22 B | 0.20 B | |

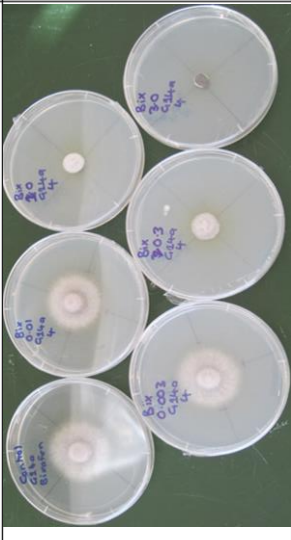
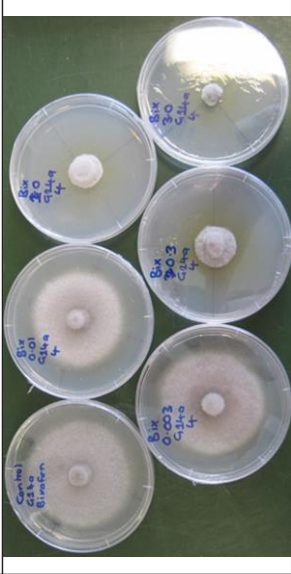
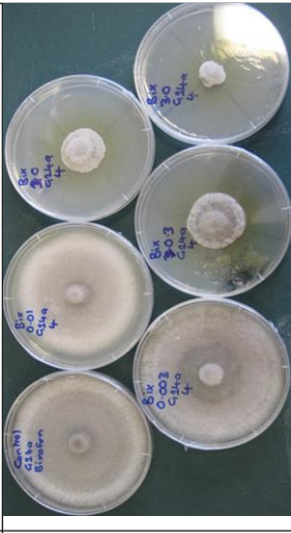


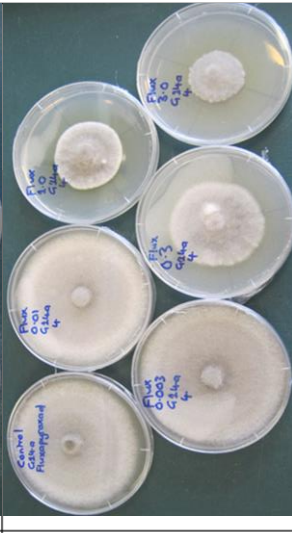
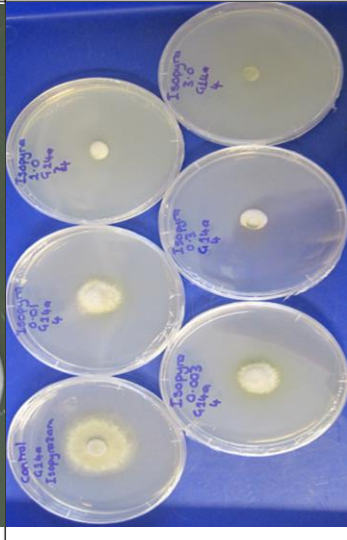
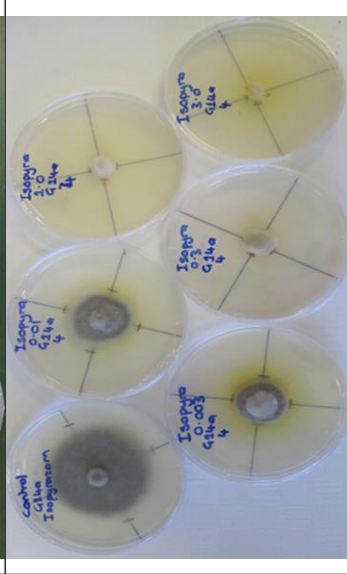

| Class | Fungicide name | Day 3 | Day 5 | Day 7 |
|---|---------------------------|--|---|---|
| Succinate dehydrogenase inhibitors (SDHI) | Bixafen + Prothioconazole |  |  |  |
| | Fluxapyroxad |  |  |  |
| | Isopyrazam |  |  |  |

Figure 4.1: Growth and appearance of *Pyrenophora tritici-repentis* on PDA amended with different concentrations of the fungicides bixafen + prothioconazole, fluxapyroxad and isopyrazam. For each fungicide and day the 6 plates represent the increasing concentrations of the fungicide from left to right.



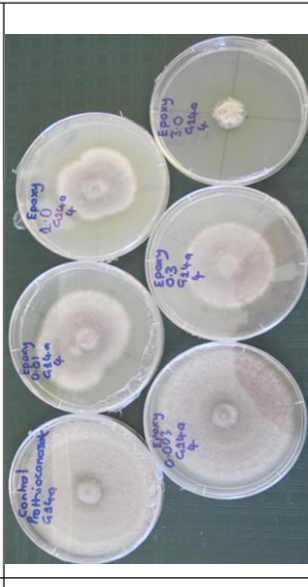

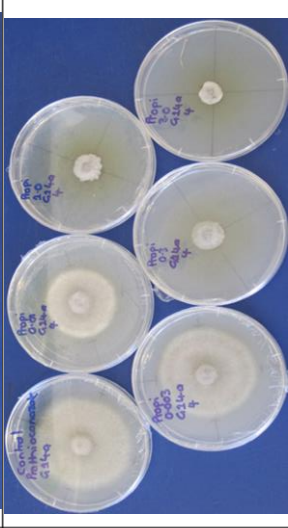
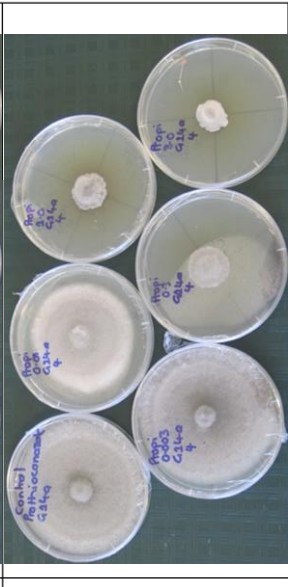
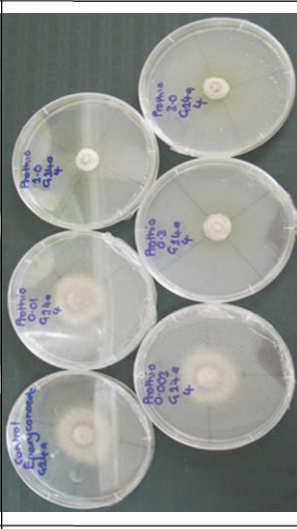
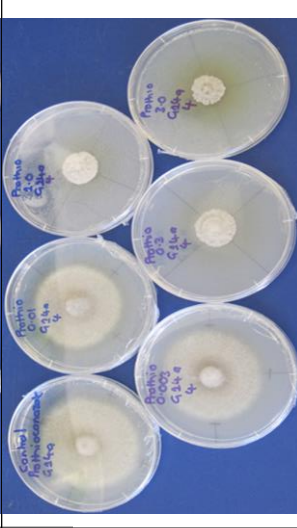

| Class | Fungicide name | Day 3 | Day 5 | Day 7 |
|--------------------------------|-----------------|---|--|--|
| Demethylation inhibition (DMI) | Epoxiconazole |  |  |  |
| | Propiconazole |  |  |  |
| | Prothioconazole |  |  |  |

Figure 4.2: Growth and appearance of *Pyrenophora tritici-repentis* on PDA amended with different concentrations of the fungicides epoxiconazole, propiconazole and prothioconazole. For each fungicide and day the 6 plates represent the increasing concentrations of the fungicide from left to right.

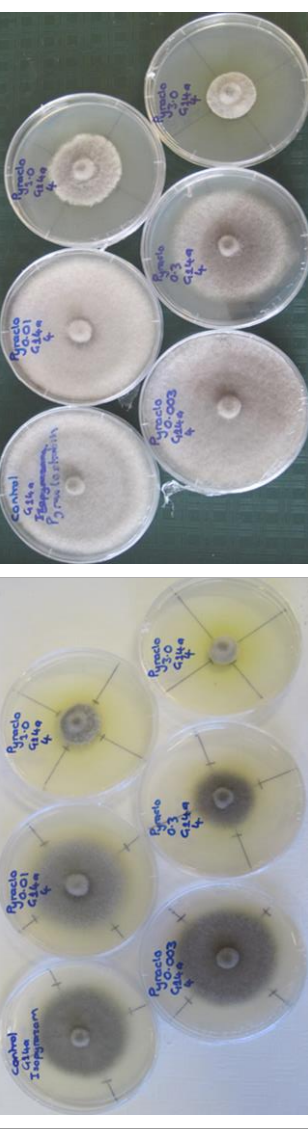


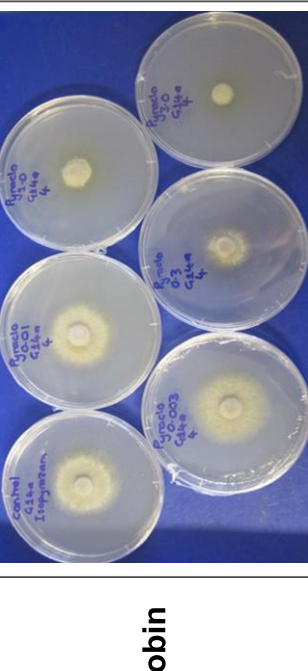
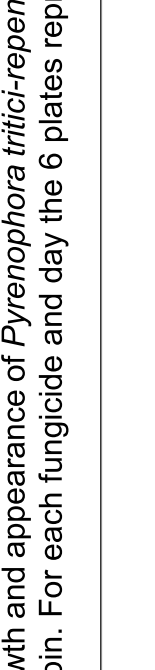

| Class | Fungicide name | Day 3 | Day 5 | Day 7 |
|----------------------------------|----------------|--|---|---|
| Quinone outside inhibitors (QoI) | Azoxystrobin |  |  |  |
| | Pyaclostrobin |  |  |  |

Figure 4.3: Growth and appearance of *Pyrenophora tritici-repentis* on PDA amended with different concentrations of the fungicides azoxystrobin and pyaclostrobin. For each fungicide and day the 6 plates represent the increasing concentrations of the fungicide from left to right.

4.4 Discussion

This study is the first investigation of the *in vitro* sensitivity of New Zealand isolates of *P. tritici-repentis* to a range of fungicides. The results showed that the New Zealand isolates screened were sensitive to all of the fungicides tested. In addition, there is no evidence of any resistance being present in the New Zealand *P. tritici-repentis* isolates tested. This is a very favourable finding because it indicates all of the tested fungicides would provide good control of tan spot infection in a field environment. Additionally, this study also provides an ideal baseline for any future studies investigating and monitoring the sensitivity of New Zealand populations of *P. tritici-repentis*.

The results of this study showed that the SDHI fungicide isopyrazam and DMI fungicide propiconazole were the most effective at reducing the *in vitro* mycelial growth of *P. tritici-repentis* below 50% with mean EC₅₀ values of 0.02 and 0.05 mg/ L respectively. In contrast, the QoI fungicides azoxystrobin and pyraclostrobin were found to be the least effective fungicides at reducing *P. tritici-repentis* growth with mean EC₅₀ values of 2.96 and 0.59 mg/ L respectively. The combination of bixafen and prothioconazole did not give any additional inhibition of *P. tritici-repentis* compared with prothioconazole alone indicating no synergistic effect between the two fungicides. However, EC₅₀ values were determined based on the total a.i. concentration of both chemicals in the combined prothioconazole and bixafen product. It would be useful to determine the effect of bixafen alone on *P. tritici-repentis*.

At present, there is a limited range of studies which have investigated the *in vitro* sensitivity of *P. tritici-repentis* to compare the results of this study against. Additionally, to date there are only reports about the *in vitro* sensitivity of *P. tritici-repentis* to triazole fungicides such as epoxiconazole and propiconazole, with there being no reports of other fungicides. Beard *et al.* (2009) compared the *in vitro* baseline sensitivities of 45 Australian *P. tritici-repentis* single spore isolates collected both between 1986-1987 and 2001-2003 to the fungicides epoxiconazole, propiconazole and tebuconazole. It was found that epoxiconazole had almost twice the activity of the other tested fungicides, with a mean EC₅₀ of 0.2 µg/ mL, in contrast to tebuconazole and propiconazole which both had EC₅₀ values of 0.4 µg/ mL. It was further concluded that there was no evidence to suggest that the sensitivity of Australian *P. tritici-repentis* populations to the tested fungicides had changed from 1986 to 2003. In contrast, the current study showed that propiconazole was more effective than epoxiconazole at reducing the growth of New Zealand *P. tritici-repentis* isolates. Tebuconazole was not tested in the current study. However, Hunger & Brown (1987) reported that ten *P. tritici-repentis* isolates from Oklahoma and Texas were most sensitive to propiconazole, with a mean EC₅₀ value of

0.43 µg/ mL, and moderately sensitive to tebuconazole which had a mean EC₅₀ of 0.19 µg/ mL.

The results of Beard *et al.* (2009) are contrasting to the current study. In the current study, propiconazole was found to be much more effective than epoxiconazole at reducing the mycelial growth of *P. tritici-repentis in vitro*. There are a number of potential reasons relating to the methodologies employed by this study, Beard *et al.* (2009) and Hunger & Brown (1987) that may explain the differences in the results obtained.

Firstly, the fact that the reported EC₅₀ values reported in Hunger & Brown (1987) and Beard *et al.* (2009) were determined using a much larger range of fungicide concentrations could be a potential factor. For example, in the current study six concentrations ranging from 0 to 3.0 mg/L for each fungicide were selected. In contrast, Beard *et al.* (2009) used different concentrations ranging from 0 to 30.0 µg/ mL (30 mg/ L). This wider range of fungicide concentrations may have provided a much larger dataset to measure and determine fungicide EC₅₀ values leading to differences between studies.

Secondly, the number of *P. tritici-repentis* isolates tested may also have affected the accuracy of determining the EC₅₀ for propiconazole and epoxiconazole. For example, in the current study the sensitivities of four New Zealand *P. tritici-repentis* isolates were determined, whereas ten and 45 isolates were tested by Hunger & Brown (1987) and Beard *et al.* (2009) respectively. Despite four isolates being small, this number of isolates was chosen to best represent the current geographic distribution and genetic diversity of *P. tritici-repentis* in New Zealand. The number of isolates (45) tested by Beard *et al.* (2009) was a good number for best representing the moderate levels of genetic variation present in Australian populations of *P. tritici-repentis*. In contrast, Hunger & Brown (1987) only tested ten different isolates of *P. tritici-repentis*. This is small considering that there are high levels of genetic diversity present within North American populations of *P. tritici-repentis* (Friesen *et al.*, 2005; Gurung *et al.*, 2013). As a result, this could have resulted in the estimated EC₅₀ of North American *P. tritici-repentis* isolates to propiconazole and epoxiconazole being inaccurate.

Thirdly, factors such as human error may have also caused the differences between the current study and highlighted studies. Variations in media preparation could have resulted in slight differences in the relative growth rates of the isolate replicates. In the current study it was observed that one isolate grew poorly in one particular replicate for one fungicide in contrast to the other replicates.

The type of agar media selected for this study may have also been another factor that led to differences between the current and highlighted studies. For example, in contrast to the PDA selected in the current study, Beard *et al.* (2009) used malt extract agar (MEA) and Hunger &

Brown (1987) used a clarified V-8 agar media. It is known that the sensitivity of different phytopathogenic fungi can often vary widely on different types of agar (Russell, 2002).

Lastly, the formulation of the tested fungicide could also lead to differences between the current and highlighted studies. Beard *et al.* (2009) used unformulated (raw) technical grade fungicides, whereas Hunger & Brown (1987) used formulated technical grade. In contrast, formulated non-technical grade fungicides were utilised for this study. Differences between the formulation and unformulated additive's may have influenced the sensitivity results obtained.

The biology of the *P. tritici-repentis* isolates studied may also have caused the differing outcomes between the current and highlighted studies. It is well known that there are moderate levels of genetic diversity present within populations of *P. tritici-repentis* in Australia (Gurung *et al.*, 2013). Therefore, it could be speculated that there is a high possibility that the sensitivity of different isolates could vary *in vitro*. Beard *et al.* (2009) concluded that the moderate variations seen in *P. tritici-repentis* isolate sensitivity to propiconazole and epoxiconazole may possibly have reflected existing natural variations in the population and was not the result of cross-resistance from natural selection. In contrast, the results of Chapter 3 suggest New Zealand has a very clonal *P. tritici-repentis* population structure. This result indicated that it was possible that New Zealand populations had undergone infrequent recombination events. Therefore, any variations in isolate sensitivity would most likely not be the result of genetic differences. In the current study, the growth of New Zealand isolates did not vary significantly to propiconazole, but there was slight (but significant) variation in sensitivity to epoxiconazole between isolates S12a and K16a. As a result of this, it is possible that Australian isolates of *P. tritici-repentis* are slightly less sensitive to propiconazole than New Zealand isolates *in vitro*. Therefore, due to Australian populations being known to have undergone genetic recombination events they have the potential to adapt more quickly to the selection pressures of fungicide application. Despite there being differences in the EC₅₀ values determined and ranking of fungicides, the results of the current and highlighted studies indicate that both propiconazole and epoxiconazole would be effective at controlling *P. tritici-repentis*.

It is difficult to correlate the EC₅₀ results of the fungicides evaluated in this study with their performances in the New Zealand field environment. One finding of the 2013-2014 farmer questionnaire was that many of the farmers surveyed had applied most of the fungicides evaluated in this study at rates below their recommended label rates. It is well known that the rate and timing of a fungicide application are extremely important factors and have the potential to influence the effectiveness a fungicide to control diseases in the field (Colson *et al.*, 2003). This factor could therefore explain why the tan spot infestations observed on the farms surveyed were very severe. For example, both South Canterbury farmer 3 and the North Canterbury farmer of the 2013-2014 survey (Section 2.3.1.1) applied azoxystrobin and epoxiconazole at a second crop stage timing (T2) at rates between 300 to 500 mL/ ha. The tan

spot infestations on these properties were severe. From the results of this study azoxystrobin and epoxiconazole were the least effective fungicides of the classes QoI and DMI respectively, in reducing the *in vitro* growth of *P. tritici-repentis*. It is possible that the rates these fungicide were applied at in these two field sites were below the concentration that would permit complete control of *P. tritici-repentis*. Other factors such as poor application method and timing could also potentially explain why the tan spot outbreaks were not successfully managed. Additionally, the long-term application of fungicides below their recommended rates can also increase the risk of fungicide resistant isolates developing (Beard *et al.*, 2009; Cools & Fraaije, 2012).

Due to tan spot only recently coming to the attention of the New Zealand wheat industry, there is limited research on evaluating the performance of fungicides to control *P. tritici-repentis* under New Zealand field conditions. However, a study by Harvey *et al.* (2015) evaluated the effectiveness of fungicides, including all of the fungicides evaluated in the current study, at controlling tan spot. A total of eight different fungicides were applied at three different timings (GS-33, GS-39 and GS-65) with there also being a single treatment at GS-65 and again 10 days later. It was found that the fungicides propiconazole and prothioconazole provided the best, although not complete, control of tan spot early (GS-33) and late (GS-39) in the season. In contrast, the fungicide difenoconazole, which was not evaluated in the current study, was found to give the least effective early control of tan spot. It was also reported that a combined application of prothioconazole at GS33 and bixafen + prothioconazole gave the best overall green leaf retention. However, it was also noted by the authors that tan spot was not well controlled in the trial due to the overall severity of the epidemic being high. Ideal weather for tan spot, susceptibility of the cultivar, in-correct timing or frequency of fungicide applications were linked as possible contributing factors to the lack of complete control observed in this study. The results obtained by this field study do correlate with the *in vitro* sensitivity results obtained in the current study, where propiconazole was found to be the most effective DMI fungicide compared to prothioconazole and epoxiconazole.

Colson *et al.* (2003) reported on the results of three separate field experiments conducted from 1988 to 1999 in Australia to investigate the effectiveness of fungicides to control *P. tritici-repentis*. Within these three experiments, a total of 12 fungicides were evaluated for their ability to reduce the effect of tan spot on grain yield. Fungicides were applied at varying rates at a single spray timing at 90% flag leaf emergence. The severity of tan spot epidemics varied significantly between each year. In one experiment, it was reported that grain yield loss was significantly lowered following the application of 72 mL a.i/ ha propiconazole, 125 mL a.i/ ha tebuconazole, 72 mL a.i/ ha epoxiconazole, 125 and 250 g a.i/ ha azoxystrobin. In another experiment, yield loss of two wheat cultivars was significantly reduced following the application of propiconazole, tebuconazole, prochloraz and flusilazol between GS 53 and 59.

It is difficult to correlate the findings of the current study with the findings of this study due the levels of tan spot varying between different experiments and the yield loss reductions being estimated on different cultivars year to year.

Overall, the findings of both of these studies do correlate with the findings of the current study. However, it is difficult to confirm the overall versatility and effectiveness of isopyrazam and bixafen in the field due to the fact they were mixed and applied at a single application timing with other fungicides by Harvey *et al.* (2015).

Alternatively, the overall sensitivity of *P. tritici-repentis* to fungicides could also be determined in future studies by investigating both the mycelial growth sensitivity and the percentage inhibition of conidial germination by fungicides. This is because conidia are the most likely inoculum source in the field (Wolf & Hoffmann, 1993). Research by Bleach (2012) demonstrated that relative susceptibility of the mycelium and germinating conidia of the black foot disease pathogen "*Cylindrocarpon*" spp. can vary *in vitro*. In this study, the main reason the effect of the fungicides was not tested on conidial germination was because of the difficulty of generating sufficient numbers of conidia *in vitro*. Mycelial growth fungicide sensitivity bioassays are considered the preferred method of choice for assessing the sensitivity of plant pathogens to fungicides due to the method being less laborious and time consuming in contrast to conidial germination based assays (Russell, 2002). Future experimental work utilizing artificially inoculated pot plants could help to investigate the effectiveness of fungicides to inhibit the growth of *P. tritici-repentis in planta*, in contrast to field experiments which are reliant on natural infection by *P. tritici-repentis* from the environment.

From the results of this study it is possible to determine what fungicides would be the most ideal for controlling tan spot in a field environment in New Zealand. However, further work is recommended in order to assess the overall effectiveness of the fungicides to validate and confirm their overall activity against *P. tritici-repentis in planta*. The SDHI fungicide isopyrazam, DMI fungicides propiconazole and prothioconazole would be the most suitable fungicides to advise to a New Zealand farmer to apply to control a tan spot infestations in wheat. Due to the recent reports of QoI resistant populations of *Z. tritici*, the causal agent of speckled leaf blotch, another major disease of wheat in New Zealand (Stewart *et al.*, 2014), the fungicides azoxystrobin and pyraclostrobin are not strongly recommended for controlling tan spot in New Zealand. In addition, epoxiconazole would also not be strongly recommended due to *Z. tritici* also being reported to have reduced *in vitro* sensitivity (Stewart *et al.*, 2014). These fungicides would either need to be applied with another class of fungicide e.g. SDHI in order to ensure that both *Z. tritici* and *P. tritici-repentis* are effectively controlled should they occur in the same wheat crop. In addition, it should also be emphasised that in order to ensure that tan spot is effectively controlled with these fungicides, they should be applied either at their respective recommended rates. In order to further ensure that the risks of *P. tritici-repentis* populations

developing resistance to these fungicides in future is low, they should also be rotated and applied at different crop development timings with other fungicides of different classes. Lastly, the sensitivity of *P. tritici-repentis* populations to all of the evaluated fungicides of this study should also be continually monitored in future by conducting regular EC₅₀ studies and comparing the obtained results to the baseline results of this study.

4.5 Conclusions

This study is the first to investigate the *in vitro* sensitivity of four New Zealand *P. tritici-repentis* isolates to a number of different fungicides. The results of this study demonstrate that all of the assessed fungicides are able to effectively reduce the mycelial growth of *P. tritici-repentis in vitro* to below 50%. The fungicides isopyrazam, propiconazole, prothioconazole had the lowest EC₅₀ values indicating they were the most effective fungicides at reducing the *in vitro* growth of *P. tritici-repentis*. In contrast, the fungicides azoxystrobin, pyraclostrobin and fluxapyroxad were the least effective at reducing the *in vitro* growth of *P. tritici-repentis*. Questionnaire results revealed that the timing and rate of application of the tested fungicides was important for effectively controlling tan spot in a field environment. The fungicides isopyrazam, propiconazole and prothioconazole would be strongly recommended for use in the field to control outbreaks of tan spot. It is also strongly advised that these fungicides be applied at their recommended rates and mixed with fungicides of other classes to preserve their efficacy and reduce the risks of resistance development in future. Further work in both greenhouse and field environments is required in order to fully investigate the overall performance and efficacy of the tested fungicides against *P. tritici-repentis in planta*. The sensitivity of *P. tritici-repentis* populations to the evaluated fungicides of this study should also be continually monitored in future.

Chapter 5

Determination of susceptibility of wheat cultivars to *Pyrenophora tritici-repentis*

5.1 Introduction

Plant diseases are ubiquitous in nature and have the potential to cause huge financial losses to the agricultural industry (Klinkowski, 1970). One of the best, and most economical, approaches for controlling disease epidemics including tan spot, is the use of genotype lines which possess tolerance or resistance (Lamari & Bernier, 1989b; Friesen & Faris, 2004). The occurrence of increasingly severe tan spot epidemics worldwide in recent years has resulted in there being an urgent need for the broadening of the genetic base of resistance and the rapid incorporation of multiple tan spot resistance genes into new wheat lines (Singh *et al.*, 2010).

The terms plant disease 'resistance' and 'tolerance' are related but also distinct concepts (Roy & Kirchner, 2000). As a result of this, care must be taken to understand the distinction between them in order to not confuse the two concepts with each other (Roy & Kirchner, 2000). The term 'resistance' is used to refer to a plant which possesses traits that allow it to prevent infection, or limit the severity of the infection e.g. plant hairs and induced localised cell death (Roy & Kirchner, 2000). In contrast, the term 'tolerance' is used to refer to traits that help a plant reduce or offset the fitness consequences which occur as a result of infection but will not eliminate or reduce the severity of the infection e.g. delayed senescence of infected tissue (Roy & Kirchner, 2000). In this study, the term 'tolerant' will be used to refer to wheat cultivars which display symptoms of tan spot, but also have reduced levels of infection in contrast to cultivars which are known to be susceptible to tan spot.

High levels of resistance to tan spot have been observed in wheat (Lamari & Bernier, 1989b). The underlying genetics of resistance to tan spot has been reported to be both quantitative (Friesen & Faris, 2004) and qualitative (Singh & Hughes, 2005; Singh *et al.*, 2006a) in nature. The underlying molecular mechanism which is responsible for resistance of wheat cultivars to tan necrosis and extensive chlorosis induced by *P. tritici-repentis* has been found to be conferred by independent loci (Lamari & Bernier, 1991). For example, resistance to necrosis induced by different races of *P. tritici-repentis* have been found to be controlled by a range of recessive genes *Tsr1* (Faris *et al.*, 1996), *Tsr2* (Singh *et al.*, 2006b), *Tsr3* (Tadesse *et al.*, 2006a) *Tsr4* (Tadesse *et al.*, 2006b) and *Tsr5* (Singh *et al.*, 2008a). In contrast,

resistance to chlorosis has been found to be controlled by a single recessive gene in hexaploid wheat known as *Tsr6* (Singh *et al.*, 2008b).

The susceptibility of cultivated wheat lines to tan spot has been extensively examined overseas. A common trend which was identified in all of these studies is that different wheat cultivars respond differently to *P. tritici-repentis* infection (Mitra, 1934; Duff, 1954; Hosford 1971). The screening of wheat seedlings for their relative susceptibility to tan spot has been predominantly undertaken by artificial inoculation within a greenhouse environment. This method is known to permit the examination of resistance under uniform disease pressure (Singh *et al.*, 2010). The utilization of a lesion-type rating scale has been the preferred method of choice for tan spot researchers because it is regarded to be the most practical and convenient method for evaluating cultivar response to *P. tritici-repentis* (Singh *et al.*, 2010). A study by Evans *et al.* (1999) reported that there is a positive correlation between assessment of tan spot resistance at the seedling stage and rating of adult plants in the field (Evans *et al.*, 1999).

An issue that is often encountered when breeding tan spot resistant and tolerant cultivars is that the levels of resistance, or tolerance, of one particular cultivar are often found to differ widely between different regions. For example, cultivars considered to be resistant in one study can often be found to be susceptible in another related study (Gilchrist *et al.*, 1984; Lamari & Bernier, 1989a). Possible reasons for these discrepancies in cultivar resistance include differences in the virulence and race of the *P. tritici-repentis* isolates selected and the methodologies employed by research workers to rate host reaction (Lamari & Bernier, 1989a). Additionally, the fact that tan spot will often occur in association with other wheat foliar diseases also makes it more difficult to do field screenings for tan spot resistance. Thus, breeding for tan spot resistance is normally undertaken by evaluating seedling plants under controlled environmental conditions (Singh *et al.*, 2010).

To date, it has not been fully established which of the wide variety of commercially available feed and milling wheat cultivars in New Zealand are resistant, tolerant, or susceptible to tan spot disease. Thus, the objective of this research was to determine the susceptibility of commonly grown New Zealand wheat cultivars to *P. tritici-repentis* under glasshouse conditions.

5.2 Materials and methods

5.2.1 Fungal isolates

Four *P. tritici-repentis* single spore isolates (S12a, G14a, G22a and K16a) were selected for inoculation of wheat plants. Isolates were selected to best represent the geographic range and

current genetic diversity of *P. tritici-repentis* populations in New Zealand based on the results of Chapter 3.

5.2.2 Selection of wheat cultivars

Cultivars were selected on the basis of the results obtained from the regional survey conducted in Objective 1 and data and information supplied by Rob Craigie (Foundation for Arable Research (FAR)). Ten New Zealand wheat cultivars were selected to assess susceptibility and response to *P. tritici-repentis* infection. The wheat cultivars selected included the following; Empress, Saracen, Reliance, Wakanui, Duchess, Viceroy, Starfire (KWW46), Inferno (KWW47) and Torch. Wheat seed was kindly provided by Plant and Food Research, PGG Wrightsons Seeds Ltd and Seedforce New Zealand Ltd.

5.2.3 Preparation of plants

Plants were grown in a glasshouse located at the Lincoln University nursery. For each wheat cultivar, five seeds were planted at 1 cm deep in a one litre pot (10 X 10 cm) containing general 3 month potting mix (80% composted pine bark, 20% pumice 1-7 mm, Osmocote exact N:P:K: 16-3.5-10 (3-4 month), 3 g/ L, Horticultural lime 1 g/ L and Hydraflo 1 g/ L). For each of the selected wheat cultivars, a total of 20 pots were prepared and consisted of 10 replicate pots for both inoculated and uninoculated treatments. The pots were placed on a bench (4 m²) and arranged in a complete randomized block design (Figure 5.1), with 10 blocks each containing one replicate for each treatment (cultivar and inoculated/ uninoculated). At GS-20, the number of seedlings per pot were thinned from five to three plants per pot.

Plants were grown at a temperature of 20-25°C during the day and 15°C at night. Plants were grown under artificial lighting (400 watt high pressure sodium lamps) to extend photoperiod to 12 hours light and 12 hours darkness. Plants were lightly watered once every day.

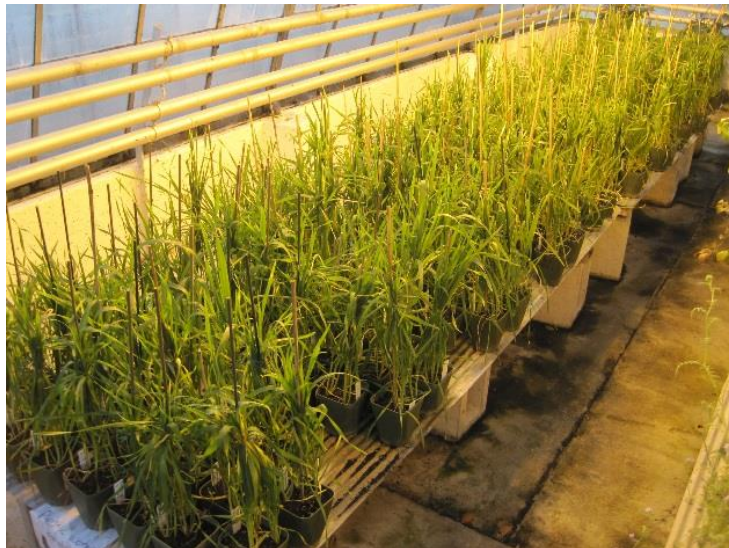


Figure 5.1: Experimental set up for the cultivar susceptibility experiment with the different wheat cultivars grown in pots arranged in a randomized complete block design in the Lincoln University glasshouse.

5.2.4 Preparation of fungal inoculum

All four single spore isolates (S12a, G14a, G22a and K16a) were grown initially for 5 days at 25°C in continuous darkness on full strength PDA (39 g/ L; Oxoid). The isolates were then plated onto V8-PDA (Appendix A.1) and grown for 4 days at 25°C in continuous darkness.

After 4 days incubation, the growing mycelium was flooded and flattened following the method described in Section 2.2.5. Plates were placed in an Adaptis CMP6010 growth cabinet (Conviron) and incubated under white light for 12 hours at 22.5°C. After 12 hours incubation under white light, plates were placed into an incubator and incubated for 20 hours in continuous darkness at 16°C.

Conidia were harvested using a modification of the method developed by Lamari & Bernier (1989a) and described in Section 2.2.5. The conidial concentration for each isolate was determined using a haemocytometer and adjusted to 3×10^3 conidia/ mL. A mixed isolate conidial suspension containing equivalent numbers of conidia (3×10^3 conidia/ mL) for each isolate was prepared by pipetting an equal volume (250 μ L) of conidial suspension from each of the isolates into a 1.7 mL Eppendorf. The conidial suspension was kept cold during transit to the glass house and during inoculation procedure by being stored on ice contained in a polystyrene container.

5.2.5 Determination of *Pyrenophora tritici-repentis* inoculum viability

In order to verify the viability of the conidial inoculum 10 µL of conidial suspension of each *P. tritici-repentis* isolate was pipetted onto three replicate water agar (15 g/ L) plates and spread using a flame sterilized hockey stick. Plates were placed in an incubator and incubated for 12 hours at 16°C in continuous darkness. After 12 hours incubation, the number of viable germinated conidia characteristic of *P. tritici-repentis* were counted under a stereo microscope to determine the percentage conidial germination.

5.2.6 Inoculation of plants






Wheat seedlings were inoculated at the tillering Zadoks growth stage (ZGS-20-29) (Zadoks *et al.*, 1974). To account for the time taken to inoculate the experiment, the seedlings of all cultivar treatments within one block were inoculated at the same time, starting from block 1 to block 10. Each wheat cultivar seedling within a pot (three seedlings per pot) was inoculated by placing a 5 µL drop of the mixed *P. tritici-repentis* isolate conidial suspension (3×10^3 conidia per mL) using a pipette on marked points located on wheat leaves 2, 3 and 4.

A plastic bag sprayed with approximately 1 mL of sterile water was placed over the three inoculated wheat plants per pot to help generate a relative humidity of 90-100%. The inoculated wheat seedlings were incubated for 48 hours at 100% RH with a 12 hour light/ dark photoperiod and a day/ night temperature of 15/ 20°C. After 48 hours, the plastic bag was removed and the plants were tied up with a cane (Figure 5.1). The inoculated plants were then grown under a 12 hour light/ dark regime in the greenhouse for 3 weeks.

5.2.7 Disease severity rating system

The inoculated plants were evaluated for the severity of their reaction to *P. tritici-repentis* infection three weeks after inoculation. The largest lesion located closest to the inoculation point was rated for leaves 2, 3 and 4 for two wheat plants per pot using a slight modification of the 0-5 lesion rating scale developed by Lamari & Bernier (1989a) as outlined in Table 5.1. The length and width of the largest lesion present on leaf 3 of one wheat plant chosen at random per pot was also measured using a digital calliper to estimate lesion area. Visual assessment of disease severity was based on figures shown in Faris *et al.* (2013) and descriptions in Singh *et al.* (2010).

Table 5.1: Disease score (0-5 scale) based on lesion size and appearance used to assess severity of *Pyrenophora tritici-repentis* lesions which developed on inoculated wheat cultivars (modified from Lamari & Bernier, 1989a).

| Disease score (0-5 scale) | Lesion size and appearance | Example picture |
|----------------------------------|--|---|
| 0 | No lesion present (indicates no infection or strong plant resistance) | |
| 1 | Small, dark brown to black singular spots (<0.5 mm diameter). Lack of any distinctive chlorotic (yellow) or necrotic zones (tan) (Moderate level of resistance/ high tolerance level) |  |
| 2 | Small dark brown to black spots (1-2 mm diameter) with very faint chlorotic borders. Some necrosis (tan to reddish brown) present. (Moderate level of tolerance) |  |
| 3 | Small to medium (2-3 mm diameter) oval to diamond shaped lesion. Dark brown to black appearance which is completely or mostly surrounded with a chlorotic halo. Distinctive necrotic zone present (tan to reddish brown). Lesion may be coalescing with surrounding singular lesions (Slight level of tolerance/ low susceptibility) |  |
| 4 | Medium sized oval/ diamond shaped lesion (3-10 mm diameter). Central eyespot may be present. Lesion has distinctive necrotic zone and chlorotic halo. (Main lesion coalescing with surrounding singular lesions) (Moderate to high level of susceptibility) |  |
| 5 | Medium to large oval/ diamond shaped lesion (10-20 mm diameter) with distinctive central eye spot being indistinguishable. Main lesion coalescing with most surrounding singular lesions. Distinct necrotic zone with a clear surrounding chlorotic halo. (High susceptibility) |  |

5.2.8 Re-isolation assessment

To confirm *P. tritici-repentis* as the causal agent, and to complete Koch's postulates isolations from symptomatic lesion tissue were carried out. One leaf from an inoculated seedling showing the characteristic tan spot symptoms (tan necrosis and yellow chlorosis) was selected from three different replicates chosen at random for six different wheat cultivars. Three randomly selected leaves from three uninoculated plants were also taken for isolation. Isolation from lesion tissues was undertaken following the methods described in Section 2.2.4. The sterilised lesions were placed onto PDA and incubated at 25°C in continuous darkness. After four days incubation, plates with colonies displaying the characteristic morphological features of *P. tritici-repentis* were observed and recorded.

5.2.9 Statistical analysis of cultivar disease scores and lesion area

The mean lesion rating scores and mean lesion area for each wheat cultivar were analysed with one-way analysis of variance (ANOVA) (with randomised blocks) using Genstat version 16. Means were separated using Tukey's 95% confidence interval test at $P \leq 0.05$.

5.3 Results

5.3.1 *Pyrenophora tritici-repentis* conidia viability

All *P. tritici-repentis* conidia observed had germinated, representing 100% viability for all isolates. However, the conidia viability counts indicated a varying range of conidia per 10 µL drop plated, being 1-57 conidia for isolate S12a, 5-33 conidia for isolate G14a, 50-128 conidia for G22a and 2-50 for K16a. This represents a potential range of 7.25-32.5 conidia / 5 µL inoculation drop or 1450-6700 conidia/ mL of a mixed isolate conidial suspension.

5.3.2 Visual observation of tan spot symptoms on cultivars

Symptoms of tan spot differed on each of the different wheat cultivars inoculated with *P. tritici-repentis* (Figure 5.2). No symptoms of tan spot were observed on any of the uninoculated wheat plants. The most characteristic diamond shaped lesions with distinctive eyespots were observed on the wheat cultivars Discovery, Reliance and Wakanui. Less characteristic and irregular shaped tan spot lesions were observed on the other inoculated wheat cultivars. For all wheat cultivars screened, very little chlorosis (yellow) was observed. The most distinctive and largest chlorotic zones were observed on the cultivars Discovery, Reliance, Wakanui and Inferno (Figure 5.2). Distinctive necrosis (tan) of varying degrees were observed on all cultivars inoculated with *P. tritici-repentis*.

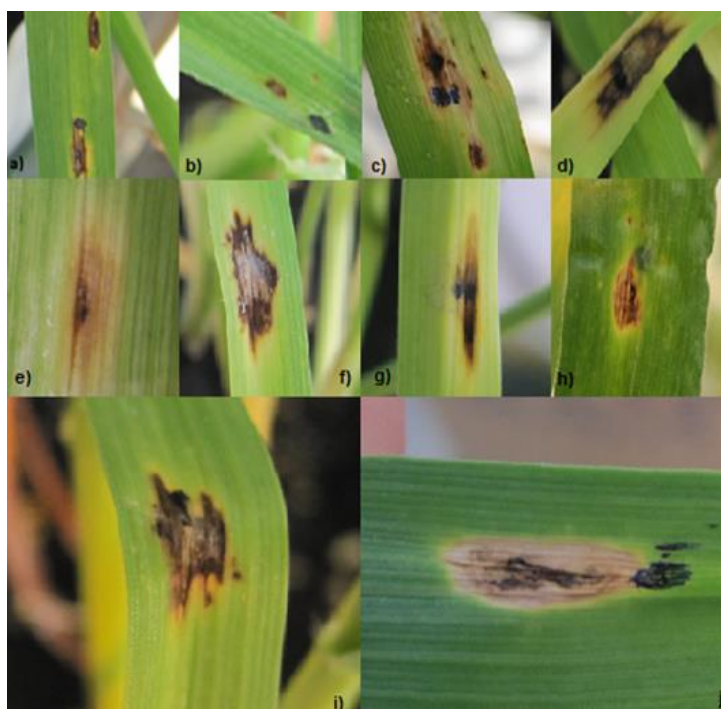


Figure 5.2: Representative tan spot symptoms observed on the different wheat cultivars inoculated with *Pyrenophora tritici-repentis*: a) Discovery, b) Empress, c) Duchess, d) Inferno, e) Saracen, f) Starfire, g) Torch, h) Reliance, i) Viceroy and j) Wakanui.

5.3.3 Determination of cultivar susceptibility to *Pyrenophora tritici-repentis*

There was a highly significant effect of cultivar on the mean disease score ($P \leq 0.001$; Appendix E.1.1) (Table 5.2). The wheat cultivar Empress had a significantly lower ($P \leq 0.05$) mean score

than Saracen, Reliance and Discovery. Of these cultivars, Discovery was also significantly ($P \leq 0.05$) different from the cultivar Duchess. None of the other cultivars differed significantly in mean disease score.

Similar results were also seen with mean lesion area (Table 5.2). There was a significant effect ($P \leq 0.001$; Appendix E.1.2) of cultivar on the mean lesion size which developed on *P. tritici-repentis* inoculated plants. The mean lesions area of Empress was significantly smaller ($P \leq 0.05$) compared to that on cultivars Saracen, Reliance and Discovery. The mean lesion area which developed on Discovery was also significantly larger ($P \leq 0.05$) compared with Duchess, Wakanui, Inferno, Viceroy and Torch.

Table 5.2: The mean disease score (0-5 scale) and mean lesion area (mm²) of ten different New Zealand wheat cultivars assessed 21 days after inoculation with *Pyrenophora tritici-repentis*.

| Cultivar | Mean disease score | Mean lesion area (mm ²) |
|-----------|--------------------|-------------------------------------|
| Empress | 1.3 a | 1.6 A |
| Duchess | 1.6 ab | 2.2 AB |
| Wakanui | 1.9 abc | 2.1 AB |
| Inferno | 1.9 abc | 2.1 AB |
| Starfire | 2.0 abc | 2.7 ABC |
| Viceroy | 2.0 abc | 2.5 AB |
| Torch | 2.3 abc | 2.1 AB |
| Saracen | 2.6 bc | 3.6 BC |
| Reliance | 2.6 bc | 3.6 BC |
| Discovery | 3.0 c | 4.6 C |

Values within columns followed by the same letter are not significantly different according to Tukeys test at $P \leq 0.05$. Susceptible mean score (a-c) was significant ($P \leq 0.001$; F = 4.01). Lesion area (A-C) was also significant ($P \leq 0.001$; F = 4.56).

Colonies characteristic of *P. tritici-repentis* were isolated from all inoculated wheat cultivar lesions plated onto PDA (Figure 5.4). No colonies characteristic of *P. tritici-repentis* or any other fungi were isolated from the tissue of un-inoculated plants on PDA.

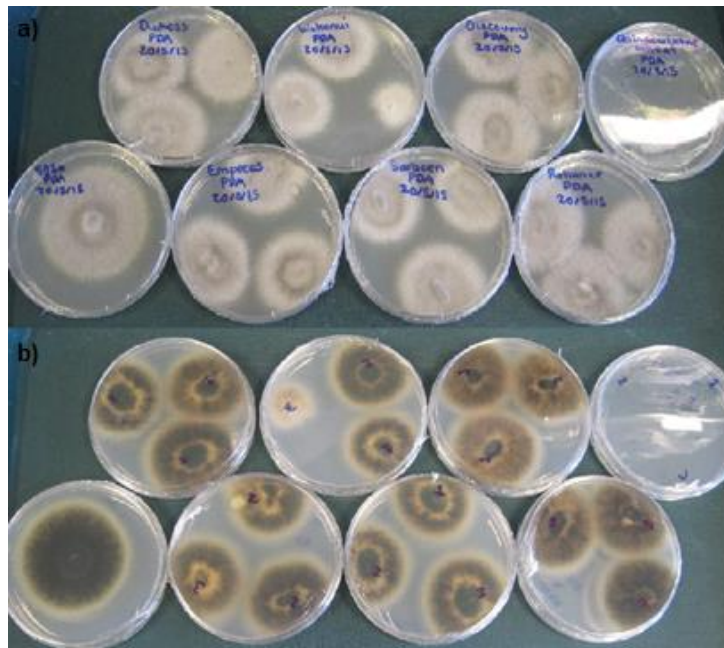


Figure 5.3: Appearance of characteristic *Pyrenophora tritici-repentis* on PDA from tan spot lesions which developed on *P. tritici-repentis* inoculated wheat plants. a) surface and b) underside colony morphology.

5.4 Discussion

This is the first study to investigate the susceptibility of New Zealand wheat cultivars to inoculation with *P. tritici-repentis*. It is also the first study on the pathogenicity of isolates representing the current New Zealand population. Additionally, the New Zealand cultivars specifically evaluated in this study had never been evaluated for their susceptibility to *P. tritici-repentis* to date. As a result of this, there was no current information available on their susceptibility, in New Zealand or other countries.

From the results, it is clear that all of the screened cultivars possess varying degrees of susceptibility to tan spot disease. Based on the results, no levels of complete tan spot resistance appear to exist in any of the wheat cultivar lines tested. However, the cultivars Empress and Duchess displayed symptoms consistent with the description of resistance in the literature (Singh *et al.*, 2010; Faris *et al.*, 2013). These results strongly indicate that at present the majority of the most commonly grown wheat cultivars commercially available in New Zealand should be considered as either susceptible, or at most, moderately resistant to tan spot. The evaluation of wheat cultivars for disease susceptibility under glasshouse conditions has been shown to provide good correlations with their relative susceptibility under field conditions in other studies (Evans *et al.*, 1999). However, the levels of susceptibility/resistance

of the cultivars evaluated in the current study should be further investigated and confirmed under New Zealand field conditions. The results of a preliminary FAR cultivar rating trial conducted during the 2012-2013 summer also provide some similar outcomes to the current study. For example, Wakanui was found to be moderately susceptible and Saracen susceptible to tan spot (FAR Trial report, Rob Craigie, Pers Comm).

Of the ten wheat cultivars assessed, the cultivars Empress and Duchess had the lowest levels of tan spot infection indicating they may possess a level resistance or high tolerance to *P. tritici-repentis*. In contrast, the wheat cultivars Discovery, Reliance and Saracen were the most susceptible cultivars to infection by *P. tritici-repentis*. Based on these results, the cultivars Empress, Duchess and potentially Wakanui and Inferno would be the best cultivars to recommend to a farmer growing in a high tan spot incidence region. However, provided that a robust fungicide program is implemented, it should be emphasised that all of the evaluated cultivars could potentially be grown in a high incidence tan spot region without high yield losses being incurred. Further work investigating the application and timing of fungicides on wheat cultivars with varying degrees of susceptibility is needed in order to help provide more information to wheat farmers.

One major finding from this study indicated that there has been a predominant trend in New Zealand to selectively breed wheat cultivars with traits that favour yield and not tolerance or resistance to diseases such as tan spot. This result is not surprising, considering that tan spot has only recently become a disease of interest to the New Zealand wheat growing industry and therefore to date wheat breeders have not been selectively breeding wheat lines to possess resistance to tan spot. As a result, the levels of potential resistance observed in the wheat lines Empress and Duchess could be presumed to have been attained without any intentional selection for resistance to *P. tritici-repentis*.

At present, wheat cultivars with resistance to tan spot have only been selectively bred in countries such as North America (Tadesse *et al.*, 2006b), where tan spot is regarded as an economically significant disease (Ali & Francl, 2003). Rees *et al.* (1988) reported at the time of their study that three quarters of the assessed wheat cultivars being grown in Australia were found to be highly susceptible to tan spot. In addition, it was also reported that very few of the wheat cultivars assessed in their study possessed any useful level of resistance to tan spot. This low amount of resistance in New Zealand cultivars therefore represents a major issue that will need to be addressed in future in order to improve the performance of newly introduced wheat cultivars against tan spot disease.

In addition to helping to reduce the need for applying fungicides, the development of resistant wheat cultivars in future could also provide an advantageous opportunity to help reduce the incidence of tan spot in New Zealand. There are two main reasons for this. Firstly, based on the results of Chapter 3, there is evidence to suggest that the fungus does not go through very

frequent recombination events. Thus, the likelihood that *P. tritici-repentis* could quickly break down the resistance of a newly introduced cultivar could be much lower in New Zealand in contrast to the risks presented by a genetically diverse population of *P. tritici-repentis* as seen overseas (Singh *et al.*, 2010; Faris *et al.*, 2013).

Secondly, the incorporation of resistance genes into new wheat lines in New Zealand is much less likely to be quickly broken down as in a classical gene-for-gene host pathogen-system. This is because the *P. tritici-repentis* pathosystem follows a toxin model, in that for disease to occur every host specific toxin must have a corresponding host specific receptor (Singh *et al.*, 2010). If a wheat line is bred to lack a specific host receptor for a specific toxin such as Ptr ToxA this would lead to the wheat line possessing resistance to *P. tritici-repentis*. The probability of *P. tritici-repentis* overcoming this resistance by producing a new toxin through gain of function via reverse mutation is considered to be very low (Singh *et al.*, 2010). Additionally, the introduction of resistance genes into new cultivar lines would also not induce high selection pressure on *P. tritici-repentis* populations in contrast to the selection pressures of other control strategies such as fungicides (Singh *et al.*, 2010).

One way the breeding and selection for tan spot resistance could be undertaken in future could be to use molecular marker-assisted selection based methods. Some examples of molecular marker methods include AFLP, microsatellite markers and expressed sequence tag PCR (Singh *et al.*, 2010). These methods could facilitate the screening and selection of cultivars which lack the known toxin sensitivity loci such as *Tsr1* gene (Singh *et al.*, 2010; Faris *et al.*, 2013). At present, New Zealand wheat breeders would potentially only need to breed cultivars possessing resistance to *P. tritici-repentis* races 1 (Ptr ToxA + Ptr ToxC) or 2 (Ptr ToxA), due to these being the only races indicated to be present in New Zealand (Section 2.3.5).

Resistance to tan spot is typically expressed on a wheat plant as small, dark brown lesions that do not increase in size (Singh *et al.*, 2010). In contrast, a susceptible wheat plant will express dark brown spots surrounded by extensive yellow chlorosis and/or tan necrosis (Lamari & Bernier, 1989b; Singh *et al.*, 2010). In this study, eight of the ten inoculated cultivars expressed characteristic symptoms of tan spot which indicated they were susceptible or tolerant to varying degrees to *P. tritici-repentis*. Based on relevant literature (Singh *et al.*, 2010; Faris *et al.*, 2013), Empress and Duchess displayed symptoms that indicated they may possess a level resistance to *P. tritici-repentis*. However, it is difficult to determine whether or not a single relatively high concentration of conidia applied at each leaf inoculation point could have potentially prevented the observation of characteristic resistance symptoms on the other inoculated cultivars in the current study.

It is possible that the inoculation of a relatively high concentration of *P. tritici-repentis* conidia at a specific site may have overloaded the resistance threshold of some of the evaluated wheat cultivar leaves. In the current study, approximately 7.25-32.5 conidia / 5 μ L was applied.

At present, the minimum number of *P. tritici-repentis* conidia required to initiate infection is unknown. However, a study by Evans *et al.* (1996), investigated the inoculum density and infection efficiency of conidia of different isolates of *P. tritici-repentis*. It was found that when conidia were inoculated at concentrations of 2000, 4600, 6100 and 10,700 conidia/ mL in a suspension separate of conidiophores the infection efficiency (lesion incidence) of conidia of three different isolates ranged from 0.91 to 0.55. Despite the numbers of conidia inoculated in the current study not being relatively high, it was apparent that this range was sufficient enough to initiate infection on the evaluated wheat cultivars. It is possible this concentration of conidia was high enough to induce the expression of large necrotic lesions with some chlorosis which were interpreted as indicating a degree of susceptibility. For example, the cultivars Empress, Inferno, Starfire and Viceroy expressed irregularly shaped very dark black lesions with little chlorosis three weeks after being inoculated with *P. tritici-repentis*. It is possible that had the whole surface of these cultivars been uniformly inoculated by a spray-based method using an atomizer they may have expressed a degree of resistance to tan spot. Further greenhouse or field-based work would need to be conducted in order to confirm this.

In addition to the conidia inoculum possibly overloading the response of cultivars, there are some other factors relating to the methodology of this study which could have led to variations in the cultivar responses observed. One issue which was encountered during the cultivar inoculation process was that it took a very long time to inoculate the plants in each replicate. This extended inoculation time may have caused the viability of conidia to be reduced over time. However, there were some methods during the inoculation procedure which were used to potentially account for this. For example, all wheat cultivars were inoculated together in an experimental randomized block design to ensure each replicate was inoculated uniformly. In addition, the statistical analysis also indicated that there was no significant block effect between each replicate. The variation in the range of the concentration of conidia per drop may have also potentially caused the response of some cultivars in different replicates to differ. However, some measures were taken in order to account for this potential source of variation. For example, during both the viability experiment and inoculation procedure the tubes containing conidia were shaken vigorously in order to ensure that conidia were consistently held in suspension. In addition, Tween 20 was also added to the conidial suspensions to ensure that the conidia would not clump together when they were inoculated onto the surfaces of the plants.

The results of this study also strongly indicate that the evaluated wheat cultivars very likely possess the HST Ptr ToxA susceptibility gene *Tsn1*. This was due to necrosis of varying levels being observed on all of the wheat cultivars inoculated with *P. tritici-repentis*. Combined with the knowledge that all of the inoculated isolates were characterised as possessing the Ptr ToxA inducing gene *ToxA* (Section 2.3.5), the necrosis observed in this study can be presumed

to be the likely result of a *Tsn1-ToxA* interaction (Faris *et al.*, 2013). This result further supports the hypothesis that the *ToxA-Tsn1* interaction plays a predominant role in the *P. tritici-repentis*-wheat interaction in New Zealand. Future work could investigate whether or not New Zealand cultivars possess the *Tsn1* gene in order to help determine whether the *ToxA-Tsn1* interaction plays a major role in New Zealand in future.

Another major finding of this study was that the degree of chlorosis observed on the cultivars inoculated with *P. tritici-repentis* appeared to be very low and not as pronounced as the levels reported on some international wheat lines (Singh *et al.*, 2010). This result may indicate that there is either a low prevalence of the chlorosis inducing *tsc1* or *tsc2* genes in the assessed wheat cultivars, or absence of *ToxB* and *ToxC* inducing *P. tritici-repentis* isolates in New Zealand (Faris *et al.*, 2013). The chlorosis observed in this study was most likely not the result of a *ToxB-tsc1* interaction due to the inoculated *P. tritici-repentis* isolates being found to lack the *ToxB* gene (Section 2.3.5). Whether this result could indicate that the *P. tritici-repentis* isolates used to inoculate the wheat cultivars in this study possess the HST Ptr *ToxC* could not be determined in this study. However, as for the *ToxA-Tsn1* gene interaction, this could be further investigated by evaluating for the presence of *tsc1* and more specifically *tsc2* in the current range of wheat cultivars in New Zealand. In addition, New Zealand isolates of *P. tritici-repentis* should also be screened for the *ToxC* gene(s) should a PCR-based method be developed in future. Together this knowledge would provide valuable information for New Zealand wheat breeders to enable the selective breeding of new wheat lines lacking the HST susceptible genes and lead to cultivars with reduced susceptibility to *P. tritici-repentis*.

The drop-based inoculation method and modified rating system used in the current study was found to provide sufficient information on the degree of each cultivars response to *P. tritici-repentis*. For this study a mean disease score and mean lesion area were measured for each inoculated wheat cultivar. The system was effective in allowing the responses of the cultivars to be rated and compared against each other to determine their overall susceptibility or tolerance. The disease score scale was designed to help quantify to some extent the large range of known responses which can be induced by *P. tritici-repentis* when inoculated onto different wheat genotypes. The reason the lesion rating scale of this study was modified from the scale designed by Lamari & Bernier (1989a) was so that it could take into account the fact that the wheat plants were being inoculated with a concentrated amount of conidia at specific points on the plant rather than the whole plant being inoculated using an atomizer as in other tan spot studies. In general, similar ratings of susceptibility were recorded with both measuring methods in the current study. However, in some instances for example with the cultivar Starfire, the results showed that it was more susceptible with the lesion area score compared with the disease score rating. This outcome may have been due to the disease score taking into account the chlorosis whereas the lesion area score did not.

It is also possible that the position of the wheat leaves when inoculated may have been a potential factor which could have affected the results obtained in this study. A study by Raymond *et al.* (1985) reported that in a glasshouse experiment investigating the responses of wheat cultivars to tan spot that the position of the leaf where conidia were inoculated significantly affected the severity score. It was found that for all cultivars younger leaves had lower disease ratings than older wheat leaves, despite both leaf types being inoculated at the same time. Hosford *et al.* (1990) reported that using a 0-5 scale the lesion length rating was higher on the lower leaves for 59 Chinese wheats cultivars inoculated with three *P. tritici-repentis* isolates. In the current study, the susceptibility of leaves 4 and 2 to *P. tritici-repentis* may have varied between the different cultivars, with some having more tolerant younger leaves than others. Future work is required in order to further investigate this.

The stages which the wheat cultivars were inoculated could also have been another factor which led to variations in cultivar response to *P. tritici-repentis* infection. In the current study, wheat cultivars were inoculated at GS-20-29 (ZGS-20-29) (Zadoks *et al.*, 1974) because of the leaf surfaces being larger and easier to inoculate with *P. tritici-repentis* conidia. In contrast, there have been a number of studies where wheat plants have been inoculated at seedling stages e.g. two to five leaf stages (GS 10-19) (Lamari & Bernier, 1989a; Lamari & Bernier, 1989b; Hosford *et al.*, 1990; Tadesse *et al.*, 2006b). However, it is unlikely that this had a significant effect on the cultivar responses observed in this study since Shabeer & Bockus (1988) reported that plants were most physiologically susceptible when inoculated in the field at booting (GS 40-49) and flowering (GS60-69) growth stages. Additionally, the researchers reported that 50% of the total yield losses occurred by the boot stage. Work by Bankina & Priekule (2011) also reported increases in the severity of tan spot in the field at the later stages of wheat development. Future work is needed to investigate the responses and susceptibility of different leaves and growth stages of the same set of wheat cultivars of this study to *P. tritici-repentis* in both glasshouse and field-based experiments.

Whether the outcome of this study would have been different had a whole plant inoculation method been employed instead of a single drop-based method is difficult to determine. One of the main reasons a drop-based inoculation method was used in this study was because of the difficulty to induce the production of high numbers of *P. tritici-repentis* conidia *in vitro*. Further work should be carried out to validate whether the results of the single drop method correlate with a whole plant spray inoculation method. However, if shown to provide similar results the single inoculation point method has several advantages including the need for less conidial inoculum and that the lesion development associated with different treatments such as conidial concentration and fungicide evaluation can be more accurately assessed. Additionally, the work of this study could be further improved by including international differential wheat cultivars, such as Glenlea, Salamouni and 6B-365 (Faris *et al.*, 2013), with different

susceptibilities/ resistances to *P. tritici-repentis* into a susceptibility study to provide a baseline to enable the susceptibility of New Zealand cultivars to be evaluated against.

5.5 Conclusions

This study represents the first attempt to investigate the susceptibility of New Zealand wheat cultivars to *P. tritici-repentis*. The results of this study showed that the wheat cultivars Discovery, Reliance and Saracen were the most susceptible, whilst Empress and Duchess displayed symptoms that indicated they may possess a level of resistance to *P. tritici-repentis*. Further work utilizing a spray-based inoculation method in future would be beneficial in order to further investigate the responses of the assessed cultivars to *P. tritici-repentis*. The results of this study also indicate that the cultivars Empress, Duchess and Wakanui would be most suitable for cultivation in regions of New Zealand with a high incidence of tan spot. However, all of the evaluated cultivars could potentially be grown in areas of high tan spot incidence provided their cultivation includes a robust fungicide program. Further work incorporating the use of resistant overseas cultivars and investigating the HST receptor genes possessed by New Zealand wheat lines would greatly benefit the breeding of resistance wheat lines in the future.

Chapter 6

Concluding discussion

This study is the first to characterise populations of *P. tritici-repentis* in New Zealand. The aim of this study was to determine the distribution and genetic structure of *P. tritici-repentis* in the South Island of New Zealand. In addition, this study also set out to investigate the sensitivity of *P. tritici-repentis* to commonly used fungicides and the susceptibility of different wheat cultivars to *P. tritici-repentis* infection.

Prior to the undertaking of this study, *P. tritici-repentis* was a poorly understood pathogen in New Zealand, despite reportedly being first isolated from wheat seed in 1976 (Hampton & Matthews, 1978). Additionally, despite the genetic structure of overseas *P. tritici-repentis* populations being well characterised (Friesen *et al.*, 2005; Aboukhaddour *et al.*, 2011; Gurung *et al.*, 2013), this is the first study to characterise New Zealand *P. tritici-repentis* isolates.

In this study, a total of 15 different populations of *P. tritici-repentis* were collected and positively identified by both molecular and morphology-based methods. The survey showed that *P. tritici-repentis* was widely distributed in the South Island of New Zealand. The estimated range of *P. tritici-repentis* currently stretches from as far south as Clinton, Southland to as far north as Greendale North Canterbury representing the main wheat growing area of the South Island. Whether or not *P. tritici-repentis* is also present in the North Island was not investigated in the current study and therefore requires further investigation in future. It could also not be determined whether or not the distribution pattern revealed in this study was the result of a recent single incursion or multiple incursions over the last 20-30 years and requires further investigation. The results of a follow up farmer questionnaire also showed that the re-use and sowing of untreated wheat seed and continuous sowing of wheat over 10-20 years may have been potentially responsible for exacerbating tan spot infestations.

The use of molecular tools to identify *P. tritici-repentis* in the New Zealand isolate collection allowed a high degree of accuracy. This isolate collection will provide a valuable resource for any future molecular-based studies in New Zealand and overseas. An additional milestone achieved by this study was, after some difficulty trying to induce *P. tritici-repentis* to sporulate *in vitro* for single spore isolations, the development of a reliable method for harvesting conidia based on slight modifications of the method described by Lamari & Bernier (1989a). This method could be utilised for any future studies in New Zealand which attempt to generate *P. tritici-repentis* single spore isolates for molecular-based studies.

Another major finding of this study was the discovery that the current race structure of the New Zealand *P. tritici-repentis* population is most likely either virulence races 1, or 2, since isolates

had the HST Ptr ToxA gene *ToxA*, but lack the Ptr ToxB gene *ToxB*. This finding was similar to other related studies where races 1 and 2 were found to be the most predominate virulence races in Australia (Antoni *et al.*, 2010), Canada (Lamari *et al.*, 1998; Lamari *et al.*, 2005; Aboukhaddour *et al.*, 2013), the USA (Friesen *et al.*, 2005) and South America (Gamba *et al.*, 2012). Further work should be carried out to determine whether New Zealand populations of *P. tritici-repentis* possess the ability to produce Ptr ToxC in order to accurately characterise the race structure of New Zealand populations.

Another finding of this study is that the *ToxA-Tsn1* gene interaction appears to be a dominating factor in the *P. tritici-repentis*-wheat interaction in New Zealand, whilst the *ToxB-Tsc2* interaction appears to be insignificant. This finding correlates with a study by Antoni *et al.* (2010) which determined that the *ToxA-Tsn1* interaction was a significant factor in the wheat *P. tritici-repentis* relationship in Australia in contrast to *ToxB-Tsc2* which was determined to be less significant. Future work could be conducted to identify which HST receptor genes the current New Zealand wheat lines possess to help facilitate the identification of susceptible and resistance wheat lines.

Representative isolates of each of the regions sampled were used as a diverse group to investigate the genetic variation of populations in New Zealand. This number of isolates was chosen to best represent the possible diversity. Three different molecular methods (UP-PCR, RAPD and microsatellite analysis) were utilised in this study with the results obtained from all three methods strongly indicating that the genetic structure of the South Island *P. tritici-repentis* isolates was very clonal. This result was unexpected, considering that *P. tritici-repentis* is a fungus which is known to frequently undergo sexual reproduction (Lepoint *et al.*, 2010). In addition, when New Zealand isolates were compared with 12 different international *P. tritici-repentis* isolates the New Zealand isolates were genetically distinct. Based on these results, the genetic structure of the New Zealand *P. tritici-repentis* isolates characterised in this study is unique when compared to other global populations. Further work characterising the genetic structure of New Zealand populations of *P. tritici-repentis* overtime should be undertaken to help determine the rate at which New Zealand populations undergo recombination events.

This study has also demonstrated the versatility and differing levels of information provided by the three different molecular methods for characterising *P. tritici-repentis*. In this study, UP-PCR, which had not been previously utilised to characterise *P. tritici-repentis*, was found to be unsuitable for detecting genetic diversity between isolates of *P. tritici-repentis*. This was due to no polymorphic bands being found to be generated for any of the New Zealand or international *P. tritici-repentis* isolates used in this study. This result was also unexpected, considering the fact that UP-PCR had been reported to detect low to high levels of genetic diversity in other pathogenic fungi which were known to either lack a sexual phase or undergo sexual reproduction infrequently (Pottinger *et al.*, 2002; Tyson *et al.*, 2002; Obanor *et al.*, 2010;

Baskathevan *et al.*, 2012). RAPD could differentiate *P. tritici-repentis* isolates based both on their race and point of geographic origin. However, it was unable to detect any genetic differences between the New Zealand isolates assessed in this study. Microsatellite analysis was found to effectively differentiate international isolates from one another, regardless of their race, or point of geographic origin. However, it also did not detect any genetic differences between the New Zealand isolates characterised in this study.

The findings of this study suggest that New Zealand populations of *P. tritici-repentis* do not undergo frequent genetic recombination events. Unfortunately, the factors which could have been contributing to this phenomenon were not identified in this study. Possible explanations which could explain this absence of genetic variation include the populations of *P. tritici-repentis* studied being part of a recent incursion and the life cycle of *P. tritici-repentis* being interrupted by both abiotic and cultural factors in the New Zealand wheat growing environment. Future work could help investigate and elucidate the nature of these unique New Zealand environmental factors.

It may however, also be possible that the environment may not be the factor which prevents sexual reproduction of *P. tritici-repentis* isolates in New Zealand. Alternatively, it could possibly be related to specific strains of *P. tritici-repentis* being present in New Zealand which lack the ability to develop pseudothecia. This hypothesis could be investigated in future by studying the relative contribution of sexual (ascospores) and asexual (conidia) reproduction on the disease cycle of tan spot in New Zealand. For example, this could be undertaken using spore trapping methods to look at what *P. tritici-repentis* spore types are predominately produced and at what times they released. Future work could also identify if *P. tritici-repentis* can produce pseudothecia and therefore ascospores under field conditions. This could be undertaken by sampling and observing wheat stubble from previously infected crops over the duration of a cropping season. Additionally, future work could also determine whether or not New Zealand *P. tritici-repentis* isolates are able to produce pseudothecia *in vitro*. For example, a study by James *et al.* (1991) developed a reliable procedure for producing *P. tritici-repentis* on wheat straw under controlled environment conditions using different substrates and nutrients.

The fungicide bioassays conducted in this study demonstrated that the *in vitro* growth of *P. tritici-repentis* could be inhibited with three different classes of fungicides. Due to the generation of conidia being difficult, only the *in vitro* inhibition of mycelial growth of four *P. tritici-repentis* isolates was investigated in this study. All of the evaluated fungicides had ability to effectively reduce the mycelial growth of *P. tritici-repentis in vitro* below 50%. Overall, this was a very favourable finding because it indicated that all of the assessed fungicides have good levels of control over *P. tritici-repentis* in New Zealand. Of the ten assessed fungicides, isopyrazam, propiconazole and prothioconazole had the lowest EC₅₀ values which indicated they were the most effective fungicides against *P. tritici-repentis*. The fungicides azoxystrobin,

pyraclostrobin and fluxapyroxad were the least effective at reducing *in vitro* growth of *P. tritici-repentis*. The fungicide EC₅₀ results obtained in this study contrasted with the results of a similar overseas study (Beard *et al.*, 2009). Factors such as media type, fungicide formulation, number of isolates tested and genetic structure of the populations tested could possibly have led to some differences between the current study and other *in vitro* studies. However, the results of this study also correlated to a recent New Zealand field study (Harvey *et al.*, 2015). Results of the farmer questionnaire also indicated that the timing and rate of application of the tested fungicides were important for helping to effectively control tan spot. Future work could further investigate the effects of fungicides on the germination of *P. tritici-repentis* conidia *in vitro* and effects of fungicides *in planta* in both glasshouse and field trials to help validate the results of this study. Additionally, the results of this study could also be used as a baseline for future studies attempting to monitor for changes in fungicide sensitivity of *P. tritici-repentis* populations.

This study also identified the susceptibility of a range of commonly cultivated New Zealand wheat cultivars to *P. tritici-repentis* infection. Of the cultivars evaluated, Empress and Duchess had the lowest susceptibility towards *P. tritici-repentis* whilst Discovery, Reliance and Saracen were the most susceptible to *P. tritici-repentis*. Empress and Duchess also displayed symptoms that indicated they may possess a level of resistance to *P. tritici-repentis*. The findings of this study indicated that there is a need to breed more new wheat lines which possess levels of tolerance or resistance to *P. tritici-repentis* in the future. In future work, New Zealand cultivars could be screened to identify whether they possess the Ptr ToxA susceptibility *Tsn1* gene and compared with cultivars from overseas which are known to be resistant to tan spot to enable the susceptibility of New Zealand cultivars to be evaluated against. Further work, depending on whether ToxC is identified as being present in the New Zealand *P. tritici-repentis* population, screening for the presence of Ptr ToxC susceptibility gene(s) could also be carried out.

A conidial drop inoculation method was developed for inoculating wheat plants in this study. Overall, the accuracy of this method for assessing cultivar response to *P. tritici-repentis* infection was difficult to determine in this study. This was due to their being no studies in New Zealand to compare the results against. In addition, there was a possibility that the relatively high concentrations of conidia (7.25-32.5 conidia per droplet) inoculated onto wheat plants may have overloaded the tolerance/ resistance thresholds of some of the wheat cultivars. This could have prevented any clear characteristic levels of tan spot resistance from being identified. Future work could further investigate the overall accuracy of this method for determining host response by comparing its effectiveness against a spray-based method using the same set of wheat cultivars evaluated in this study at different growth stages and leaf positions. If this method is found to provide similar results, this method could have several

advantages including the need for less conidial inoculum and it could allow the lesion development associated with different treatments such as conidial concentration and/or fungicide evaluation to be more accurately assessed.

Since this study showed widespread distribution of this pathogen in wheat growing areas, the control of tan spot will be very important in future in order to ensure good wheat yields can be achieved. Based on all of the results of this study, it is possible to envision how a potential tan spot management program could be designed to be recommended to farmers in the future. Results of the farmer questionnaire strongly indicated that the continuous sowing of wheat season after season was a major contributing factor controlling the incidence of tan spot epidemics in New Zealand. Therefore, based on this finding it should be strongly recommended that farmers continuously rotate wheat crops around their farm annually. This farm practice would help to break the life cycle of *P. tritici-repentis* which is strongly dependent on the residues of infected wheat plants being retained in the same field season to season. The incorporation of farm practices such as direct drilling would also not be strongly recommended due to the fact that they encourage the retention of infected wheat stubble. In addition to rotating wheat crops, the burning of wheat stubble and other infected plant residues could also be very effective in facilitating the destruction of *P. tritici-repentis* inoculum season to season. Similar work by Wegulo (2011) recommended that tan spot could be managed in the USA by the use of rotations with non-host plants and destruction of infected residues by burial or burning.

The discovery that *P. tritici-repentis* appears to infrequently undergo genetic recombination events in the New Zealand environment could have a number of potentially positive implications for the control of tan spot in future. Firstly, the ability of New Zealand *P. tritici-repentis* isolates to quickly adapt to selective pressures such as fungicides could be low. Secondly, the low genotypic diversity, and therefore race structure, of *P. tritici-repentis* isolates could make it much easier to manage tan spot outbreaks in future through the breeding and introduction of resistance wheat lines.

The fungicides isopyrazam, propiconazole and prothioconazole would be the most strongly recommended fungicides for use in the field to control severe outbreaks of tan spot. The application of the fungicides azoxystrobin, pyraclostrobin and epoxiconazole is not strongly advised. This is because these fungicides lack the ability to effectively control the economically significant disease speckled leaf blotch (*Z. tritici*) in the field due to the development of increased insensitivity or resistance (Stewart *et al.*, 2014). It is also strongly advised that these fungicides be applied at their recommended rates and mixed with fungicides of other classes to preserve their efficacy and reduce the risks of resistance development. Finally, the cultivars Empress and Duchess would be the most recommended cultivars for farmers growing in regions of the South Island with a high incidence of tan spot. However, all of the evaluated

cultivars could potentially be grown in areas of high tan spot incidence provided their cultivation includes a robust fungicide program.

In summary, this study has provided new information on the genetic structure, distribution and incidence of tan spot in the South Island of New Zealand. This study has also provided the first insight into the sensitivity of *P. tritici-repentis* isolates to a broad range of fungicides and the susceptibility of commonly cultivated wheat cultivars to tan spot. Overall, this study has provided New Zealand wheat growers with valuable knowledge that will facilitate the development and implementation of control strategies to help manage tan spot into the foreseeable future.

References

- Aboukhaddour, R., Cloutier, S., Lamari, L., Strelkov, S.E. (2011). Simple sequence repeats and diversity of globally distributed populations of *Pyrenophora tritici-repentis*. *Canadian Journal of Plant Pathology*. **33**: 389-399.
- Aboukhaddour, R., Turkington, T.K., Strelkov, S.E. (2013). Race structure of *Pyrenophora tritici-repentis* (tan spot of wheat) in Alberta, Canada. *Canadian Journal of Plant Pathology*. **35**: 256-268.
- Adhikari, T.B., Ali, S., Burlakoti, R.R., Singh, P.K., Mergoum, M., Goodwin, S.B. (2008). Genetic structure of *Phaeosphaeria nodorum* populations in the north, central and midwestern United States. *Phytopathology*. **98**: 101-107.
- Ali, S., Francl, L.J. (2003). Population race structure of *Pyrenophora tritici-repentis* prevalent on wheat and noncereal grasses in the great plains. *Plant Disease*. **87**: 418-422.
- Ali, S., Gurung, S., Adhikari, T.B. (2010). Identification and characterization of novel isolates of *Pyrenophora tritici-repentis* from Arkansas. *Plant Disease*. **94**: 229-235.
- Andrie, R.M., Pandelova, I., Ciuffetti, L.M. (2007). A combination of phenotypic and genotypic characterization strengthens *Pyrenophora tritici-repentis* race identification. *Phytopathology*. **97**: 694-701.
- Antoni, E.A., Rybak, K., Tucker, M.P., Hane, J.K., Solomon, P.S., Drenth, A., Shanker, M., Oliver, R.P. (2010). Ubiquity of ToxA and absence of ToxB in Australian populations of *Pyrenophora tritici-repentis*. *Australasian Plant Pathology*. **39**: 63-68.
- Aung, T.S.G. (2001). *Molecular polymorphism and virulence in Pyrenophora tritici-repentis*. M.Sc. Thesis. University of Manitoba, Winnipeg, Canada.
- Ballance, G.M., Lamari, L., Bernier, C.C. (1989). Purification and characterization of a host-selective necrosis toxin from *Pyrenophora tritici-repentis*. *Physiological and Molecular Plant Pathology*. **35**: 203-213.
- Baskarathevan, J. (2011). *Botryosphaeriaceous infection in New Zealand vineyards: Identification, population structure and genetic diversity*. Unpublished PhD thesis, Lincoln University, New Zealand.
- Baskarathevan, J., Jaspers, M.V., Jones, E.E., Cruickshank, R.H., Ridgway, H.J. (2012). Genetic and pathogenic diversity of *Neofusicoccum parvum* in New Zealand vineyards. *Fungal Biology*. **116**: 276-288.

- Bankina, B. (2005). Tan spot – the most harmful wheat leaf disease in Latvia. *Acta Agrobotanica*. **58**: 5-10.
- Bankina, B., Priekule, I. (2011). A review of tan spot research in the Baltic countries: occurrence, biology and possibilities of control. *Žemdirbyste= Agriculture*. **98**: 3-9.
- Beard, C., Loughman, R., Smith, A., Speijers, J. (2009). Baseline sensitivity to three triazole fungicides in *Pyrenophora tritici-repentis*. *Australian Plant Pathology*. **38**: 168-172.
- Bleach, C.M. (2012). *Management of Cylindrocarpon black foot disease in New Zealand nurseries and vineyards*. Unpublished PhD thesis, Lincoln University, New Zealand.
- Bulat, S.A., Mironenko, N.V., Lapteva, M.N., Strelchenko, P.P. (1994). Polymerase chain reaction with universal primers (UP-PCR) and its applications to plant genome analysis: In: Adams, R.P., Millar, J.S., Golenberg, E.M., Adams, J.E. (eds), *Conservation of Plant Genes II: utilization of ancient and modern DNA*, pp. 113-129.
- Bulat, S.A., Lübeck, M., Mironenko, N., Jensen, D.F., Lübeck, P.S. (1998). UP-PCR analysis and ITS1 ribotyping of strains of *Trichoderma* and *Gliocladium*. *Mycological Research*. **102**: 933-943.
- Cabral, A., Groenewald, J.Z, Rego, C., Oliveira, H., Crous, P.W. (2012). *Cylindrocarpon* root rot: multi-gene analysis reveals novel species within the *Ilyonectria radicolola* species complex. *Mycological Progress*. **11**: 655-688.
- Ciuffetti, L.M., Tuori, R.P., Gaventa, M. (1997). A single gene encodes a selective toxin causal to the development of tan spot on wheat. *Plant Cell*. **9**: 135-144.
- Ciuffetti, L.M., Tuori, R.P. (1999). Advances in the characterization of the *Pyrenophora tritici-repentis*-wheat interaction. *Phytopathology*. **89**: 444-449.
- Colson, E.S., Platz, G.J., Usher, T.R. (2003). Fungicidal control of *Pyrenophora tritici-repentis* in wheat. *Australasian Plant Pathology*. **32**: 241-246.
- Cools, H.J., Fraaije, B.A. (2012). Resistance to azole fungicides in *Mycosphaerella graminicola*: mechanism and management. In *Fungicide Resistance in Crop Protection: Risk and Management*. pp. 64-77. Ed. Thind, T.S. CAB International. Wallingford, UK.
- Cox, C. M., Garrett, K. A., Bowden, R. L., Fritz, A. K., Dendy, S. P., Heer, W. F. (2004). Cultivar mixtures for the simultaneous management of multiple diseases: tan spot and leaf rust of wheat. *Phytopathology*. **94**: 961-969.
- Cromey, M.G., Francis, G.S., Trimmer, L.A., Tabley, F.J., Gillespie, P.M., Fraser, A.J., Pearson, R.C., Butler, R.C., Curtin, D., Bithell, S.L. (2008). Influences of crop rotation,

- tillage, residue management and winter cover crop on take-all in spring wheat. *New Zealand Plant Protection*. **61**: 261-269.
- Cumagun, C.J.R., Hockenhull, J., Lubeck, M. (2000). Characterization of *Trichoderma* isolates from Philippine rice fields by UP-PCR and rDNA-ITS1 analysis: identification of UP-PCR markers. *Journal of Phytopathology-Phytopathologische Zeitschrift*. **148**: 109-115.
- Cunfer, B.M. (2000). *Stagonospora* and *Septoria* diseases of barley, oat and rye. *Canadian Journal of Plant Pathology*. **22**: 332-348.
- De Wolf, E. (2008). *Tan spot*. Plant Pathology. <http://www.ksre.k-state.edu/bookstore/pubs/EP134.pdf>. (Retrieved 16/7/2013).
- Duff, A.D.S. (1954). A new disease of wheat in Kenya caused by a species of *Pyrenophora*. *East African Agricultural Journal*. **18**: 225-229.
- Effertz, R.J., Meinhardt, S.W., Anderson, J.A., Jordahl, J.G., Francl, L.J. (2002). Identification of a chlorosis-inducing toxin from *Pyrenophora tritici-repentis* and the chromosomal location of an insensitivity locus in wheat. *Phytopathology*. **92**: 527-533.
- Entz, M. H., Berg, C. V. D., Stobbe, E. H., Rossnagel, B. G., Lafond, G. P., Austenson, H. M. (1990). Effect of late-season fungicide application on grain yield and seed size distribution in wheat and barley. *Canadian Journal of Plant Science*. **70**: 699-706.
- Evans, C.K., Hunger, R.M., Siegerist, W.C. (1996). Inoculum density and infection efficiency of conidia and conidiophores of isolates of *Pyrenophora tritici-repentis*. *Plant Disease*. **80**: 502-512.
- Evans, C.K., Hunger, R.M., Siegerist, W.C. (1999). Comparison of greenhouse and field testing to identify wheat resistant to tan spot. *Plant Disease*. **83**: 269-273.
- Faris, J.D., Anderson, J.A., Francl, L.J., Jordahl, J.G. (1996). Chromosomal location of a gene conditioning insensitivity in wheat to a necrosis-inducing culture filtrate from *Pyrenophora tritici-repentis*. *Phytopathology*. **86**: 459-463.
- Faris, J.D., Anderson, J.A., Francl, L.J., Jordahl, J.G. (1997). RFLP mapping of resistance to chlorosis induction by *Pyrenophora tritici-repentis* in wheat. *Theoretical and Applied Genetics*. **94**: 98-103.
- Faris, J.D., Friesen, T.L. (2005). Identification of quantitative trait loci for non-specific resistance to tan spot of wheat. *Theoretical and Applied Genetics*. **111**: 386-392.
- Faris, J.D., Liu, Z., Xu, S.S. (2013). Genetics of tan spot resistance in wheat. *Theoretical and Applied Genetics*. **126**: 2197-2217.

- FRAC, 2013. *List of resistant plant pathogenic organisms, February 2013*.
<http://www.frac.info/>. (Retrieved 25th February 2014).
- Friesen, T.L., Faris, J.D. (2004). Molecular mapping of resistance to *Pyrenophora tritici-repentis* race 5 and sensitivity to *Ptr* ToxB in wheat. *Theoretical and Applied Genetics*. **109**: 464-471.
- Friesen, T.L., Ali, S., Klein, K.K., Rasmussen, J.B. (2005). Population genetic analysis of a global collection of *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat. *Phytopathology*. **95**: 1144-1150.
- Friesen, T.L., Stukenbrock, E.H., Liu, Z., Meinhardt, S., Ling, H., Faris, J.D., Rasmussen, J.B., Solomon, P.S., McDonald, B.A., Oliver, R.P. (2006). Emergence of a new disease as a result of intraspecific virulence gene transfer. *Nature Genetics*. **38**: 953-956.
- Gamba, F.M., Strelkov, S.E., Lamari, L. (2012). Virulence of *Pyrenophora tritici-repentis* in the Southern Cone Region of South America. *Canadian Journal of Plant Pathology*. **34**: 545-550,
- Gilchrist, S.L., Fuentes, S.F., de Bauer, M.de L.de la Isla. (1984). Determination de fuentes de Resistencia contra *Helminthosporium tritici-repentis* bajo conditions de campo e invernadero. *Agrociencia*. **56**: 95-105.
- Gurung, S., Goodwin, S.B., Kabbage, M., Bockus, W.W., Adhikari, T.B. (2011). Genetic differentiation at microsatellite loci among populations of *Mycosphaerella graminicola* from California, Indiana, Kansas and North Dakota. *Phytopathology*. **101**: 1251-1259.
- Gurung, S., Short, D.P.G., Adhikari, T.B. (2013). Global population structure and migration patterns suggest significant population differentiation among isolates of *Pyrenophora tritici-repentis*. *Fungal Genetics and Biology*. **52**: 32-41.
- Hampton, J.G., Matthews, D. (1978). *Drechslera* spp. on New Zealand certified cereal seed. *New Zealand Journal of Agricultural Research*. **21**: 539-542.
- Harvey, I.C., Craigie, R.A., McCloy, B. (2015). The control of tan spot of wheat (caused by *Pyrenophora tritici-repentis*): a possible emerging disease in New Zealand. *New Zealand Plant Protection*. **68**: 428-433.
- Haen, K.M., Lu, H.J., Friesen, T.L., Faris, J.D. (2004). Genomic targeting and high resolution mapping of the *Tsn1* gene in wheat. *Crop Science*. **44**: 951-962.
- Ho, W.C., Ko, W.H. (1997). A simple method for obtaining single-spore isolates of fungi. *Botanical Bulletin of Academia Sinica*. **38**: 41-44.

- Hosford, R.M. (1971). A form of *Pyrenophora trichostoma* pathogenic to wheat and other grasses. *Phytopathology*. **61**: 28-32.
- Hosford, R.M., Larez, C.R., Hammond, J.J. (1987). Interaction of wet period and temperature on *Pyrenophora tritici-repentis* infection and development in wheats of differing resistance. *Phytopathology*. **77**: 1021-1027.
- Hosford, R.M., Jordahl, J.G., Hammond, J.J. (1990). Effect of wheat genotype, leaf position, growth stage, fungal isolate, and wheat period on tan spot lesions. *Plant Disease*. **74**: 385-390.
- Hunger, R.M., Brown, D.A. (1987). Colony color, growth, sporulation, fungicide sensitivity and pathogenicity of *Pyrenophora tritici-repentis*. *Plant Disease*. **71**: 907-910.
- James, T.D.W., Summerell, B.A., Burgess, L.W. (1991). Production of pseudothecia and conidia by *Pyrenophora tritici-repentis* in relation to nutrients and substrate. *Australasian Plant Pathology*. **20**:92-96.
- Jorgensen, L.N., Olsen, L.V. (2007). Control of tan spot (*Drechslera tritici-repentis*) using cultivar resistance, tillage methods and fungicides. *Crop Protection*. **26**: 1606-1616.
- Kader, K. A. (2010). *Fitness, virulence and genetic variability in Pyrenophora tritici-repentis isolates causing tan spot of wheat in Oklahoma*. Unpublished PhD thesis, Oklahoma State University, USA.
- Krupinsky, J. M. (1992). Grass hosts of *Pyrenophora tritici-repentis*. *Plant disease*. **76**: 92-95.
- Klinkowski, M. (1970). Catastrophic plant diseases. *Annual Review of Phytopathology*. **8**: 37-60.
- Lamari, L., Bernier, C.C. (1989a). Evaluation of wheat lines and cultivars to tan spot (*Pyrenophora tritici-repentis*) based on lesion type. *Canadian Journal of Plant Pathology*. **11**: 49-56.
- Lamari, L., Bernier, C.C. (1989b). Virulence of isolates of *Pyrenophora tritici-repentis* on 11 wheat cultivars and cytology of differential host reactions. *Canadian Journal of Plant Pathology*. **11**: 284-290.
- Lamari, L., Bernier, C.C. (1991). Genetics of tan necrosis and extensive chlorosis in tan spot of wheat caused by *Pyrenophora tritici-repentis*. *Phytopathology*. **81**: 1092-1095.
- Lamari, L., Bernier, C.C. (1994). Temperature-induced resistance to tan spot (*Pyrenophora tritici-repentis*) of wheat. *Canadian Journal of Plant Pathology*. **16**: 279-288.

- Lamari, L., Bernier, C.C. (1995). Seed transmission of *Pyrenophora tritici-repentis*, causal fungus of tan spot of wheat. *European Journal of Plant Pathology*. **101**: 81-91.
- Lamari, L., Sayoud, R., Boulif, M., Bernier, C.C. (1995). Identification of a new race of *Pyrenophora tritici-repentis*: implications for the current pathotype classification system. *Canadian Journal of Plant Pathology*. **17**: 312-318.
- Lamari, L., Gilbert, J., Tekauz, A. (1998). Race differentiation in *Pyrenophora tritici-repentis* and survey of physiologic variation in western Canada. *Canadian Journal of Plant Pathology*. **20**: 396-400.
- Lamari, L., Strelkov, S.E., Yahyaoui, A., Orabi, J., Smith, R.B. (2003). The identification of two new races of *Pyrenophora tritici-repentis* from the host center of diversity confirms a one-to-one relationship in tan spot of wheat. *Phytopathology*. **93**: 391-396.
- Lamari, L., Strelkov, S.E., Yahyaoui, A., Amedov, M., Saidov, M., Djunusova, M., Koichibayev, M. (2005). Virulence of *Pyrenophora tritici-repentis* in the countries of the Silk Road. *Canadian Journal of Plant Pathology*. **27**: 383-388.
- Larez, C. R., Hosford Jr, R. M., Freeman, T. P. (1986). Infection of wheat and oats by *Pyrenophora tritici-repentis* and initial characterization of resistance. *Phytopathology*. **76**: 931-938.
- Leisova, L., Minarikova, V., Kucera, L., Ovesna, J. (2005). Genetic diversity of *Pyrenophora teres* isolates as detected by AFLP analysis. *Journal of Phytopathology*. **153**: 569-578.
- Leisova, L., Hanzalova, A., Kucera, L. (2008). Genetic diversity of *Pyrenophora tritici-repentis* isolates revealed by AFLP analysis. *Journal of Plant Pathology*. **90**: 233-245.
- Lepoint, P., Renard, M.E., Legrève, A., Duveiller, E., Maraite, H. (2010). Genetic diversity of the mating type and toxin production genes in *Pyrenophora tritici-repentis*. *Phytopathology*. **100**: 474-483.
- Lübeck, P.S., Alekhina, I.A., Lübeck, M., Bulat, S.A. (1998). UP-PCR genotyping and rDNA analysis of *Ascochyta pisi* Lib. *Journal of Phytopathology*. **146**: 51-55.
- Lübeck, P.S., Alekhina, I.A., Stephensen Lübeck, P., Funck Jensen, D., Bulat, S.A. (1999). Delineation of *Trichoderma harzianum* into two different genotypic groups by a highly robust fingerprinting method, UP-PCR, and UP-PCR product cross-hybridization. *Mycological Research*. **103**: 289-298.
- Manning, V.A., Pandelova, I., Dhillon, B., Wilhelm, L.J., Goodwin, S.B., Berlin, A.M., Figueroa, M., Freitag, M., Hane, J.K., Henrissat, B., Holman, W.H., Kodira, C.D., Martin, J., Oliver, R.P., Robbertse, B., Schackwitz, W., Schwartz, D.C., Spatafora, J.W., Turgeon,

- B.G., Yandava, C., Young, S., Zhou, S., Zeng, Q., Grigoriev, I.V., Ma, L.J., Ciuffetti, L.M. (2013). Comparative genomics of a plant-pathogenic fungus, *Pyrenophora tritici-repentis*, reveals transduplication and the impact of repeat elements on pathogenicity and population divergence. *G3: Genes| Genomes| Genetics*. **3**: 41-63.
- Martinez, J.P., Oesch, N.W., Ciuffetti, L.M. (2004). Characterization of the multiple-copy host-selective toxin gene, *ToxB*, in pathogenic and nonpathogenic isolates of *Pyrenophora tritici-repentis*. *Molecular Plant-Microbe Interactions*. **17**: 467-474.
- Mereno, M.V., Stenglein, S.A., Perello, A.E. *Pyrenophora tritici-repentis*, causal agent of tan spot: A review of intraspecific genetic diversity. (2012). In: *The Molecular Basis of Plant Genetic Diversity*. pp. 297-330. Eds Caliskan, M. Intechopen.
- Millner, J. P., Roskruge, N. R., Dymond, J. R. The New Zealand arable industry. (2013). In: *Ecosystem services in New Zealand: conditions and trends* pp. 102-114.
- Mironenko, N., Timopheeva, E., Mikhailova, L., Kopahnke, D., Krämer, I., Ordon, F. (2007). Intraspecific genetic diversity of *Pyrenophora tritici-repentis* (Died.) Drechs. (*Drechslera tritici-repentis* [Died.] Shoem.) detected by random amplified polymorphic DNA assays. *Archives of Phytopathology and Plant Protection*. **40**: 431-440.
- Mitra, M. (1934). A leaf spot disease of wheat caused by *Helminthosporium tritici-repentis* Died. *Indian Journal of Agricultural Science*. **4**: 692-700.
- Obanor, F.O., Walter, M., Jones, E.E., Candy, J., Jaspers, M.V. (2010). Genetic variation in *Spilocaea oleagina* populations from New Zealand olive groves. *Australasian Plant Pathology*. **39**: 508-516.
- Oliver, R.P., Lord, M., Rybak., Faris, F.D., Solomon, P.S. (2007). Emergence of tan spot disease caused by toxigenic *Pyrenophora tritici-repentis* in Australia is not associated with increased deployment of toxin-sensitive cultivars. *Phytopathology*. **98**: 488-491.
- Patel, J.S., Gudmestad, N.C., Meinhardt, S., Adhikari, T.B. (2012). Pyraclostrobin sensitivity of baseline and fungicide exposed isolates of *Pyrenophora tritici-repentis*. *Crop Protection*. **34**:37-41.
- Pathrose, B., Jones, E.E., Jaspers, M.V. Ridgway, H.J. (2014). High genotypic and virulence diversity in *Ilyonectria liriodendri* isolates associated with black foot disease in New Zealand vineyards. *Plant Pathology*. **63**: 613-624.
- Perello, A., Mereno, V., Simón, M.R., Sisterna, M. (2003). Tan spot of wheat (*Triticum aestivum* L.) infection at different stages of crop development and inoculum type. *Crop Protection*. **22**: 157-169.

- Peever, T.L., Milgroom, M.G. (1994). Lack of correlation between fitness and resistance to sterol biosynthesis-inhibiting fungicides in *Pyrenophora teres*. *Phytopathology*. **84**: 515-519.
- Pottinger, B., Stewart, A., Carpenter, M., Ridgway, H.J. (2002). Low genetic variation detected in New Zealand populations of *Phaeoconiella chlamydospora*. *Phytopathologia Mediterranea*. **41**: 199-211.
- Raymond, P.J., Bockus, W.W., Norman, B.L. (1985). Tan spot of winter wheat: Procedures to determine host response. *Phytopathology*. **75**: 686-690.
- Reeleder, R.D. (2003). Fungal plant pathogens and soil biodiversity. *Canadian Journal of Soil Science*. **83**: 331-336.
- Rees, R.G., Platz, G.J., Mayer, R.J., (1982). Yield losses in wheat from yellow spot: comparison of estimates derived from single tillers and plots. *Australian Journal of Agricultural Research*. **33**: 899-908.
- Rees, R.G., Platz, G.J., Mayer, R.J. (1988). Susceptibility of Australian wheats to *Pyrenophora tritici-repentis*. *Australian Journal of Agricultural Research*. **39**: 141-151.
- Rees, R. G., Platz, G. J. (1990). Sources of resistance to *Pyrenophora tritici-repentis* in bread wheats. *Euphytica*. **45**: 59-69.
- Reide, C.R., Francl, L.J., Anderson, J.A. Jordahl, J.G., Meinhardt, S.W. (2003). Additional sources of resistance to tan spot of wheat. *Crop Science*. **36**: 771-777.
- Reimann, S., & Deising, H. B. (2005). Inhibition of efflux transporter-mediated fungicide resistance in *Pyrenophora tritici-repentis* by a derivative of 4'-hydroxyflavone and enhancement of fungicide activity. *Applied and Environmental Microbiology*. **71**:3269-3275.
- Roy, B.A., Kirchner, J.W. (2000). Evolutionary dynamics of pathogen resistance and tolerance. *Evolution*. **54**: 51-63.
- Russell, P. E. (2002). *Sensitivity baselines in fungicide resistance research and management* (pp. 1-53). Brussels: Crop Life International.
- dos Santos, A.M.P.V., Santos Matsumura, A.T., Van Der Sand, S.T. (2002). Intraspecific genetic diversity of *Drechslera tritici-repentis* as detected by random amplified polymorphic DNA analysis. *Genetics and Molecular Biology*. **25**: 243-250.
- Schilder, A.M.C., Bergstrom, G.C. (1992). The dispersal of conidia and ascospores of *Pyrenophora tritici-repentis*. In: *Advances in Tan Spot Research, NDSU Agric. Exp. Sta. Pub.* pp. 96-99. Eds. Francl, L.J., Krupinsky, J.M., McMullen, M.P.

- Schilder, A.M.C., Bergstrom, G.C. (1995). Seed transmission of *Pyrenophora tritici-repentis*, causal fungus of tan spot of wheat. *European Journal of Plant Pathology*. **101**: 81-91.
- Shabeer, A., Bockus, W.W. (1988). Tan spot effects on yield and yield components relative to growth stage in winter wheat. *Plant Disease*. **72**: 599-602.
- Sheridan, J.E., Nendick, D.K. (1987). Strains of *Pyrenophora teres* in New Zealand barley crops with reduced sensitivity to ergosterol biosynthesis inhibitor fungicides. In: *Proceedings of the 40th New Zealand weed and pest control conference*, No. 40, Nelson, New Zealand. Eds. Popay, A.J. pp. 172-175. The New Zealand Weed and Pest Society, Inc.
- Singh, P.K., Hughes, G.R. (2006). Genetic similarity among isolates of *Pyrenophora tritici-repentis*, Causal agent of tan spot of wheat. *Journal of Phytopathology*. **154**: 178-184.
- Singh, P.K., Mergoum, M., Ali, S., Adhikari, T.B., Elias, E.M., Hughes, G.R. (2006a). Identification of new sources of resistance to tan spot *Stagonospora nodorum* blotch and *Septoria tritici* blotch of wheat. *Crop Science*. **46**: 2047-2053.
- Singh, P.K., Gonzalez-Hernandez, J.L., Mergoum, M., Ali, S., Adhikari, T.B., Kianian, S.F., Elias, E.M., Hughes, G.R. (2006b). Identification and molecular mapping of a gene in tetraploid wheat conferring resistance to *Pyrenophora tritici-repentis* race 3. *Phytopathology*. **96**: 885-889.
- Singh, P.K., Mergoum, M., Gonzalez-Hernandez, J.L. Ali, S., Adhikari, T.B., Kianian, S.F., Elias, E.M., Hughes, G.R. (2008a). Genetics and molecular mapping of resistance to necrosis inducing race 5 of *Pyrenophora tritici-repentis* in tetraploid wheat. *Molecular Breeding*. **21**: 293-304.
- Singh, P.K., Mergoum, M., Ghavami, F., Ali, S., Adhikari, T.B., Kianian, S.F. (2008b). Genetic analysis and mapping of tan spot resistance genes using DArT markers. *Phytopathology*. **98**: S147.
- Singh, P.K., Singh, R.P., Duveiller, E., Mergoum, M., Adhikari, T.B., Elias, E.M. (2010). Genetics of wheat-*Pyrenophora tritici-repentis* interactions. *Euphytica*. **171**: 1-13.
- Staub, R. (2008). A Short History of Fungicides. <http://www.apsnet.org/publications/apsnetfeatures/Pages/Fungicides.aspx>. (Retrieved 19/7/2015)
- Stewart, T.M., Perry, A.J., Evans, M.J. (2014). Resistance of *Zymoseptoria tritici* to azoxystrobin and epoxiconazole in the lower North Island of New Zealand. *New Zealand Plant Protection*. **67**: 304-314.

- Strelkov, S.E., Lamari, L., Sayoud, R., Smith, R.B. (2002). Comparative virulence of chlorosis-inducing races of *Pyrenophora tritici-repentis*. *Canadian Journal of Plant Pathology*. **24**: 29-35.
- Strelkov, S.E., Lamari, L. (2003). Host-parasite interactions in tan spot (*Pyrenophora tritici-repentis*) of wheat. *Canadian Journal of Plant Pathology*. **25**: 339-349.
- Strukenbrock, E.H., Quaedvlieg, Javen-Nikhah, Zala, M., Crous, P.W., McDonald. (2012). *Zymoseptoria ardabiliae* and *Z. pseudotritici*, two progenitor species of the Septoria tritici leaf blotch fungus *Z. tritici* (synonym: *Mycosphaerella graminicola*). *Mycologia*. **104**: 1397-1407.
- Summerall, B.A., Burgess, L.W. (1989). Factors influencing the survival of *Pyrenophora tritici-repentis*: stubble management. *Mycological Research*. **93**: 38-40.
- Sutton, J.C., Vyn, T.J. (1990). Crop sequences and tillage practices in relation to diseases of winter wheat in Ontario. *Canadian Journal of Plant Pathology*. **77**: 1333-1340.
- Tautz, D., Renz, M. (1984). Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic acids research*. **12**: 4127-4138.
- Tadesse, W., Hsam, S.L.K., Wenzel, G., Zeller, F.J. (2006a). Identification and monosomic analysis of tan spot resistance genes in synthetic wheat lines (*Triticum turgidum* L. x *Aegilops tauschii* Coss.). *Crop Science*. **46**: 1212-1217.
- Tadesse, W., Hsam, S.L.K., Zeller, F.J. (2006b). Evaluation of common wheat (*Triticum aestivum* L.) cultivars for tan spot resistance and chromosomal location of a resistance gene in cultivar 'Salamouni'. *Plant Breeder*. **125**: 318-322.
- Tomás, A., Feng, G.H., Reeck, G.R., Bockus, W.W., Leach, J.E. (1990). Purification of a cultivar-specific toxin from *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat. *Molecular Plant-Microbe Interactions*. **3**: 221-224.
- Tommerup, I. C., Barton, J. E., O'Brien, P. A. (1995). Reliability of RAPD fingerprinting of three basidiomycete fungi, *Laccaria*, *Hydnangium* and *Rhizoctonia*. *Mycological Research*. **99**, 179-186.
- Tuori, A., Wolpert, T.J., Ciuffetti, L.M. (1995). Purification and immunological characterization of toxic components from cultures of *Pyrenophora tritici-repentis*. *Molecular Plant-Microbe interactions*. **8**: 41-48.
- Tyson, J.L., Ridgway, H.J., Fullerton, R.A., Stewart, A. (2002). Genetic diversity in New Zealand populations of *Sclerotium cepivorum*. *New Zealand Journal of Crop and Horticultural Science*. **30**: 37-48.

- Wegulo, S.N. (2011). *Tan spot of cereals*.
<http://www.apsnet.org/edcenter/intropp/lessons/fungi/ascomycetes/Pages/TanSpot.aspx>
- Wegulo, S.N., Klein, R.N., Harveson, R.M. (2012). *Tan spot of wheat*.
<http://www.ianrpubs.unl.edu/pages/publication> (Retrieved 15/7/2013).
- Weise, M.V. (1987). *Compendium of Wheat Diseases* (Second edition). APS Press. The American Phytopathological Society. St. Paul, MN, USA. pp. 112.
- White, T.J., Bruns, T., Lee, S., Taylor, J.W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: a guide to methods and applications. (Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., eds). *Academic Press*, San Diego, USA, 315-322.
- Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic acids research*. **18**: 6531-6535.
- Williams, R., Poole, N., Fraser, T., Grant, D., Hanson, N., Orson, J., Rolston, P., Sim, J. (2013). Review of the role and practices of stubble burning in New Zealand, including alternative options and possible improvements. Foundation for Arable Research, New Zealand.
- Wolf, P. F. J., Hoffmann, G. M. (1993). Biological studies on *Drechslera tritici-repentis* (Died.) Shoem. (teleomorph *Pyrenophora tritici-repentis* (Died.) Drechsler) the causal agent of a leaf spot disease on wheat. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz*. **100**: 33-48.
- Wolf, P.H.J., Hoffmann, G.M. (1994). Epidemiological development *Drechslera tritici-repentis* in wheat crops. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz*. **101**: 22-37.
- Yli-Mattila, T., Mironenko, N.V., Alekhina, I.A., Hannukkala, A., Bulat, S.A. (1997). Universally primed polymerase chain reaction analysis of *Fusarium avenaceum* isolated from wheat and barley in Finland. *Agricultural and Food Science in Finland*. **6**:25-36.
- Zadoks, J.C., Chang, T.T., Konzak, C.F. (1974). A decimal code for the growth stages of cereals. *Weed Research*. **14**: 415-421.
- Zietkiewicz, E., Rafalski, A., Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics*. **20**:176-183.

Appendix A

A.1 V8-PDA Recipe

150 mL V8 juice, 10 g Davis agar, 10g PDA, 3 g CaCO_3 and 850 mL distilled water

A.2 V8 recipe

200 mL V8 juice, 15 g Davis agar, 6 mL 1.0M NaOH and 800 mL distilled water

A.3 TAE used for gel electrophoresis

To prepare 50X TAE:

242 g Tris-base

57.1 mL Glacial acetic acid

100 mL 0.5 M EDTA (pH 8)

Make up to 1 L with sterile water

Appendix B

B.1 Questionnaire form sent to farmers

2014 Tan Spot Farmer Questionnaire

Name _____

Farm name/Address _____

Phone number _____

Total size of farm (in hectares) _____

1) As far back as you can remember how long have you been growing wheat on your property?

2) Aside from wheat, what other cereal/grass crops do you currently grow on your property?

3) What wheat cultivars have you sown in the past and for the 2013-14 growing season? (Please include whether or not you re-sow your wheat seed and the merchant(s) who provided the seed)

4) When exactly was the tan spot sampled wheat crop on your property sown?

5) What is the past cropping history (as far as you can remember) of the paddock for which tan spot (*Pyrenophora tritici-repentis*) was recovered?

6) What post-harvest practices do you use after the harvesting of a wheat crop on your property i.e. Burn stubble, direct drilling etc.

7) In a single paddock, do you either continuously sow wheat or frequently rotate wheat around your property year to year?

8) What were the fungicides (either common name or chemical name) which were applied onto the tan spot infected paddock during the 2013-2014 growing season? (Please provide the rates and timings e.g. T1 if known and whether or not you apply pre-sowing fungicide seed treatments).

B.2 Details of the New Zealand *Pyrenophora tritici-repentis* isolates isolated from wheat used in the study

| Isolate Code | Race | Date collected | Collection Location |
|--------------|----------|--------------------------------|---------------------------------|
| S12a | R1 or R2 | 16 th January 2014 | Wedonside, Southland |
| S22a | R1 or R2 | 22 nd November 2013 | Clinton, Southland |
| S26a | R1 or R2 | 22 nd November 2013 | Clinton, Southland |
| M13c | R1 or R2 | 31 st December 2014 | Methven, Mid-Canterbury |
| M14d | R1 or R2 | 31 st December 2014 | Methven, Mid-Canterbury |
| G14a | R1 or R2 | 1 st November 2013 | Greendale, North Canterbury |
| G22a | R1 or R2 | 24 th November 2013 | Geraldine, South Canterbury |
| F12d | R1 or R2 | 19 th November 2013 | Fairlie basin, South Canterbury |
| W12a | R1 or R2 | 19 th November 2013 | Waimate, South Canterbury |
| W12c | R1 or R2 | 19 th November 2013 | Waimate, South Canterbury |
| W15a | R1 or R2 | 19 th November 2013 | Waimate, South Canterbury |
| K16a | R1 or R2 | 4 th December 2013 | Kakanui, North Otago |

B.3 Codes allocated to name *Pyrenophora tritici-repentis* obtained in the 2013-2014 survey

| Place of collection | Code |
|----------------------------|-------------|
| Southland | S |
| Kakanui | K |
| Waimate | W |
| Fairlie | F |
| Methven | M |
| Geraldine | G2 |
| Greendale | G1 |

B.4 Race, toxin produced and origin of the international *Pyrenophora tritici-repentis* isolates used in study

| Isolate code | Race | Toxins | Region/ Country collected | Supplied by |
|-------------------|----------|--------|---|----------------------|
| 90-2 | R4 | - | Canada | Dr Reem Aboukhaddour |
| ASC1 | R1 | A | Canada | Dr Reem Aboukhaddour |
| AB47-10 | R1 | A | Morley region (Canada) | Dr Reem Aboukhaddour |
| Alg3-24 | R5 | B | Algeria | Dr Reem Aboukhaddour |
| AlGH2 | R6 | BC | Algeria | Dr Reem Aboukhaddour |
| AB33-1 | R1 | A | Wilson region (Canada) | Dr Reem Aboukhaddour |
| AB39-2 | R3 | C | Wilson region (Canada) | Dr Reem Aboukhaddour |
| AB39-8 | R1 | A | Wilson region (Canada) | Dr Reem Aboukhaddour |
| TS93-71B | R8 | ABC | Turkey-Syria | Dr Reem Aboukhaddour |
| CCDM-Meck4 | R1 or R2 | A | Meckering, Western Australia (Australia) | Dr Caroline Moffat |
| CCDM-239 | R1 or R2 | A | Sea Lake, Victoria (Australia) | Dr Caroline Moffat |
| CCDM-134 | R1 or R2 | A | Biloela, Queensland (Australia) | Dr Caroline Moffat |

Appendix C

C.1 DNA sequences of *Pyrenophora tritici-repentis* isolates: ITS, β -tubulin, *ToxA* and PtrUnique primer sites

C.1.1 ITS sequences of *Pyrenophora tritici-repentis* isolates

S12a ITS forward

```
1      AGGTCAAAAG GTTGAAAGAA GCTTCATGGA CGCGCGACCG CGGCTGGACA
51     AGAGCGCAA TAATGTGCTG CGCTCCGAAA CCAGTAGGCC GGCTGCCAAT
101    GATTTTAAGG CGAGTCTCGG GAGAGAGACA AGACGCCCAA CACCAAGCAA
151    AGCTTGAGGG TACAAATGAC GCTCGAACAG GCATGCCCTT TGGAATACCA
201    AAGGGCGCAA TGTGCGTTCA AAGATTGAT GATTCACTGA ATTCTGCAAT
251    TCACACTACT TATCGCATTT CGCTGCGTTC TTCATCGATG CCAGAACCAA
301    GAGATCCGTT GTTGAAAGTT GTAATTGATT ACATTTGTTT TGCTGACGCT
351    GATTGCAACT GAAAAAAGG TTTGAATAAG GTCCAATTGG CGGGCGGACC
401    CGCCAAGGAA ACAAGTAGTA CGCAAAAAGA CATGGGTGAA TATGGCGCCA
451    GACGGGCAAG GCAGCGAGGC CCTATCCCAG TCCGGCTTCA TATTTGTGTA
501    ATGATCCCTC CGCAGGTTCA CCTACAGAGA CCTTGTTACG AT
```

S12a ITS reverse

```
1      ACATTACACA AATATGAAGC CGGACTGGGA TAGGGCCTCG CTGCCTTGCC
51     CGTCTGGCGC CATATTCACC CATGTCTTTT TGCGTACTAC TTGTTTCCTT
101    GGCGGGTCCG CCCGCCAATT GGACCTTATT CAAACCTTTT TTTGAGTTGC
151    AATCAGCGTC AGCAAACAA ATGTAATCAA TTACAACCTT CAACAACGGA
201    TCTCTTGTTT CTGGCATCGA TGAAGAACGC AGCGAAATGC GATAAGTAGT
251    GTGAATTGCA GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC
301    CCTTTGGTAT TCCAAAGGGC ATGCCTGTTT GAGCGTCATT TGTACCCTCA
351    AGCTTTGCTT GGTGTTGGGC GTCTTGTTCT TCTCCCGAGA CTCGCCTTAA
401    AATCATTGGC AGCCGGCCTA CTGGTTTCGG AGCGCAGCAC ATTATTTGCG
451    CTCTTGTTCA GCCGCGGTCG CGCGTCCATG AAGCTTCTTT CAACCTTTTG
501    ACCTCGGATC AGGTAGGGAT ACCCGCTGAA CTTAAGCATA TCAATA
```

S22a ITS forward

```
1      CCTGATCGAG GTCAAAGGTT GAAGAAGCTT CATGGACGCG CGACCGCGGC
51     TGGACAAGAG CGCAAATAAT GTGCTGCGCT CCGAAACCAG TAGGCCGGCT
101    GCCAATGATT TTAAGGCGAG TCTCGGGAGA GAGACAAGAC GCCCAACACC
151    AAGCAAAGCT TGAGGGTACA AATGACGCTC GAACAGGCAT GCCCTTTGGA
201    ATACCAAAGG GCGCAATGTG CGTTCAAAGA TTCGATGATT CACTGAATTC
251    TGCAATTCAC ACTACTTATC GCATTTGCTT GCGTTCTTCA TCGATGCCAG
301    AACCAAGAGA TCCGTTGTTG AAAGTTGTAA TTGATTACAT TTGTTTTGCT
351    GACGCTGATT GCAACTGAAA AAAAGGTTTG AATAAGGTCC AATTGGCGGG
401    CGGACCCGCC AAGGAAACAA GTAGTACGCA AAAAGACATG GGTGAATATG
451    GCGCCAGACG GGCAAGGCAG CGAGGCCCTA TCCCAGTCCG GCTTCATATT
501    TGTGTAATGA TCCCTCCGCA GGTTACCTA CGGAGACCTT GTTACGA
```

S22a ITS reverse

1 ACATTACACA AATATGAAGC CGGACTGGGA TAGGGCCTCG CTGCCTTGCC
51 CGTCTGGCGC CATATTCACC CATGTCTTTT TGCCTACTAC TTGTTTCCTT
101 GGCGGGTCCG CCCGCCAATT GGACCTTATT CAAACCTTTT TTTAGTTGTC
151 AATCAGCGTC AGCAAAACAA ATGTAATCAA TTACAACCTT CAACAACGGA
201 TCTCTTGTTT CTGGCATCGA TGAAGAACGC AGCGAAATGC GATAAGTAGT
251 GTGAATTGCA GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC
301 CCTTTGGTAT TCCAAAGGGC ATGCCTGTTT GAGCGTCATT TGTACCCTCA
351 AGCTTTGCTT GGTGTTGGGC GTCTTGTCTC TCTCCCGAGA CTCGCCTTAA
401 AATCATTGGC AGCCGGCCTA CTGGTTTCGG AGCGCAGCAC ATTATTTGCG
451 CTCTTGTCCA GCCGCGGTCG CGCGTCCATG AAGCTTCTTT CAACCTTTTG
501 ACCTCGGATC AGGTAGGGAT ACCCGCTGAA CTTAAGCATA TCAATAA

S26a ITS forward

1 AGGTCAAAGG TTGAAAGAAG CTCATGGAC GCGCGACCGC GGCTGGACAA
51 GAGCGCAAAT AATGTGCTGC GCTCCGAAAC CAGTAGGCCG GCTGCCAATG
101 ATTTTAAGGC GAGTCTCGGG AGAGAGACAA GACGCCCAAC ACCAAGCAA
151 GCTTGAGGGT ACAAATGACG CTCGAACAGG CATGCCCTTT GGAATACCAA
201 AGGGCGCAAT GTGCGTTCAA AGATTCGATG ATTCACTGAA TTCTGCAATT
251 CACACTACTT ATCGCATTTC GCTGCGTTCT TCATCGATGC CAGAACCAAG
301 AGATCCGTTG TTGAAAGTTG TAATTGATTA CATTGTGTTT GCTGACGCTG
351 ATTGCAACTG AAAAAAGGT TTGAATAAGG TCCAATTGGC GGGCGGACCC
401 GCCAAGGAAA CAAGTAGTAC GCAAAAAGAC ATGGGTGAAT ATGGCGCCAG
451 ACGGGCAAGG CAGCGAGGCC CTATCCCAGT CCGGCTTCAT ATTTGTGTAA
501 TGATCCCTCC GCAGGTTTAC CTACGGAGAC CTTGTTA

S26a ITS reverse

1 ACATTACACA AATATGAAGC CGGACTGGGA TAGGGCCTCG CTGCCTTGCC
51 CGTCTGGCGC CATATTCACC CATGTCTTTT TGCCTACTAC TTGTTTCCTT
101 GGCGGGTCCG CCCGCCAATT GGACCTTATT CAAACCTTTT TTTAGTTGTC
151 AATCAGCGTC AGCAAAACAA ATGTAATCAA TTACAACCTT CAACAACGGA
201 TCTCTTGTTT CTGGCATCGA TGAAGAACGC AGCGAAATGC GATAAGTAGT
251 GTGAATTGCA GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC
301 CCTTTGGTAT TCCAAAGGGC ATGCCTGTTT GAGCGTCATT TGTACCCTCA
351 AGCTTTGCTT GGTGTTGGGC GTCTTGTCTC TCTCCCGAGA CTCGCCTTAA
401 AATCATTGGC AGCCGGCCTA CTGGTTTCGG AGCGCAGCAC ATTATTTGCG
451 CTCTTGTCCA GCCGCGGTCG CGCGTCCATG AAGCTTCTTT CAACCTTTTG
501 ACCTCGGATC AGGTAGGGAT ACCCGCTGAA CTTAAGCATA TCA

M13c ITS forward

1 ATCCGAGGTC AAAGGTTGAA AGAAGCTTCA TGGACGCGCG ACCGCGGCTG
51 GACAAGAGCG CAAATAATGT GCTGCGCTCC GAAACCAGTA GGCCGGCTGC
101 CAATGATTTT AAGGCGAGTC TCGGGAGAGA GACAAGACGC CCAACACCAA
151 GCAAAGCTTG AGGGTACAAA TGACGCTCGA ACAGGCATGC CCTTTGGAAT
201 ACCAAAGGGC GCAATGTGCG TTCAAAGATT CGATGATTCA CTGAATTCTG
251 CAATTCACAC TACTTATCGC ATTTGCTGTC GTTCTTCATC GATGCCAGAA
301 CCAAGAGATC CGTTGTTGAA AGTTGTAATT GATTACATTT GTTTTGTCTG
351 CGCTGATTGC AACTGAAAAA AAGGTTTGA TAAGGTCCAA TTGGCGGGCG
401 GACCCGCCAA GGAAACAAGT AGTACGCAA AAGACATGGG TGAATATGGC
451 GCCAGACGGG CAAGGCAGCG AGGCCCTATC CCAGTCCGGC TTCATATTTG
501 TGTAATGATC CCTCCGACG TTCACCTACG GAGACCTTGT TACGACT

M13c ITS reverse

```
1      ACATTACACA AATATGAAGC CGGACTGGGA TAGGGCCTCG CTGCCTTGCC
51     CGTCTGGCGC CATATTCACC CATGTCTTTT TGCCTACTAC TTGTTTCCTT
101    GGCGGGTCCG CCCGCCAATT GGACCTTATT CAAACCTTTT TTTAGTTGTC
151    AATCAGCGTC AGCAAAACAA ATGTAATCAA TTACAACCTT CAACAACGGA
201    TCTCTTGGTT CTGGCATCGA TGAAGAACGC AGCGAAATGC GATAAGTAGT
251    GTGAATTGCA GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC
301    CCTTTGGTAT TCCAAAGGGC ATGCCTGTTT GAGCGTCATT TGTACCTCA
351    AGCTTTGCTT GGTGTTGGGC GTCTTGTCTC TCTCCCGAGA CTCGCCTTAA
401    AATCATTGGC AGCCGGCCTA CTGGTTTCGG AGCGCAGCAC ATTATTTGCG
451    CTCTTGTCCA GCCGCGGTCG CGCGTCCATG AAGCTTCTTT CAACCTTTTG
501    ACCTCGGATC AGGTAGGGAT ACCCGCTGAA CTTAAGCATA TCAATAA
```

M14d ITS forward

```
1      AGGTCAAAAG GTTCAAAGAA GCTTCATGGA CGCGCGACCG CGGCTGGACA
51     AGAGCGCAA TAATGTGCTG CGCTCCGAAA CCAGTAGGCC GGCTGCCAAT
101    GATTTTAAGG CGAGTCTCGG GAGAGAGACA AGACGCCCAA CACCAAGCAA
151    AGCTTGAGGG TACAAATGAC GCTCGAACAG GCATGCCCTT TGGAATACCA
201    AAGGGCGCAA TGTGCGTTCA AAGATTGAT GATTACTGA ATTCTGCAAT
251    TCACACTACT TATCGCATTT CGCTGCGTTC TTCATCGATG CCAGAACCAA
301    GAGATCCGTT GTTCAAAGTT GTAATTGATT ACATTTGTTT TGCTGACGCT
351    GATTGCAACT GAAAAAAGG TTTGAATAAG GTCCAATTGG CGGGCGGACC
401    CGCCAAGGAA ACAAGTAGTA CGCAAAAAGA CATGGGTGAA TATGGCGCCA
451    GACGGGCAAG GCAGCGAGGC CCTATCCCAG TCCGGCTTCA TATTTGTGTA
501    ATGATCCCTC CGCAGGTTCA CCTACGGAGA CTTGTTTACG A
```

M14d ITS reverse

```
1      ACATTACACA AATATGAAGC CGGACTGGGA TAGGGCCTCG CTGCCTTGCC
51     CGTCTGGCGC CATATTCACC CATGTCTTTT TGCCTACTAC TTGTTTCCTT
101    GGCGGGTCCG CCCGCCAATT GGACCTTATT CAAACCTTTT TTTAGTTGTC
151    AATCAGCGTC AGCAAAACAA ATGTAATCAA TTACAACCTT CAACAACGGA
201    TCTCTTGGTT CTGGCATCGA TGAAGAACGC AGCGAAATGC GATAAGTAGT
251    GTGAATTGCA GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC
301    CCTTTGGTAT TCCAAAGGGC ATGCCTGTTT GAGCGTCATT TGTACCTCA
351    AGCTTTGCTT GGTGTTGGGC GTCTTGTCTC TCTCCCGAGA CTCGCCTTAA
401    AATCATTGGC AGCCGGCCTA CTGGTTTCGG AGCGCAGCAC ATTATTTGCG
451    CTCTTGTCCA GCCGCGGTCG CGCGTCCATG AAGCTTCTTT CAACCTTTTG
501    ACCTCGGATC AGGTAGGGAT ACCCGCTGAA CTTAAGCATA TCAATAA
```

G14a ITS forward

```
1      CCTGATCGAG GTCAAAGGTT GAAGAAGCTT CATGGACGCG CGACCGCGGC
51     TGGACAAGAG CGCAAATAAT GTGCTGCGCT CCGAAACCAG TAGGCCGGCT
101    GCCAATGATT TTAAGGCGAG TCTCGGGAGA GAGACAAGAC GCCCAACACC
151    AAGCAAAGCT TGAGGGTACA AATGACGCTC GAACAGGCAT GCCCTTTGGA
201    ATACCAAAGG GCGCAATGTG CGTTCAAAGA TTCGATGATT CACTGAATTC
251    TGCAATTCAC ACTACTTATC GCATTTGCTT GCGTTCTTCA TCGATGCCAG
301    AACCAAGAGA TCCGTTGTTG AAAGTTGTAA TTGATTACAT TTGTTTGTCT
351    GACGCTGATT GCAACTGAAA AAAAGGTTTG AATAAGGTCC AATTGGCGGG
401    CGGACCCGCC AAGGAAACAA GTAGTACGCA AAAAGACATG GGTGAATATG
451    GCGCCAGACG GGCAAGGCAG CGAGGCCCTA TCCCAGTCCG GCTTCATATT
501    TGTGTAATGA TCCCTCCGCA GTTTACCTA CGGAGACCTT GTTACGA
```

G14a ITS reverse

```
1      ACATTACACA AATATGAAGC CGGACTGGGA TAGGGCCTCG CTGCCTTGCC
51     CGTCTGGCGC CATATTCACC CATGTCTTTT TGCGTACTAC TTGTTTCCTT
101    GGCGGGTCCG CCCGCCAATT GGACCTTATT CAAACCTTTT TTTCAGTTGC
151    AATCAGCGTC AGCAAAACAA ATGTAATCAA TTACAACCTT CAACAACGGA
201    TCTCTTG GTT CTGGC ATCGA TGAAGAACGC AGCGAAATGC GATAAGTAGT
251    GTGAATTGCA GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC
301    CCTTTGGTAT TCCAAAGGGC ATGCCTGTTT GAGCGTCATT TGTACCCTCA
351    AGCTTTGCTT GGTGTTGGGC GTCTTGTCTC TCTCCCGAGA CTCGCCTTAA
401    AATCATTGGC AGCCGGCCTA CTGGTTTCGG AGCGCAGCAC ATTATTTGCG
451    CTCTTGTCCA GCCGCGGTCTG CGCGTCCATG AAGCTTCTTT CAACCTTTTG
501    ACCTCGGATC AGGTAGGGAT ACCCGCTGAA CTTAAGCATA TCAATA
```

G22a ITS forward

```
1      ATCCGAGGTC AAAGGTTGAA GAAGCTTCAT GGACGCGCGA CCGCGGCTGG
51     ACAAGAGCGC AAATAATGTG CTGCGCTCCG AAACCAGTAG GCCGGCTGCC
101    AATGATTTTA AGGCGAGTCT CGGGAGAGAG ACAAGACGCC CAACACCAAG
151    CAAAGCTTGA GGTACAAAT GACGCTCGAA CAGGCATGCC CTTTGGAATA
201    CCAAAGGGCG CAATGTGCGT TCAAAGATTC GATGATTCAC TGAATTCTGC
251    AATTCACACT ACTTATCGCA TTTCGCTGCG TTCTTCATCG ATGCCAGAAC
301    CAAGAGATCC GTTGTGAAA GTTGTAAATTG ATTACATTTG TTTTGCTGAC
351    GCTGATTGCA ACTGAAAAAA AGGTTTGAAT AAGGTCCAAT TGGCGGGCGG
401    ACCCGCCAAG GAAACAAGTA GTACGCAAAA AGACATGGGT GAATATGGCG
451    CCAGACGGGC AAGGCAGCGA GGCCCTATCC CAGTCCGGCT TCATATTTGT
501    GTAATGATCC CTCCGCAGGT TCACCTACAG AGACCTTGTT ACGACT
```

G22a ITS reverse

```
1      ACATTACACA AATATGAAGC CGGACTGGGA TAGGGCCTCG CTGCCTTGCC
51     CGTCTGGCGC CATATTCACC CATGTCTTTT TGCGTACTAC TTGTTTCCTT
101    GGCGGGTCCG CCCGCCAATT GGACCTTATT CAAACCTTTT TTTCAGTTGC
151    AATCAGCGTC AGCAAAACAA ATGTAATCAA TTACAACCTT CAACAACGGA
201    TCTCTTG GTT CTGGC ATCGA TGAAGAACGC AGCGAAATGC GATAAGTAGT
251    GTGAATTGCA GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC
301    CCTTTGGTAT TCCAAAGGGC ATGCCTGTTT GAGCGTCATT TGTACCCTCA
351    AGCTTTGCTT GGTGTTGGGC GTCTTGTCTC TCTCCCGAGA CTCGCCTTAA
401    AATCATTGGC AGCCGGCCTA CTGGTTTCGG AGCGCAGCAC ATTATTTGCG
451    CTCTTGTCCA GCCGCGGTCTG CGCGTCCATG AAGCTTCTTT CAACCTTTTG
501    ACCTCGGATC AGGTAGGGAT ACCCGCTGAA CTTAAGCATA TCATA
```

F12d ITS forward

```
1      CCTGATCGAG GTCAAAGGTT GAAGAAGCTT CATGGACGCG CGACCCGCGC
51     TGGACACAGA GCGCAAATAA TGTGCTGCGC TCCGAAACCA GTAGGCCGGC
101    TGCCAATGAT TTTAAGGCGA GTCTCGGGAG AGAGACAAGA CGCCCAACAC
151    CAAGCAAAGC TTGAGGGTAC AAATGACGCT CGAACAGGCA TGCCCTTTGG
201    AATACCAAAG GCGCAATGT GCGTTCAAAAG ATTCGATGAT TCACTGAATT
251    CTGCAATTCA CACTACTTAT CGCATTTTCGC TCGTTCCTT ATCGATGCCA
301    GAACCAAGAG ATCCGTTGTT GAAAGTTGTA ATTGATTACA TTTGTTTTGC
351    TGACGCTGAT TGCAACTGAA AAAAAGGTTT GAATAAGGTC CAATTGGCGG
401    GCGGACCCGC CAAGGAAACA AGTAGTACGC AAAAAGACAT GGGTGAATAT
451    GGC GCCAGAC GGGCAAGGCA GCGAGGCCCT ATCCCAGTCC GGCTTCATAT
501    TTGTGTAATG ATCCCTCCGC AGGTTACCTT ACGGAGACCT TGTTA
```

F12d ITS reverse

1 TTACACAAAT ATGAAGCCGG ACTGGGATAG GGCCTCGCTG CCTTGCCCGT
51 CTGGCGCCAT ATTCACCCAT GTCTTTTTGC GTACTACTTG TTTCTTGCC
101 GGGTCCGCC GCCAATTGGA CCTTATTCAA ACCTTTTTTT CAGTTGCAAT
151 CAGCGTCAGC AAAACAAATG TAATCAATTA CAACTTTCAA CAACGGATCT
201 CTTGGTTCTG GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAGTGTG
251 AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC ATTGCGCCCT
301 TTGGTATTCC AAAGGGCATG CCTGTTGAGC CGTCATTTGT ACCCTCAAGC
351 TTTGCTTGGT GTTGGGCGTC TTGTCTCTCT CCCGAGACTC GCCTTAAAAT
401 CATTGGCAGC CGGCCTACTG GTTTCGGAGC GCAGCACATT ATTTGCGCTC
451 TTGTCCAGCC GCGGTCGCGC GTCCATGAAG CTTCTTTCAA CCTTTTGACC
501 TCGGATCAGG TAGGGATACC CGCTGAACCT AAGCATATCA AT

W12a ITS forward

1 ACCTGATCCG AGGTCAAAAG GTTGAAGAA GCTTCATGGA CGCGCGACCG
51 CGGCTGGACA AGAGCGCAA TAATGTGCTG CGCTCCGAAA CCAGTAGGCC
101 GGCTGCCAAT GATTTTAAGG CGAGTCTCGG GAGAGAGACA AGACGCCCAA
151 CACCAAGCAA AGCTTGAGGG TACAAATGAC GTCGAACAG GCATGCCCTT
201 TGGAATACCA AAGGGCGCAA TGTGCGTTCA AAGATTGAT GATTCACTGA
251 ATTCTGCAAT TCACACTACT TATCGCATTT CGCTGCGTTC TTCATCGATG
301 CCAGAACCAA GAGATCCGTT GTTGAAGTT GTAATTGATT ACATTTGTTT
351 TGCTGACGCT GATTGCAACT GAAAAAAGG TTTGAATAAG GTCCAATTGG
401 CGGGCGGACC CGCCAAGGAA ACAAGTAGTA CGCAAAAAGA CATGGGTGAA
451 TATGGCGCCA GACGGGCAAG GCAGCGAGGC CCTATCCCAG TCCGGCTTCA
501 TATTTGTGTA ATGATCCCTC CGCAGGTTCA CCTACGGAGA CCTTGTTACG
551 A

W12a ITS reverse

1 TCATTACACA AATATGAAGC CGGACTGGGA TAGGGCCTCG CTGCCTTGCC
51 CGTCTGGCGC CATATTCACC CATGTCTTTT TGCCTACTAC TTGTTTCTT
101 GGCGGGTCCG CCCGCCAATT GGACCTTATT CAAACCTTTT TTTAGTTGC
151 AATCAGCGTC AGCAAAACAA ATGTAATCAA TTACAACCTT CAACAACGGA
201 TCTCTTGGTT CTGGCATCGA TGAAGAACGC AGCGAAATGC GATAAGTAGT
251 GTGAATTGCA GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC
301 CCTTTGGTAT TCCAAAGGGC ATGCCTGTTT GAGCGTCATT TGTACCCTCA
351 AGCTTTGCTT GGTGTTGGGC GTCTTGTCTC TCTCCCGAGA CTCGCCTTAA
401 AATCATTGGC AGCCGGCCTA CTGGTTTCGG AGCGCAGCAC ATTATTTGCG
451 CTCTTGTCCA GCCGCGGTCG CGCGTCCATG AAGCTTCTTT CAACCTTTTG
501 ACCTCGGATC AGGTAGGGAT ACCCGCTGAA CTTAAGCATA TCAATA

W12c ITS forward

1 CCTACCTGAT CGAGGTCAAA GGTGAAGAA GCTTCATGGA CGCGCGACCG
51 CGGCTGGACA AGAGCGCAA TAATGTGCTG CGCTCCGAAA CCAGTAGGCC
101 GGCTGCCAAT GATTTTAAGG CGAGTCTCGG GAGAGAGACA AGACGCCCAA
151 CACCAAGCAA AGCTTGAGGG TACAAATGAC GTCGAACAG GCATGCCCTT
201 TGGAATACCA AAGGGCGCAA TGTGCGTTCA AAGATTGAT GATTCACTGA
251 ATTCTGCAAT TCACACTACT TATCGCATTT CGCTGCGTTC TTCATCGATG
301 CCAGAACCAA GAGATCCGTT GTTGAAGTT GTAATTGATT ACATTTGTTT
351 TGCTGACGCT GATTGCAACT GAAAAAAGG TTTGAATAAG GTCCAATTGG
401 CGGGCGGACC CGCCAAGGAA ACAAGTAGTA CGCAAAAAGA CATGGGTGAA
451 TATGGCGCCA GACGGGCAAG GCAGCGAGGC CCTATCCCAG TCCGGCTTCA
501 TATTTGTGTA ATGATCCCTC CGCAGGTTCA CCTACGGAGA CCTTGTTACG
551 A

W12c ITS reverse

1 ACATTACACA AATATGAAGC CGGACTGGGA TAGGGCCTCG CTGCCTTGCC
51 CGTCTGGCGC CATATTCACC CATGTCTTTT TGCCTACTAC TTGTTTCCTT
101 GCGGGTCCG CCCGCCAATT GGACCTTATT CAAACCTTTT TTTCAGTTGC
151 AATCAGCGTC AGCAAAACAA ATGTAATCAA TTACAACCTT CAACAACGGA
201 TCTCTTGGTT CTGGCATCGA TGAAGAACGC AGCGAAATGC GATAAGTAGT
251 GTGAATTGCA GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC
301 CCTTTGGTAT TCCAAAGGGC ATGCCTGTTT GAGCGTCATT TGTACCTCA
351 AGCTTTGCTT GGTGTTGGGC GTCTTGTCTC TCTCCGAGA CTCGCCTTAA
401 AATCATTGGC AGCCGGCCTA CTGGTTTCGG AGCGCAGCAC ATTATTTGCG
451 CTCTTGTCCA GCCGCGGTCG CGCGTCCATG AAGCTTCTTT CAACCTTTTG
501 ACCTCGGATC AGGTAGGGAT ACCCGCTGAA CTTAAGCATA TCAATAA

W15a ITS forward

1 ATCGAGGTCA AAGGTTGAAG AAGCTTCATG GACGCGGAC CGCGGCTGGA
51 CAAGAGCGCA AATAATGTGC TGCGCTCCGA AACCAGTAGG CCGGCTGCCA
101 ATGATTTTAA GGCGAGTCTC GGGAGAGAGA CAAGACGCC AACACCAAGC
151 AAAGCTTGAG GGTACAAATG ACGCTCGAAC AGGCATGCC TTTGGAATAC
201 CAAAGGGCGC AATGTGCGTT CAAAGATTCG ATGATTCACT GAATTCTGCA
251 ATTCACACTA CTTATCGCAT TTCGCTGCGT TCTTCATCGA TGCCAGAACC
301 AAGAGATCCG TTGTTGAAAG TTGTAATTGA TTACATTTGT TTTGCTGACG
351 CTGATTGCAA CTGAAAAAAAA GTTTTGAATA AGGTCCAATT GCGGGCGGA
401 CCCGCCAAGG AAACAAGTAG TACGCAAAAA GACATGGGTG AATATGGCGC
451 CAGACGGGCA AGGCAGCGAG GCCCTATCCC AGTCCGGCTT CATATTTGTG
501 TAATGATCCC TCCGCAGGTT CACCTACGGA GACCTTGTTA CGAC

W15a ITS reverse

1 ACATTACACA AATATGAAGC CGGACTGGGA TAGGGCCTCG CTGCCTTGCC
51 CGTCTGGCGC CATATTCACC CATGTCTTTT TGCCTACTAC TTGTTTCCTT
101 GCGGGTCCG CCCGCCAATT GGACCTTATT CAAACCTTTT TTTCAGTTGC
151 AATCAGCGTC AGCAAAACAA ATGTAATCAA TTACAACCTT CAACAACGGA
201 TCTCTTGGTT CTGGCATCGA TGAAGAACGC AGCGAAATGC GATAAGTAGT
251 GTGAATTGCA GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC
301 CCTTTGGTAT TCCAAAGGGC ATGCCTGTTT GAGCGTCATT TGTACCTCA
351 AGCTTTGCTT GGTGTTGGGC GTCTTGTCTC TCTCCGAGA CTCGCCTTAA
401 AATCATTGGC AGCCGGCCTA CTGGTTTCGG AGCGCAGCAC ATTATTTGCG
451 CTCTTGTCCA GCCGCGGTCG CGCGTCCATG AAGCTTCTTT CAACCTTTTG
501 ACCTCGGATC AGGTAGGGAT ACCCGCTGAA CTTAAGCATA TCAATAA

K16a ITS forward

1 ATCCGAGGTC AAAGGTTGAA GAAGCTTCAT GGACGCGGA CCGCGGCTGG
51 ACAAGAGCGC AAATAATGTG CTGCGCTCCG AAACCAGTAG GCCGGCTGCC
101 AATGATTTTA AGGCGAGTCT CGGGAGAGAG ACAAGACGCC CAACACCAAG
151 CAAAGCTTGA GGGTACAAAT GACGCTCGAA CAGGCATGCC CTTTGAATA
201 CCAAAGGGCG CAATGTGCGT TCAAAGATTC GATGATTCAC TGAATTCTGC
251 AATTCACACT ACTTATCGCA TTTCGCTGCG TTCTTCATCG ATGCCAGAAC
301 CAAGAGATCC GTTGTGAAA GTTGTAAATTG ATTACATTTG TTTTGCTGAC
351 GCTGATTGCA ACTGAAAAAAAA AGTTTTGAAT AAGGTCCAAT TGGCGGGCGG
401 ACCCGCCAAG GAAACAAGTA GTACGCAAAA AGACATGGGT GAATATGGCG
451 CCAGACGGGC AAGGCAGCGA GGCCCTATCC CAGTCCGGCT TCATATTTGT
501 GTAATGATCC CTCCGCAGGT TCACCTACGG AGACCTTGTT ACGACT

K16a ITS reverse

```
1      ACATTACACA AATATGAAGC CGGACTGGGA TAGGGCCTCG CTGCCTTGCC
51     CGTCTGGCGC CATATTCACC CATGTCTTTT TGCGTACTAC TTGTTTCCTT
101    GCGGGTCCG CCCGCCAATT GGACCTTATT CAAACCTTTT TTTCAGTTGC
151    AATCAGCGTC AGCAAAACAA ATGTAATCAA TTACAACCTT CAACAACGGA
201    TCTCTTGGTT CTGGCATCGA TGAAGAACGC AGCGAAATGC GATAAGTAGT
251    GTGAATTGCA GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC
301    CCTTTGGTAT TCCAAAGGGC ATGCCTGTTT GAGCGTCATT TGTACCTCA
351    AGCTTTGCTT GGTGTTGGGC GTCTTGTCTC TCTCCCGAGA CTCGCCTTAA
401    AATCATTGGC AGCCGGCCTA CTGGTTTCGG AGCGCAGCAC ATTATTTGCG
451    CTCTTGTTCCA GCCGCGGTCG CGCGTCCATG AAGCTTCTTT CAACCTTTTG
501    ACCTCGGATC AGGTAGGGAT ACCCGCTGAA CTTAAGCATA TCAATAA
```

C.1.2 β -Tubulin sequences of *Pyrenophora tritici-repentis* isolates

S12a Bt2b forward

```
1      AGAGCTGACC GAAGGGTCCA GCGCGGACGG CGTCCATGGT ACCGGGCTCG
51     AGATCGACAA GGACGGCACG GGCACGAAC TTGTTGTTGG ACGCCTATTG
101    CATGTTAGTA TTGACCGCTT ATCGGGAGCT TGGGGAGAAG CGGTTGACGT
151    ACTTCGTTGA AGTAGACATT CATGCGCTCG AGCTGGAGGT CTGAGGTGCC
201    GTTGTAGACA CCGGAGCCGT CGAGGCCATG CTCGCCGAA ATGGTCTGCC
251    AGAAGGCGGC ACCAATTTGG TTACCCTGGA GAGTCGTTAG ATGTCATCTC
301    AATGAGCGTC GAAGAAGCAA AACTTACGCA TTGGCCGGTC TGGAGGTGAA
351    CCTGTGGTAG AGAAACCGCG TTAGCTATAC TTCCCAATCT GGCTTTGTCC
401    CTGAATCTGG GCGCGGGGCA ACAACGCGCC ACAGTCTCTG TT
```

S12a T1 reverse

```
1      AGATTCAGGG ACAAAGCCAG ATTGGGAAGT ATAGCTAACG CGGTTTCTCT
51     ACCACAGGTT CACCTCCAGA CCGGCAATG CGTAAGTTT GCTTCTTCGA
101    CGCTCATTGA GATGACATCT AACGACTCTC CAGGGTAACC AAATTGGTGC
151    CGCTTCTGG CAGACCATTT CCGGCGAGCA TGGCCTCGAC GGCTCCGGTG
201    TCTACAACGG CACCTCAGAC CTCCAGCTCG AGCGCATGAA TGTCTACTTC
251    AACGAAGTAC GTCAACCGCT TCTCCCCAAG CTCCCGATAA GCGGTCAATA
301    CTAACATGCA ATAGGCGTCC AACAACAAGT TCGTGCCCCG TGCCGTCTTT
351    GTCGATCTCG AGCCCGGTAC CATGGACGCC GTCCGCGCTG GACCCTTCGG
401    TCAGCTCTTC CGTCCCGACA ACTTCGTTTT CGGCCAGTCT GGTGCTGGTA
451    ACAA
```

M14d Bt2b forward

```
1      GGGACGGAAG AGCTGACCGA AGGGTCCAGC GCGGACGGCG TCCATGGTAC
51     CGGGCTCGAG ATCGACAAGG ACGGCACGGG GCACGAACTT GTTGTGGAC
101    GCCTATTGCA TGTTAGTATT GACCGCTTAT CGGGAGCTTG GGGAGAAGCG
151    GTTGACGTAC TTCGTTGAAG TAGACATTCA TGCCTCGAG CTGGAGGTCT
201    GAGGTGCCGT TGTAGACACC GGAGCCGTCG AGGCCATGCT CGCCGAAAT
251    GGTCTGCCAG AAGGCGGCAC CAATTTGGTT ACCCTGGAGA GTCGTTAGAT
301    GTCATCTCAA TGAGCGTCGA AGAAGCAAAA CTTACGCATT GGCCGGTCTG
351    GAGGTGAACC TGTGGTAGAG AAACCGCGTT AGCTATACTT CCCAATCTGG
401    CTTTGTCCCT GAATCTGGGC GCGGGGCAAC AACGCGCCAC AGTCTCTGTT
451    C
```


M14d T1 reverse

```
1      ATTCAGGGAC AAAGCCAGAT TGGGAAGTAT AGCTAACGCG GTTTCTCTAC
51     CACAGGTTCA CCTCCAGACC GGCCAATGCG TAAGTTTTGC TTCTTCGACG
101    CTCATTGAGA TGACATCTAA CGACTCTCCA GGGTAACCAA ATTGGTGCCG
151    CCTTCTGGCA GACCATTTCC GGCAGCATG GCCTCGACGG CTCCGGTGTG
201    TACAACGGCA CCTCAGACCT CCAGCTCGAG CGCATGAATG TCTACTTCAA
251    CGAAGTACGT CAACCGCTTC TCCCCAAGCT CCCGATAAGC GGTCAATACT
301    AACATGCAAT AGGCGTCCAA CAACAAGTTC GTGCCCCGTG CCGTCCTTGT
351    CGATCTCGAG CCCGGTACCA TGGACGCCGT CCGCGCTGGA CCCTTCGGTC
401    AGCTCTTCCG TCCCGACAAC TTCGTTTTTCG GCCAGTCTGG TGCTGGTAAC
451    AA
```

G22a Bt2b forward

```
1      ACGGAAGAGC TGACCGAAGG GTCCAGCGCG GACGGCGTCC ATGGTACCGG
51     GCTCGAGATC GACAAGGACG GCACGGGGCA CGAACTTGTT GTTGGACGCC
101    TATTGCATGT TAGTATTGAC CGCTTATCGG GAGCTTGGGG AGAAGCGGTT
151    GACGTACTTC GTTGAAGTAG ACATTCATGC GCTCGAGCTG GAGGTCTGAG
201    GTGCCGTTGT AGACACCGGA GCCGTCGAGG CCATGCTCGC CGGAAATGGT
251    CTGCCAGAAG GCGGCACCAA TTTGGTTACC CTGGAGAGTC GTTAGATGTC
301    ATCTCAATGA GCGTCGAAGA AGCAAAACTT ACGCATTGGC CGGTCTGGAG
351    GTGAACCTGT GGTAGAGAAA CCGCGTTAGC TATACTTCCC AATCTGGCTT
401    TGTCCCTGAA TCTGGGCGCG GGGCAACAAC GCGCCACAGT CTCTGTTGCG
451    AGCG
```

G22a T1 reverse

```
1      TTCAGGGACA AAGCCAGATT GGGAAGTATA GCTAACGCGG TTTCTCTACC
51     ACAGGTTTAC CTCCAGACCG GCCAATGCGT AAGTTTTGCT TCTTCGACGC
101    TCATTGAGAT GACATCTAAC GACTCTCCAG GGTAACCAA TTGGTGCCGC
151    CTTCTGGCAG ACCATTTCCG GCGAGCATGG CCTCGACGGC TCCGGTGTCT
201    ACAACGGCAC CTCAGACCTC CAGCTCGAGC GCATGAATGT CTACTTCAAC
251    GAAGTACGTC AACCGTTTCT CCCAAGCTC CCGATAAGCG GTCAATACTA
301    ACATGCAATA GCGTCCAAC AACAAGTTCG TGCCCCGTGC CGTCCTTGTC
351    GATCTCGAGC CCGGTACCAT GGACGCCGTC CCGCTGGAC CCTTCGGTCA
401    GCTCTTCCGT CCCGACAAC TCGTTTTTCG CCAGTCTGGT GCTGGTAACA
451    AC
```

C.1.3 *ToxA* sequences of *Pyrenophora tritici-repentis* isolates

S12a *ToxA* forward

```
1      TCGTTAAACT TTTCGAAGCC GCCAACTCTT CTGAACTCGA CGCGCGCGGG
51     ACTCTCTCTC GACTGGACCC TCAAACCGCG CGGACTCCTA CAGGAGCGGC
101    AGGGAAGCTG CATGTCAATC ACAATCAACC CTAGTCGTCC GTCTGTCAAC
151    AACATCGGCC AAGTCGACAT TGACAGTGTT ATACTCGGAC GACCTGGTGC
201    TATAGGCTCG TGGGAAGTGA ACAACTTTAT TACCATTGGA TTGAACCGCG
251    TAAACGCCGA TACAGTGCGA GTCAACATCC GAAACACCGG CAGGACTAAT
301    CGCCTCATTA TTACTIONG GGAATAAGTC TCCCCACCAT TTTATTAGAT
351    TACTTATTAC TAACTTATAC TACTAATACT GTCACTC
```

S12a ToxA reverse

```
1      GAGTGACAGT ATTAGTAGTA TAAGTTAGTA ATAAGTAATC TAATAAAATG
51     GTGGGGAGAC TTATTCCCAT TGAGTAATAA TGAGGCGATT AGTCCTGCCG
101    GTGTTTCGGA TGTTGACTCG CACTGTATCG GCGTTTACGG GGTCAATCCA
151    ATGGTAATAA AGTTGTTTCA TTCCCACGAG CCTATAGCAC CAGGTCGTCC
201    GAGTATAACA CTGTCAATGT CGACTTGGCC GATGTTGTTG ACAGACGGAC
251    GACTAGGGTT GATTGTGATT GACATGCAGC TTCCCTGCCG CTCCTGTAGG
301    AGTCCGCGCG GTTTGAGGGT CCAGTCGAGA GAGAGTCCGC GCGCGTCGAG
351    TTCAGAAGAG TTGGCGGCTT CGAAAAGTTT AACGATTTTC TAACCGGGAT
401    CGGCTTCAGG CGTTGGGGCA GCAAGCACAG CAGCGGCGCT
```

M14d ToxA forward

```
1      AAACTTTTTCG AAGCCGCCAA CTCTTCTGAA CTCGACGCGC GCGGACTCTC
51     TCTCGACTGG ACCCTCAAAC CGCGCGGACT CCTACAGGAG CGGCAGGGAA
101    GCTGCATGTC AATCACAATC AACCTAGTC GTCCGTCTGT CAACAACATC
151    GGCCAAGTCG ACATTGACAG TGTTATACTC GGACGACCTG GTGCTATAGG
201    CTCGTGGGAA CTGAACAAC TATTACCAT TGGATTGAAC CGCGTAAACG
251    CCGATACAGT GCGAGTCAAC ATCCGAAACA CCGCAGGAC TAATCGCCTC
301    ATTATTACTC AATGGGAATA AGTCTCCCA CCATTTTATT AGATTACTTA
351    TTACTIONACT ATACTACTAA TACTGTCACT C
```

M14d ToxA reverse

```
1      GAGTGACAGT ATTAGTAGTA TAAGTTAGTA ATAAGTAATC TAATAAAATG
51     GTGGGGAGAC TTATTCCCAT TGAGTAATAA TGAGGCGATT AGTCCTGCCG
101    GTGTTTCGGA TGTTGACTCG CACTGTATCG GCGTTTACGG GGTTCATCC
151    AATGGTAATA AAGTTGTTCA GTTCCCACGA GCCTATAGCA CCAGGTCGTC
201    CGAGTATAAC ACTGTCAATG TCGACTTGGC CGATGTTGTT GACAGACGGA
251    CGACTAGGGT TGATTGTGAT TGACATGCAG CTCCCTGCC GCTCCTGTAG
301    GAGTCCGCGC GGTTTGAGGG TCCAGTCGAG AGAGAGTCCG CGCGCGTCGA
351    GTTCAGAAGA GTTGGCGGCT TCGAAAAGTT TAACGATTTT GTAACCGGGA
401    TCGGCTTC
```

G22a ToxA forward

```
1      CCCGGTTACG AAATCGTTAA ACTTTTCGAA GCCGCCAACT CTTCTGAACT
51     CGACGCGCGC GGACTCTCTC TCGACTGGAC CCTCAAACCG CGCGGACTCC
101    TACAGGAGCG GCAGGGAAGC TGCATGTCAA TCACAATCAA CCCTAGTCGT
151    CCGTCTGTCA ACAACATCGG CCAAGTCGAC ATTGACAGTG TTATACTCGG
201    ACGACCTGGT GCTATAGGCT CGTGGGAACT GAACAACCTT ATTACCATTG
251    GATTGAACCG CGTAAACGCC GATACAGTGC GAGTCAACAT CCGAAACACC
301    GGCAGGACTA ATCGCCTCAT TATTACTCAA TGGGAATAAG TCTCCCCACC
351    ATTTTATTAG ATTACTTATT ACTAACTTAT ACTACTAATA CTGTCACTCG
401    GGGGGACGTT TATGAGCTTT TTGGTGATTA CGCTTTAATT CAAG
```

G22a ToxA reverse

```
1      GAGTGACAGT  ATTAGTAGTA  TAAGTTAGTA  ATAAGTAATC  TAATAAAATG
51     GTGGGGAGAC  TTATTTCCCAT  TGAGTAATAA  TGAGGCGATT  AGTCCTGCCG
101    GTGTTTCGGA  TGTGACTCG   CACTGTATCG  GCGTTTACGG  GTTCAATCCA
151    ATGGTAATAA  AGTTGTTCAG  TTCCCACGAG  CCTATAGCAC  CAGGTCGTCC
201    GAGTATAACA  CTGTCAATGT  CGACTTGGCC  GATGTTGTTG  ACAGACGGAC
251    GACTAGGGTT  GATTGTGATT  GACATGCAGC  TTCCCTGCCG  CTCCTGTAGG
301    AGTCCGCGCG  GTTTGAGGGT  CCAGTCGAGA  GAGAGTCCGC  GCGCGTCGAG
351    TTCAGAAGAG  TTGGCGGCTT  CGAAAAGTTT  AACGATTTTCG  TAACCGGGAT
401    CGGCTTCAGG  CGTTGGGGCA  GCAAGCACAG  CAGCGGCGCT  GAAAA
```

C.1.4 PtrUnique primer sequences of *Pyrenophora tritici-repentis* isolates

S12a PtrUnique forward

```
1      TTTGTTTATG  AGCTGCTAGA  ATTAGAAATA  ACAACGTTTA  TACGTACACT
51     ACATTGCTTC  TTACTATCCT  AATTTTGCAT  TTGACAAAAT  GCGATCAATA
101    TTGACGAGAT  GCAATCTTCA  AGCCTTGATT  GCATTAATAG  TTTTCGACAGT
151    TGGAAATACG  TACTCCCCAT  TTGTGAAGGC  CTCCGCTGGC  GAGTAAAACC
201    GTATCGAGTA  CAGAAGCTTA  CCACCTCCTG  CTGGGGCCGG  AAGCCAGCTG
251    TACAAAAGGT  CTCAAGTCAG  TCCATGTTCT  GTTGCACCGA  AACAAATCGCA
301    TGATGCTACA  ATGGGTAAAC  TTACTTGCTA  GTCCAGTTAT  CCGGTGGGGG
351    AATATCAGCC  GGTTGCAATA  GGACCTGAAA  TCTCCCATCC  ACAGTTTCGT
401    TCCCAGGATT  TGCACCATCT  TC
```

S12a PtrUnique reverse

```
1      GTCCTATTGC  ACCGGGTGAT  ATTCCCCCAC  CGGATAACTG  GACTAGCAAG
51     TAAGTTTACC  CATTGTAGCA  TCATGCGATT  GTTTTCGGTG  AACAGAACAT
101    GGACTGACTT  GAGACCTTTT  GTACAGCTGG  CTTCCGGCCC  CAGCAGGAGG
151    TGGTAAGCTT  CTGTACTCGA  TACGGTTTTA  CTCGCCAGCG  GAGGCCTTCA
201    CAAATGGGGA  GTACGTATTT  CCAA
```

M14d PtrUnique forward

```
1      GAGCTGCTAG  AATTAGAAAT  AACAACGTTT  ATACGTACAC  TACATTGCTT
51     CTTACTATCC  TAATTTTGCA  TTTGACAAAA  TGCGATCAAT  ATTGACGAGA
101    TGCAATCTTC  AAGCCTTGAT  TGCATTAATA  GTTTCGACAG  TTGGAAATAC
151    GTACTCCCCA  TTTGTGAAGG  CCTCCGCTGG  CGAGTAAAAC  CGTATCGAGT
201    ACAGAAGCTT  ACCACCTCCT  GCTGGGGCCG  GAAGCCAGCT  GTACAAAAGG
251    TCTCAAGTCA  GTCCATGTTT  TGTTGCACCG  AAACAATCGC  ATGATGCTAC
301    AATGGGTAAA  CTTACTTGCT  AGTCCAGTTA  TCCGGTGGGG  GAATATCAGC
351    CGGTTGCAAT  AGGACCTGAA  ATCTCCCATC  CACAGTTTCG  TTCCCAGGAT
401    TTGCACCATC  TTC
```

M14d PtrUnique reverse

```
1      GGCCTATTG  CACCGGGTGA  TATTTCCCCA  CCGGATAACT  GACTAGCAA
51     GTAAGTTTAC  CCATTGTAGC  ATCATGCGAT  TGTTTCGGTG  CAACAGAACA
101    TGGACTGACT  TGAGACCTTT  TGTACAGCTG  GCTTCCGGCC  CCTGCAGGAG
151    GTGGTAAGCT  TCTGTACTCG  ATACGGTTTT  ACT
```

G22a PtrUnique forward

```
1      GAGCTGCTAG AATTAGAAAT AACAAACGTTT ATACGTACAC TACATTGCTT
51     CTTACTATCC TAATTTTGCA TTTGACAAAA TGCGATCAAT ATTGACGAGA
101    TGCAATCTTC AAGCCTTGAT TGCATTAATA GTTTCGACAG TTGGAAATAC
151    GTACTCCCCA TTTGTGAAGG CCTCCGCTGG CGAGTAAAAC CGTATCGAGT
201    ACAGAAGCTT ACCACCTCCT GCTGGGGCCG GAAGCCAGCT GTACAAAAGG
251    TCTCAAGTCA GTCCATGTTC TGTTGCACCG AAACAATCGC ATGATGCTAC
301    AATGGGTAAA CTTACTTGCT AGTCCAGTTA TCCGGTGGGG GAATATCAGC
351    CGGTTGCAAT AGGACCTGAA ATCTCCCATC CACAGTTTCG TTCCCAGGAT
401    TTGCACCATC TTC
```

G22a PtrUnique reverse

```
1      CCTATTGCAC CGGGTGATAT TCCCCACCG GATAACTGGA CTAGCAAGTA
51     AGTTTACCCA TTGTAGCATC ATGCGATTGT TTCGGTGCAA CAGAACATGG
101    ACTGACTTGA GACCTTTTGT ACAGCTGGCT TCCGGCCCCT GCAGGAGGTG
151    GTAAGCTTCT GTACTCGATA CGGTTTTACT CGCCAGCGGA
```

C.2 Darkened and inverted RAPD agarose gel fingerprints

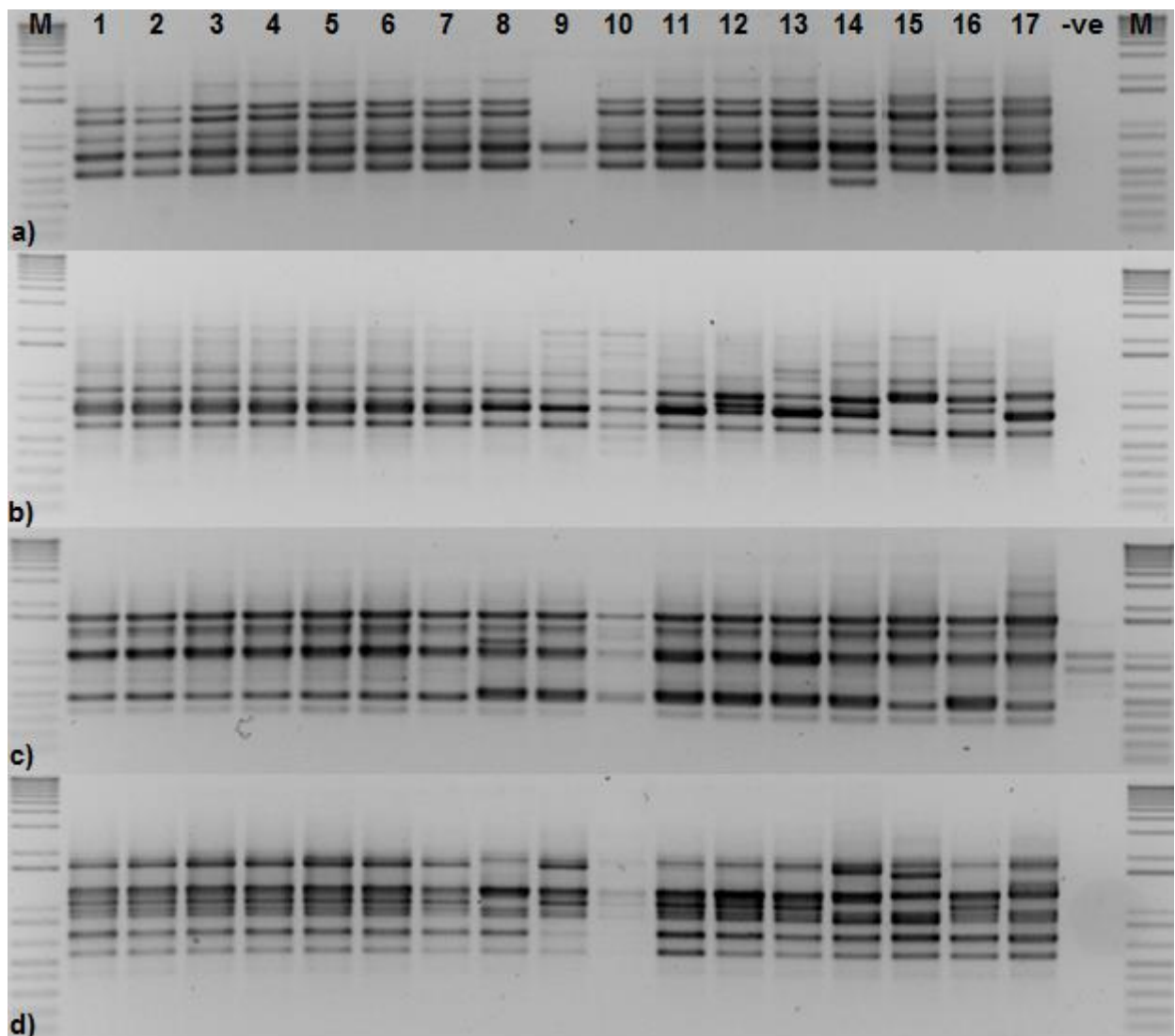


Figure 3.3: 1.3% agarose gels of RAPD fingerprints generated by genomic DNA of New Zealand and international *P. tritici-repentis* isolates. a) UBC598 primer, b) UBC600 primer, c) Operon H-19 primer and d) UBC517 primer. From left to right, lanes 1-17 are *P. tritici-repentis* isolates S12a, S26a, M13c, G14a, G22a, F12d, K16a, CCDM Meck4, CCDM 239, CCDM 134, ASC1, AB47-10, AB33-1, Alg3-24, AB39-2, AB39-8, TS93-71B. M = 1 kb plus DNA ladder, -ve = negative control.

Appendix D

D.1 Analysis of variance of fungicide sensitivity of *Pyrenophora tritici-repentis*

D.1.1 Two way ANOVA results on EC₅₀ values of fungicides on *in vitro* mycelial growth

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|----------------------|------|----------|---------|--------|-------|
| Isolate | 3 | 0.43994 | 0.14665 | 7.52 | <.001 |
| fungicide | 7 | 55.62911 | 7.94702 | 407.26 | <.001 |
| Isolate fungicide | 21 | 1.54530 | 0.07359 | 3.77 | <.001 |
| Residual | 96 | 1.87326 | 0.01951 | | |
| Total | 127 | 59.48761 | | | |

D.1.2 Mean EC₅₀ (mg a.i/ L) values with transformed log₁₀ means for eight different fungicides on the *in vitro* inhibition of mycelial growth of four *Pyrenophora tritici-repentis* single spore isolates

| Fungicide | <i>Pyrenophora tritici-repentis</i> isolate | | | | Fungicides mean EC ₅₀ |
|----------------------------------|---|-------------------------|-------------------------|-------------------------|----------------------------------|
| | S12a | G14a | G22a | K16a | |
| Isopyrazam | 0.01 b (-1.8475) | 0.02 bc (-1.6612) | 0.03 cd (-1.5770) | 0.01 a (-2.1688) | 0.02 A (-1.8136) |
| Propiconazole | 0.04 de (-1.4355) | 0.05 ef (-1.3213) | 0.05 ef (-1.2725) | 0.06 efg (-1.2432) | 0.05 B (-1.3181) |
| Bixafen + Prothioconazole | 0.09 gh (-1.0660) | 0.09 gh (-1.0665) | 0.14 l (-0.8645) | 0.12 hi (-0.9212) | 0.10 C (-0.9795) |
| Prothioconazole | 0.07 fg (-1.1760) | 0.13 hi (-0.8971) | 0.14 l (-0.8397) | 0.16 ij (-0.7935) | 0.12 C (-0.9266) |
| Epoxiconazole | 0.18 ijk (-0.7558) | 0.24 jkl (-0.6261) | 0.23 jkl (-0.6428) | 0.27 klm (-0.5616) | 0.23 D (-0.6466) |
| Fluxapyroxad | 0.31 lm (-0.5040) | 0.51 no (-0.2950) | 0.57 no (-0.2447) | 0.52 no (-0.2858) | 0.47 E (-0.3324) |
| Pyraclostrobin | 0.68 o (-0.1676) | 0.71 o (-0.1487) | 0.58 no (-0.2369) | 0.43 mn (-0.3707) | 0.59 F (-0.2309) |
| Azoxystrobin | 2.53 p (0.4032) | 2.56 p (0.4082) | 2.47 p (0.3935) | 4.78 q (0.6787) | 2.96 G (0.4709) |
| Isolate mean effect | 0.15 A (-0.8187) | 0.20 B (-0.7010) | 0.22 B (-0.6606) | 0.20 B (-0.7082) | |

Values within a column followed by the same letter are not significantly different according to Fisher's protected LSD at $P \leq 0.05$. Fungicide main effect (A-G) was significant ($P \leq 0.001$; LSD = 0.098). Isolate effect (A-B) was significant ($P \leq 0.001$; LSD = 0.034). The isolate x fungicide effect (a-q) which was also significant ($P \leq 0.001$; LSD = 0.196).

Appendix E

E.1 ANOVA of mean disease score and lesion area (mm) which develop on different wheat cultivars after inoculation with *Pyrenophora tritici-repentis*

E.1.1 One way ANOVA results for mean disease score for wheat cultivar susceptibility

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|--------------------------|------|----------|--------|------|-------|
| Block stratum | 9 | 42.2344 | 4.6927 | 7.43 | |
| Block.*Units* stratum | | | | | |
| Cultivar | 9 | 22.8067 | 2.5341 | 4.01 | <.001 |
| Residual | 81 | 51.1711 | 0.6317 | | |
| Total | 99 | 116.2122 | Total | | |

E.1.2 One way ANOVA results for lesion area on different wheat cultivars

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. |
|--------------------------|------|---------|-------|------|-------|
| Block stratum | 9 | 66.281 | 7.365 | 3.95 | |
| Block.*Units* stratum | | | | | |
| Cultivar | 9 | 76.524 | 8.503 | 4.56 | <.001 |
| Residual | 81 | 150.974 | 1.864 | | |
| Total | 99 | 293.779 | | | |